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Role of EDG-like lysophospholipid receptors in the regulation of the vascular tone

PhD Dissertation

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1. List of abbreviations

1-AGPAT: 1-acylgylcerol 3-phosphate acyltransferase

AA: Abdominal aorta

ABC: Adenosine trisphosphate-binding cassette

AC: Adenylyl cyclase

ACh: Acetylcholine

AD: Alzheimer's disease

ATX: Autotaxin

Aβ: β-amyloid

BAEC: Bovine aortic endothelial cell

C1P: Ceramide 1-phosphate

cAMP: cyclic adenosine monophosphate

CCL2: chemokine ligand 2, also known as MCP1: Monocyte chemoattractant protein-1

CIB1: Calcium and integrin-binding protein 1

COX1: Cyclooxygenase-1

COX2: Cyclooxigenase-2

CPA: Cyclic phosphatidic acid

$cPLA_2$: Ca^{2+}-dependent intracellular phospholipase A_2

CTRL: Control

DGPP: Diacylglycerol pyrophosphate

DMSO: Dimethyl sulfoxide

DSS: dextran sulfate sodium

ECL: Extracellular loop

EDG: Endothelial differentiation gene

EGF: Epidermal growth factor

eNOS: Endothelial nitric oxide synthase

ENPP: Ectonucleotide pyrophosphatase/phosphodiesterase

ER: Endoplasmic reticulum

ERK1/2: Extracellular signal-regulated kinase 1/2

GPCR: G protein-coupled receptor

GP-PDE/GDE: Glycerophosphodiester phosphodiesterase

HDAC1/2: Histone H3-histone deacetylase 1/2

HDL: High-density lipoprotein

HEV: High endothelial venule

HIF-1: Hypoxia inducible factor 1

hTERT: The human telomerase reverse transcriptase

HUVEC: Human umbilical vein endothelial cell

IBD: Inflammatory bowel disease

ICAM-1: Intercellular adhesion molecule-1

IgE: Immunglobulin E

IL-1β: Interleukin-1β

iPLA$_2$: Ca^{2+}-independent intracellular phospholipase A$_2$

JNK: c-Jun N-terminal kinase

KLF2: Krüppel-like factor 2

KO: Knockout

LDL: Low-density lipoprotein

LFABP: Liver fatty acid binding protein

LPA: Lysophosphatidic acid

LPAAT: Lysophosphatidic acid acyltransferase

LPC: Lysophosphatidyl-choline

LPhoE: Lysophosphatidyl-ethanolamine

LPP: Lipid phosphate phosphohydrolase

Lyso-PLD: Lysophospholipase D

mm-LDL: Minimally modified low density lipoprotein

mox-LDL: Mildly oxidized low density lipoprotein

MS: Multiple sclerosis

nat-LDL: Native low-density lipoprotein

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHERF2: N^+/H^+ exchange regulatory factor 2

NK cell: Natural killer cell

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

NPP: Nucleotide pyrophosphatase/phosphodiesterase

NUC: Nuclease-like domain

PA: Phosphatidic acid

PAR1: Protease-activated receptor 1

PC: Phosphatidyl-choline

PDE: Phosphodiesterase domain

PDGF: Platelet-derived growth factor

PE: Phenylephrine

PECAM-1: Platelet endothelial cell adhesion marker-1

PG: Prostaglandin

PhoE: Phosphatidyl-ethanolamine

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PLA_1: Phospholipase A_1

PLA_2: Phospholipase A_2

PLC: Phospholipase C

PLD: Phospholipase D

PLD_2: Phospholipase D_2

PPARγ: Peroxisome proliferator-activated receptor γ

PS: Phosphatidyl-serine

PSNL: Partial sciatic nerve ligation

PTEN: Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase

PTX: Pertussis toxin

RA: Rheumatoid arthritis

RBC: Red blood cell(s)

RGD: Arg-Gly-Asp integrin binding motif

ROCK: Rho kinase

S1P: Sphingosine 1-phosphate

SC: Schwann cell

SK1: Sphingosine kinase type 1

SK2: Sphingosine kinase type 2

SM: Sphingomyelin

SMase: Sphingomyelinase

SMB: Somatomedin B-like domain

SP: Substance P

SPC: Sphingosylphosphorylcholine

SPL: Sphingosine 1-phosphate lyase

$sPLA_2$: Secreted phospholipase A_2

SPP: S1P phosphatase

TA: Thoracic Aorta

TGFβ: Transforming growth factor β

TM: Transmembrane region

TNFα: Tumor necrosis factor α

TP: Thromboxane prostanoid receptor

TRAF2: Tumor necrosis factor receptor-associated factor 2

TRIP6: Thyroid receptor-interacting protein 6

TXA_2: Thromboxane A_2

TXB_2: Thromboxane B_2

VCAM-1: Vascular cell adhesion molecule-1

VLDL: Very low-density lipoprotein

VSM: Vascular smooth muscle

VSMC: Vascular smooth muscle cell(s)

WT: Wild type

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2. Introduction

The term lysophospholipid includes two lipid families with numerous members: the glycerophospholipids and the sphingolipids (1). The best-characterized representatives of these two groups are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) respectively.

The first LPA-related publication is from 1957, when Vogt isolated a mixture of lipid nature, which elicited contractions in the intestine of rabbits (2). The exact compound and structure remained however undiscovered that time. It was only in 1978, when Akira Tokumura and his group isolated and identified LPA from soybean lecithin, a new lipid mediator that induced hypertension in rats and other species (3, 4). At the beginning of the 1990s, Tigyi and Miledi identified albumin-bound LPA as an endogenous regulator of cellular functions and proposed that its actions are mediated by plasma membrane receptor(s) (5-8).

Since then, the number of reports on LPA increased rapidly. However, the molecular target of LPA actions was unclear until 1996, when Chun and colleagues identified the first G protein-coupled receptor (GPCR) of LPA, LPA_1 (9). Since that time, five other LPA receptors have been described and confirmed (LPA_{2-6}). Moreover, an intracellular receptor of the same mediator has also been reported in 2003 (10).

The first studies with S1P date back to the early 1990s as S1P was established a regulator of cell proliferation, growth, and survival (11, 12). Although the first S1P receptor itself was described already in 1990 by Timothy Hla and his group (13), it took eight years for $S1P_1$ to be de-orphaned and confirmed as an S1P GPCR in 1998 (14, 15). During the past 20 years, four other S1P receptors were reported ($S1P_{2-5}$).

Interestingly, three of the six LPA and all S1P GPCRs belong to the endothelial differentiation gene (EDG) family of receptors, sharing several similarities in structure and function as well. Studies conducted with human umbilical vein endothelial cells (HUVECs) showed, that the transcription of these genes increases upon the administration of phorbol-12-myristate-13-acetate, a compound that induces endothelial differentiation, hence the name EDG. Subsequently, it was revealed, that the structure of the EDG encoded protein shows great similarity to the GPCRs (13).

Following the early reports, the lysophospholipid field developed rapidly. The application of genetically modified animals gave another boost; hence, both LPA and

S1P became well-established mediators in several physiological and pathological processes (16, 17).

Our workgroup has been investigating the vasoactive actions of both mediators since the late 2000s. Interestingly, the literature available on both lipids is contradictory (see chapter 2.4.5.4.). In case of LPA, we described dual vasoactive effects in isolated murine vessels depending on the presence or absence of the endothelium. On the other hand, S1P, that had a nearly negligible impact on the diameter of vessels applied alone, potentiated the effect of other constrictors.

Our aim was to describe these phenomena and to explore, with the aid of pharmacological and genetic methods, which lysophospholipid receptors and downstream signaling pathways mediate these processes.

In the following, after a summary of our present knowledge on lysophospholipids, their receptors and roles in physiology and pathology, I would like to present our results on the vasoactive effects of these mediators with special attention on the GPCRs involved.

2.1. Lysophospholipids: nomenclature, molecular structure and natural analogues

2.1.1. Lysophosphatidic acid (LPA)

LPA (1-O-acyl-2-hydroxy-sn-glycerol-3-phosphate) was first identified as a key component of "Darmstoff", a smooth muscle stimulating substance in 1957 (2, 18). This simple, small lipid derives from the plasma membrane. LPA consists of a polar phosphate headgroup, a glycerol backbone and a hydrophobic fatty acid tail. Based on the acyl-group, LPA can be divided into saturated (e.g.: 16:0, 18:0) and unsaturated (e.g.: 16:1, 18:1, 18:2, 20:4) molecular species (Figure 1).

Because the acyl chains can bind to the glycerol backbone in either sn-1 or sn-2 position, sn-1 and sn-2 regioisomers can be differentiated respectively. Yet sn-2 isomers have a short half-life in vivo, as a relative rapid acyl-migration occurs towards the sn-1 position resulting in a 9:1 (sn-1:sn-2) equilibrium ratio (19, 20).

Studies around the millennium revealed the existence of alkyl-ether (21, 22) and alkenyl-ether (23) analogues of LPA (Figure 1). These naturally less abundant forms proved to be weaker agonists than LPA on its GPCRs (24, 25) with an exception: LPA_5

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showed marked preference for 1-O-alkyl-gylcerophosphate to acyl-LPA of the same chain length (26).

Cyclic phosphatidic acid (1-acyl-sn-glycerol-2,3-cyclic phosphate, CPA) is also a naturally occurring analogue of LPA, present in blood (27), however its origin is still obscure. CPA also acts as a second messenger, inhibiting the peroxisome proliferator-activated receptor γ (PPARγ) (28). CPA is proved to be a weak agonist of LPA GPCRs (25, 26).

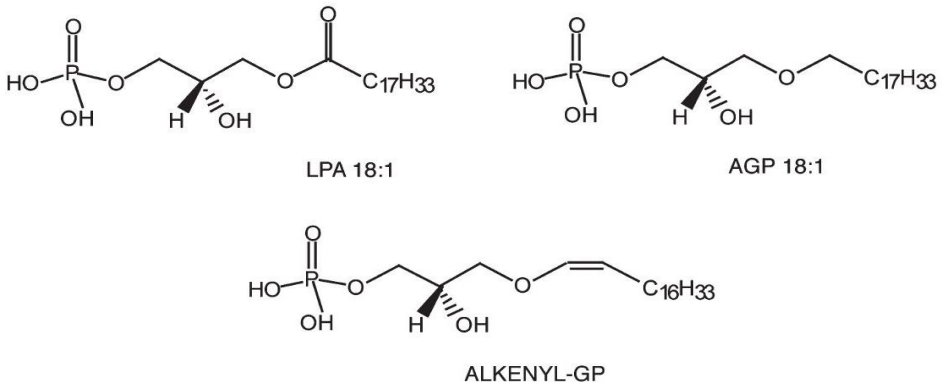

Figure 1. A few natural analogues of LPA; LPA 18:1: 1-oleoyl lysophosphatidic acid, AGP 18:1: 1-O-octadecyl glycerophosphate, Alkenyl-GP: Alkenyl glycerophosphate, modified after G. Tigyi (16)

LPA is present in human plasma in a low nanomolar concentration, however in serum it increases to the micromolar range (29, 30). The rank order of LPA species in plasma is 18:2>18:1≥18:0>16:0>20:4, whereas in serum 20:4>18:2>16:0≥18:1>18:0 (31). It is of note that LPA composition of the plasma alters with pregnancy. Palmitoyl-LPA (16:0) becomes the dominant species, although total LPA concentration of the plasma remains unaltered (32). Accumulation of this form is attributed to alteration in lysophospholipase D (lyso-PLD) activity, while the unaltered total LPA concentration may be a result of the increased general metabolism during pregnancy (32).

Considering its hydrophobic nature, LPA binds to carrier proteins in biological fluids as well as intracellularly (33). These bindings may clarify the contradiction between the facts, that, although the plasma concentration of LPA exceeds the K_d of LPA GPCRs, LPA-induced biological actions are lacking under resting conditions (31).

Albumin is the most abundant carrier of LPA in blood plasma, binding up to three mols of LPA per mol protein. It is noteworthy, that albumin also binds lysophosphatidyl-choline (LPC) and lysophosphatidyl-ethanolamine (LPhoE), although on a different binding site than LPA (33). Albumin is the most widespread carrier of LPA used under laboratory conditions.

Gelsolin is a protein, discovered in 1979, that has intracellular and secretory forms as well. It circulates in human and rodent blood in a concentration of 250±50 mg/l. Formerly considered an exclusively actin-binding protein, later proved to be able to bind LPA with nanomolar affinity (33). Lind and colleagues proposed a novel, remarkable yet speculative hypothesis about the role of plasma gelsolin in inflammation (34). In site of injury, activated platelets and leukocytes produce LPA while actin is released upon cell lysis. The actin released from dying cells binds to gelsolin, depleting it, which makes possible for LPA to act in free, unbound form on defense and repair. The same group showed that plasma gelsolin levels decrease dramatically in case of critical tissue damage, as in adult respiratory distress syndrome (34).

Aside of gelsolin another intracellular binding molecule of LPA has been identified: liver fatty acid binding protein (LFABP). This protein binds LPA on two distinct sites, on which other lysophospholipids (e.g. LPC, LPhoE and lysphosphatidyl-gylcerol) can be bound with micromolar affinity. Intracellular concentrations of LFABP range from 0.2 to 0.4 mM. Besides hepatocytes and intestinal cells, LFABP is also expressed in the cells of proximal tubules, where it is assumed to play a role in the reabsorption of lysophospholipids (33).

2.1.2. Sphingosine 1-phosphate (S1P)

The first reports of S1P were published in the early 1990s, proposing a role in intracellular calcium mobilization and cell proliferation (12) in cell growth regulation (11) and apoptosis inhibition (35). In contrast to LPA, S1P depicts a single molecular species (2S-amino-1-(dihydrogen phosphate)-4E-octadecene-1,3R-diol, Figure 2). S1P forms a zwitterionic structure at physiological pH, because the amine group of the terminal serine of the sphingosine base is basic at this pH, whilst the terminal esterified phosphate group bears negative charge. Besides this zwitterionic head group, S1P, similarly to LPA, has a long hydrophobic, aliphatic chain at the other side of the sphingosine base (36).

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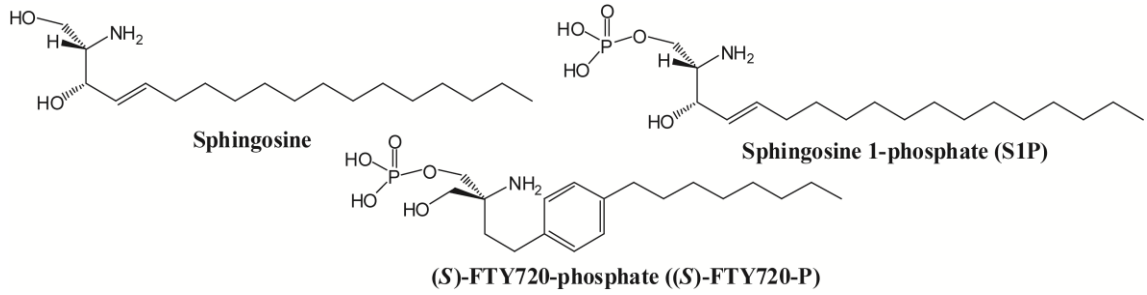

Figure 2. Structure of sphingosine 1-phosphate, its precursor sphingosine and its analogue FTY720-phosphate (Fingolimod-phosphate) (37)

Measured with multiple types of individually developed methods, S1P concentrations are estimated to range between 200-400 nM and 500-900 nM in plasma and serum respectively (38-41). Being a lipid with a considerably large hydrophobic tail, S1P traffic in plasma also requires binding molecules. For a long time albumin was postulated to be the sole carrier of S1P, till it has been revealed, that S1P binds predominantly to lipoproteins in a rank order of high-density lipoprotein (HDL) > low-density lipoprotein (LDL) > very low-density lipoprotein (VLDL) > lipoprotein-deficient plasma (mainly albumin) (42, 43). Other sources consider the amount of S1P bound to LDL and VLDL negligible (44). For a half decade, it remained enigmatic, which of the several components of HDL binds S1P, until in 2011 Christoffersen and colleagues reported it to be apolipoprotein M that binds approximately 65% of plasma S1P (45). HDL is not only a simple carrier of S1P, moreover it seems that HDL, which binds approximately 100-200 pmol/mg S1P (42), and S1P form a functional unit with distinct functions and signaling (42, 46, 47). Firstly, the K_ds of S1P for S1P GPCRs are within the 2 to 30 nM range (14, 48), which is markedly lower, than S1P concentrations found in plasma and serum. Based on these facts, it has been suggested that, HDL-binding might prevent full S1P GPCR activation and acts in a protective manner (42, 49). On the other hand, HDL-bound S1P has been reported to have four times longer half-life than that bound to albumin (42, 50), suggesting a protective role for HDL against ectoenzymes degrading S1P (49).

Later it has been revealed, that S1P is at least partly responsible for the anti-atherogenic, and cytoprotective features of HDL (42, 46). S1P inhibits cell migration in rat vascular smooth muscle cells (VSMC) via $S1P_2$ (51), moreover, HDL-bound S1P

exerts more sustained $S1P_1$ agonism, than the albumin-bound form, decreasing tumor necrosis factor α (TNFα)-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and intercellular adhesion molecule-1 expression (52). This type of agonism of $S1P_1$ might involve other structures, which facilitate the docking and entrapment of HDL-bound S1P and prevent endocytosis. However, this aspect of the S1P area requires further investigations, as the role of scavenger receptor class B type 1 occurred in the docking process (53). Besides, the fact, that oxidation of LDL, which is well-known to promote pro-atherogenic features of this particle, decreases the S1P- and reciprocally, elevates the LPC content of LDL, is also in favor of the anti-atherogenic role of S1P (50).

2.2. Biosynthesis and degradation of lysophospholipids

Although both mediators can be synthesized intra- as well as extracellularly the significance of the two sites differs in case of each lipid. While the bulk of S1P is produced intracellularly, with negligible extracellular production, the majority of plasma LPA is formed extracellularly (54-57).

Intracellularly, in mitochondria and microsomes, LPA is an intermediate of phospholipid synthesis, thus it is a product of glycerophosphate acyl transferase, which esterifies glycerol 3-phopshate with acyl-CoA or that of monoacyl-glycerol kinase as well (33).

Extracellular LPA is a result of three stimulus-coupled synthetic pathways (33). In spite of the recent advances in the field of lysophospholipid metabolism, regulation and the amount of contribution to extracellular LPA levels of each pathway are still questions to be resolved (Figure 3).

LPA can be generated from phosphatidic acid (PA) by the action of numerous enzymes. Although PA is a natural component of biological membranes, it can be a product of the cleavage of other phospholipid precursors, such as phosphatidyl-choline (PC), phosphatidyl-ethanolamine (PhoE) or phosphatidyl-serine (PS) by phospholipase D (PLD). Formation of LPA from PA is catalyzed by phospholipase A_1 (PLA_1) or phospholipase A_2 (PLA_2) (58, 59). PA specific variants of these two enzymes have been described in human (31, 60, 61), porcine (62) and horse (63) thrombocytes. Depending on the cleaving enzyme, sn-1 and sn-2 regioisomers are produced by PLA_2 and PLA_1 respectively. The ratio of the isomers is approximately constant (19, 20). PLA_2 has

small molecular weight secreted ($sPLA_2$) and intracellular Ca^{2+}-dependent and –independent ($cPLA_2$ and $iPLA_2$ respectively) isoforms, hence this pathway can also contribute to intracellular LPA production (64, 65). Our current knowledge implies that PA cleavage to LPA contributes up to 10% of extracellular LPA production (31). It is assumed that the feasible role of PLA enzymes is to create a pool lysophospholipids for Lyso-PLD activity, described in detail below (33).

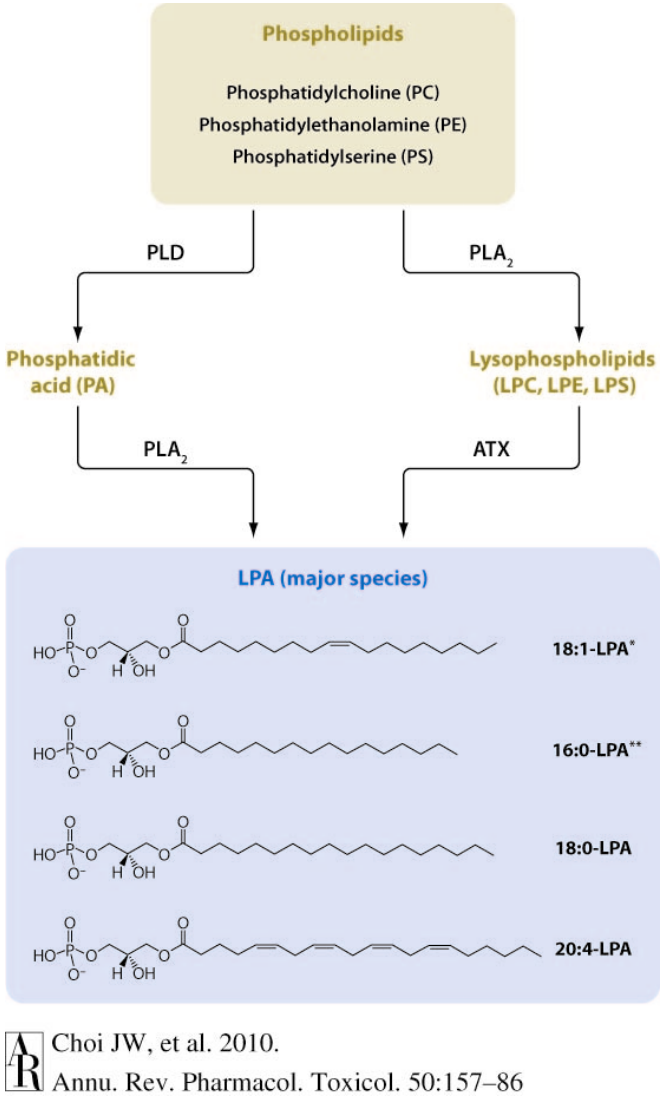

Figure 3. The major synthetic pathways of extracellular LPA; PLD: phospholipase D, PLA_2: phospholipase A_2, ATX: autotaxin (66)

Siess and colleagues proposed a still obscure pathway of LPA formation (67). In their study, mildly oxidized low-density lipoprotein (mox-LDL) and minimally

modified LDL (mm-LDL) treatment of human platelets resulted in shape-change, which proved to be mediated by LPA, was however absent in case of the native, unoxidized form (nat-LDL). Analysis of the lipid composition of nat-, mox- and mm-LDL revealed the presence of LPA in the mox- and mm- forms but not in nat-LDL. Thus, LPA generation occurs during oxidation of LDL in a Cu^{2+}-dependent manner and LPA is the main prothrombotic mediator in mox-LDL (67, 68). Besides, the LPA generated in this process can also influence the LDL uptake of atherosclerotic plaques via PPARγ activation (33).

Tokumura and colleagues raised first the possibility of LPA generation through lyso-PLD activity in rat plasma in the 1980s (69), which also gained confirmation in rabbit (70) and human (31, 71). The responsible enzyme remained enigmatic until the early 2000s, when two groups independently reported it to be autotaxin (ATX) (72, 73).

ATX was first identified as an autocrine motility factor in human A2058 melanoma cells, which stimulated motility in numerous tumor cell lines in a pertussis toxin (PTX)-dependent manner (74, 75). Besides that, high expression of ATX was found in neuroblastoma, hepatocellular carcinoma, breast cancer, renal cell carcinoma, glioblastoma, non-small cell lung cancer, B cell lymphoma, and thyroid carcinoma cells (75). ATX expression proved to be regulated diversely depending on cell lines. Studies imply the role of epidermal growth factor (EGF), basic fibroblast growth factor, transforming growth factor beta (TGFβ), v-Jun, β-catenin, Wnt-1, $\alpha_6\beta_4$ integrin, and Epstein-Barr virus infection in this process (76-82).

The crucial role of ATX in mammals is well emphasized by the fact, that mice deficient in ATX die around embryonic day 9.5 due to fatal vascular defects in embryo proper and yolk sack (83). This phenotype is interestingly recapitulated by $G\alpha_{13}$ knockout (KO) mice (84-86). ATX KO embryos also showed severe deficiency in neural tube formation and closure (83). Absence in expression of hypoxia inducible factor 1 (HIF-1) and its positive regulator Akt proved to have a role of high significance in neural tube malformations (75). It is of note, that ATX null embryos have abnormal lysosome formation in the visceral endoderm cells of the yolk sack (87). Morpholino oligonucleotide studies in zebrafish revealed a direct role of ATX in the formation of left-right asymmetry (88).

Since ATX heterozygous mice show 50% plasma LPA levels of that in wild types (WT), it has been established, that the bulk of LPA in biological fluids originates from ATX-mediated production (83). Multiple types of precursor lysophospholipids (LPC, LPhoE, LPS), of which LPC is the most abundant, can serve as substrate to ATX, which cleaves the phosphate group, thus forms LPA.

ATX has been identified as a member of the mammalian ectonucleotide pyrophosphatase/phosphodiesterase family (ENPPs or NPPs), which includes seven enzymes designated ENPP 1-7. All members of the family are capable of pyrophosphate bond hydrolysis, while ATX is unique by also having a lyso-PLD activity (72, 73, 89).

ATX is a rigid, multidomain structure glycoprotein of 125 kDa, consisting of five domains (89) (Figure 4). It includes two N-terminal somatomedin B-like (SMB1 and SMB2) domains, a central phosphodiesterase (PDE) domain, a lasso-loop domain, and a C-terminal nuclease-like (NUC) domain. Protein-protein interaction among the SMB domains and the PDE domain, an N-linked glycan and an interdomain disulfide bridge between the PDE and NUC domains, furthermore the fact, that the lasso-loop wraps tightly around the NUC domain maintain a high structural rigidity for the catalytic PDE domain (90, 91). Similarly to the other members of the family ATX has a conserved amino acid residue at Thr^{210} as the substrate binding site and two proximal Zn^{2+} ions contribute to the lytic activity (91). Unlike any other ENPP, ATX has a deep, hydrophobic lipid binding pocket of 15 Å, situated inside the catalytic domain (91). This pocket is suitable for the acceptance of mono- but not of diacyl phospholipids (91). Later on, it turned out, that the lack of a 18 amino acid sequence made the formation of this pocket possible, which is absent in every other ENPP (90, 91). Conversely, insertion of this sequence did not alter the pyrophosphatase activity of ATX but significantly alleviated its LPC hydrolysis (91). It is noteworthy, that ENPP 6 hydrolyses LPC to LPA, while having the 18 amino acid sequence, although it is possible that LPC binds to ENPP6 in a different orientation (89). ATX has one other feature, missing in other ENPPs, is having a tunnel close to the catalytic domain, which forms a so-called T-junction with the substrate binding pocket to which SMB1 also contributes (90, 91). The function of this tunnel is still ambiguous. A remarkable hypothesis suggests that it could be an exit-site for LPA generated by ATX, releasing its product directly to its cognate receptors on the cell surface (91). In support of this

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hypothesis, ATX has a relatively flat surface around the opening of the tunnel which can attach to biological membranes (89). In spite of this appealing hypothesis, the idea of this tunnel being an entry-site for the substrate LPC has also been suggested (89). Further investigations are required to clarify the role of this unique structure.

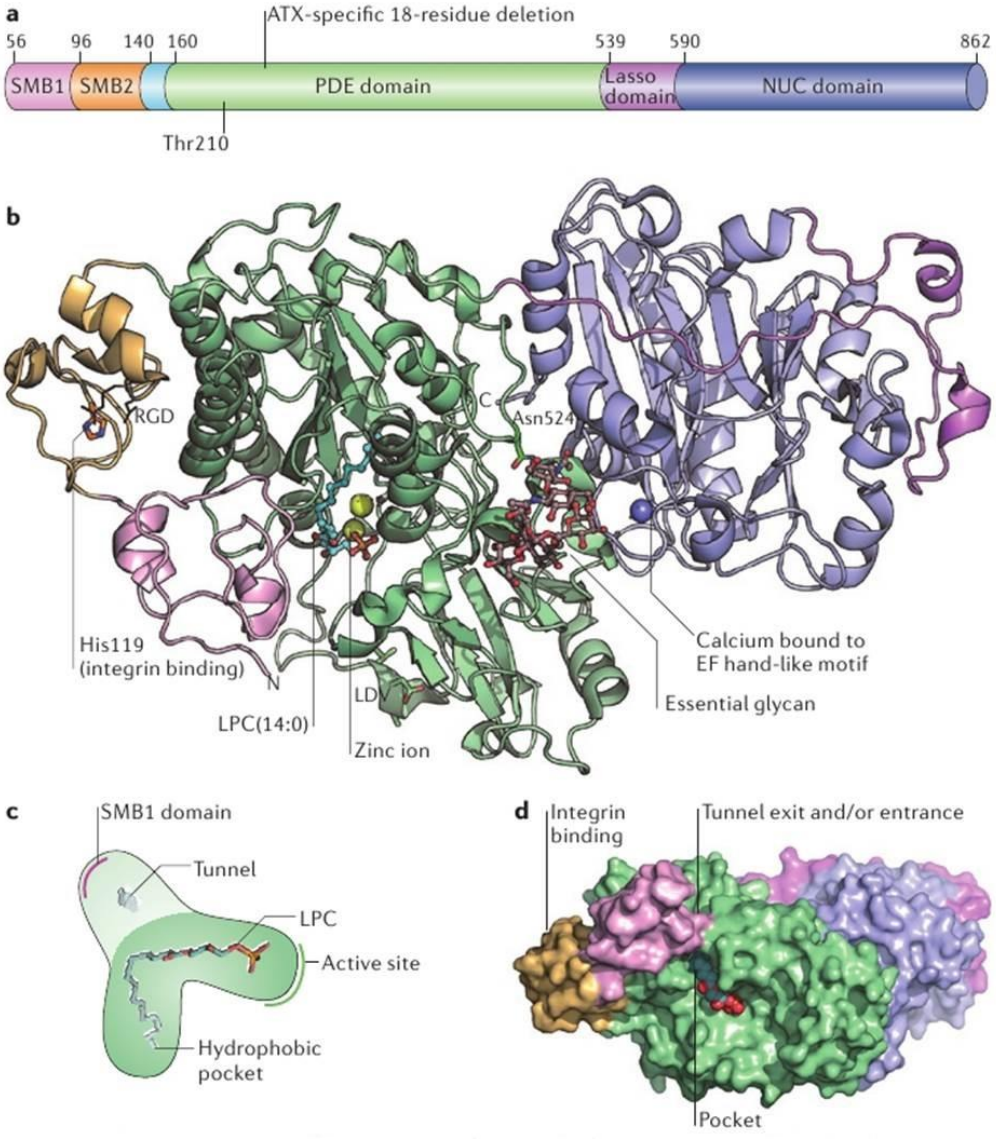

Figure 4. Autotaxin, a: Domains of ATX, b: Crystal structure of ATX while binding LPC, c: Schematic presentation of the active site, the hydrophobic pocket and the tunnel, d: Spherical model of ATX binding LPC; modified after W. H. Moolenaar and A. Perrakis

ATX is known to bind to activated platelets through β_3 integrin receptors (54). This interaction is attributed to the SMB domains, especially to SMB2, which although involves the canonical integrin binding motif (Arg-Gly-Asp (RGD)), it seems, ATX binds β_3 integrins at a distinct site (90). Besides, ATX binds to chemokine-activated lymphocytes via $\alpha_4\beta_1$ integrin (92). The detailed properties of this attachment is still elusive, however the PDE domain contains a canonical $\alpha_4\beta_1$ binding motif (Leu-Asp-Val). Considering the fact, that integrins are not only sole binding molecules but can promote intracellular signaling pathways, it is a conceivable assumption that ATX could mediate LPA-independent effects through integrins (89). Purification of ATX with heparin column chromatography revealed that ATX is able to bind heparin and heparan sulfate (89, 93), allowing this versatile enzyme to have further interactions with the cell surface and the components of the extracellular matrix.

Regulation of ATX activity is a field still flooded with questions. In vitro assays show ATX a relatively slow enzyme, which does not correlate to the rapid changes of LPA levels in biological fluids (89). β_3 integrin interactions emerged as potent regulators, as overexpression of this kind of integrin in Chinese hamster ovary cells resulted in markedly increased LPA production (89). Feedback regulation of ATX is also present in vitro by LPA and S1P (89), however this possibility seems to play a minor role in vivo (58).

Unlike to that of LPA, generation of S1P cannot be taken out of context, and should be described together with the production of other sphingolipids (Figure 5). De novo synthesis of sphingolipids occurs in the endoplasmic reticulum (ER), where ceramide is formed via multiple reactions with a rate-limiting step catalyzed by serine palmitoyltransferase (94). Ceramide can be phosphorylated by ceramide kinases to ceramide 1-phosphate (C1P) or can be converted to sphingosine by ceramidase. Sphingosine is the direct precursor of S1P. The reaction is catalyzed by two kinases called sphingosine kinase type 1 and 2 (SK1 and SK2 respectively), which are discussed below in detail. Conversely, ceramide can be formed from membrane sphingomyelin (SM) by sphingomyelinases (SMases), while sphingomyelin synthase catalyzes the inverted reaction. Ceramide can also be transformed to complex glycosphingolipids, which are highly abundant in biological membranes. As seen above, S1P synthesis can

start either de novo or form membrane lipids and since nearly every reaction is reversible structural and mediator sphingolipids can rapidly transform to each other.

It is of interest, while S1P and C1P elicit primarily mitogenic effects (95, 96), ceramide and sphingoid bases have mostly pro-apoptotic impacts (97), making the regulation of their balance fundamental in view of the cell life-cycle. The entire network of these thoroughly regulated processes is described in literature as the "sphingolipid rheostat" (Figure 5).

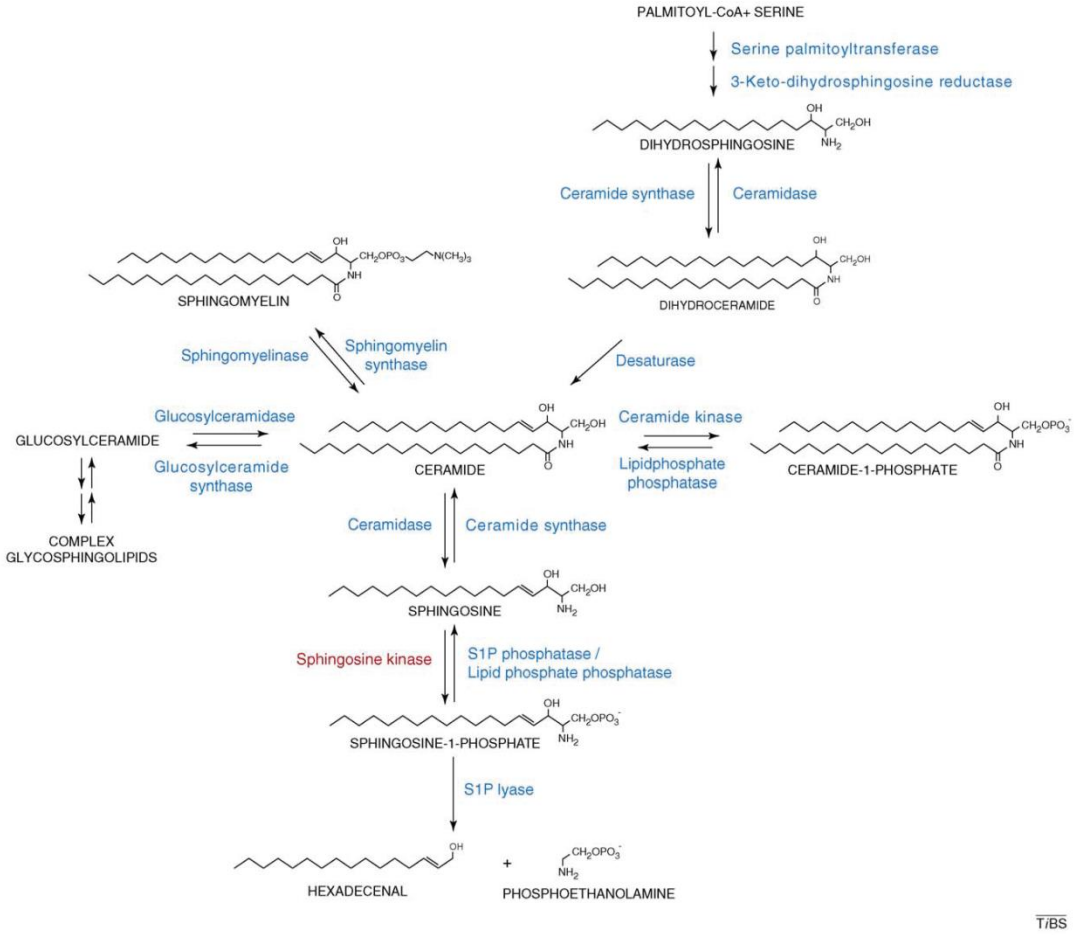

Figure 5. Sphingolipid biosynthetic and degradation pathways after Pitson (98)

SK1 and SK2 are the two enzyme isoforms that catalyze the formation of S1P from its direct precursor sphingosine. Although, the two isoforms have overlapping functions, and each can compensate the absence of the other, highlighted by reports of mice deficient in either of the two enzymes display no obvious phenotypical alteration (99-101), SK1/SK2 double KO mice die in utero due to severe disruption in angiogenesis, neurogenesis and neural tube closure (101).

Both SKs have splice variants. SK1 has three of them (named SK1a, -b and -c), which differ at their N-termini. SK1b has an additional 14 amino acid residue in comparison with SK1a, one of which is a cysteine, a putative palmytoilation site, which might give an explanation to its constitutive localization to the plasma membrane. The role of SK1c, which has an 86 amino-acid long additional residue at its N terminus, requires further investigation (57, 102). SK2 has two confirmed splice isoforms (SK2a and -b), of which SK2b possesses an additional 36 amino acid-long residue and shows higher abundance in a broad range of human tissues. The existence if a third SK2 splice variant is reported, however awaits further confirmation (57, 103, 104). It is of note, that SK2 contains a 116 amino acid-long insert in its central part, close to the sphingosine binding site, which alteration might explain the wider spectrum of artificial substrates utilized by this enzyme (105).

Despite of their importance in sphingolipid metabolism, crystal structure of both SKs have not been clarified yet, our knowledge of their structure, motifs and residues are based on homology studies with other lipid kinases, mainly diacylglycerol kinases and ceramide kinase. SKs cloned from different species contain five conserved regions (named C1-C5), which seem to be of grave importance in substrate binding and catalysis (106). Inhibitor and homology studies revealed multiple motifs in C1-C3, that are critical for nucleotide binding, whilst C5 is assumed to be involved in the catalysis of the nucleotide transfer (98). Since C4 is not conserved in diacylglycerol kinases and ceramide kinase, it appears to have a role in sphingosine binding (107, 108). Investigation with the selective SK1 inhibitor PF-543 clarified the sphingosine binding hydrophobic site, named “J-tunnel” due to its shape, which shows only slight differences in SK2 (47). More in-depth reviews on the structure of SKs are available (47, 98).

Albeit the two SKs catalyze the same reaction, they exhibit considerable differences in subcellular localization and regulation by external stimuli. SK1 normally localizes in the cytoplasm. Cytokine or growth factor mediated activation of the extracellular signal-regulated kinase 1 and 2 (ERK 1 and 2 respectively) results in phosphorylation at Ser226, activation and relocalization of SK1 to the plasma membrane (109). Translocation of SK1 occurs via interactions with the calcium and integrin-binding protein 1 (CIB1), which reaches out to SK1 in a calcium-dependent manner at a site

previously assumed to bind calmodulin (110, 111). Notwithstanding the active translocation of SK1 is CIB1-dependent, its retention is mediated by the interaction with plasma membrane phospholipids PS and PA (98). S1P produced by membrane-bound, activated SK1 either can be exported to the extracellular space, where it can bind to S1P GPCRs, or activates intracellular targets, such as tumor necrosis factor receptor-associated factor 2 (TRAF2) and thus activates NF-κB, exerting pro-survival signs (112). In most cases, ERK-mediated activation of SK1 is transient, as protein phosphatase 2A dephosphorylates SK1 at phospho-Ser225 (113). Several agonists have been associated with SK1 translocation including platelet-derived growth factor (PDGF) (114), nerve growth factor (115), insulin-like growth factor (116), TNFα (109), immunoglobulin E (IgE) (117), LPA (118), and phorbol-esters (109, 119). Besides, numerous protein-protein interactions have been revealed which regulate SK1 activity. δ-catenin (120), Lyn kinase (121), Fyn kinase (122), and eukaryotic elongation factor 1A (123) have been reported to activate, while SK-interacting protein (124), aminoacylase 1 (125), platelet endothelial cell adhesion marker-1 (PECAM-1) (126), and four-and-a-half LIM only protein-2 (127, 128) inhibit it.

SK2 activity is also rapidly increased by several agonists, such as TNFα (129), interleukin-1β (IL-1β) (129), EGF (130), and FcεRI cross-linking (122). Although the SK1 ERK regulatory site Ser225 is not conserved in SK2, it seems, that ERK1/2-mediated phosphorylation has an activator effect on SK2, however the putative site of this action is either Ser351 or Thr578 or both (131).

SK2 is generally most abundant in cytoplasm and the nucleus, though serum starvation and protein kinase C (PKC) activation are reported to facilitate its relocalization to the ER (132, 133). Molecular mechanism of this transport is still obscure; however, the N-terminus of the kinase appears to have a role in it (134). S1P produced by ER-bound SK2 is rapidly transformed to ceramide due to the high abundance of the degrading enzyme S1P phosphatase and ceramide synthase in ER (132, 135, 136).

SK2 contains nuclear localization and export signals regulating its translocation into and out of the nucleus, of which the latter is activated by protein kinase D-dependent phosphorylation at either Ser383 or Ser 385 (133, 135).

It is of note, that SK2 has been reported to induce apoptosis in an S1P-independent manner. SK2 contains a BH3 domain, which may be involved in interaction with B-cell lymphoma-extra large, release of cytochrome c and caspase-3 activation (137).

As seen before, regulation of SK2 is quite complex, and albeit a considerable knowledge is already available, the bulk of the work of deciphering involved processes remains to be done. This is well emphasized by the findings, where phosphoproteome analysis of cultured HeLa cells and murine liver tissue conceded at least five new phosphorylation sites on SK2 (Ser351, Ser363, Ser368, Ser378 and Ser448) of yet elusive function and significance (138-140).

As both types of SK localize intracellularly, the bulk of S1P is also produced there, however more and more studies indicate, that SKs can be released to the extracellular environment (141). Constitutive (57, 142) as well as heat stress- (143) and oxidized LDL immune complex-induced (144) secretion of SK1 has been reported. On the other hand, caspase-cleaved forms of SK2 are released from several types of cells during apoptosis (145). Besides, ATX-mediated cleavage of sphingosylphosphorylcholine (SPC) has been described (146), which thus leads to extracellular S1P generation, however the amount of S1P produced this way is limited due to low plasma SPC concentrations (147).

Although S1P is present in plasma at high nanomolar concentrations (38-41), S1P levels are extremely low in most tissues, what generates an in vivo S1P gradient between plasma and tissues (148). For a long period, platelets were proposed to be the major source of plasma S1P. S1P is stored in these blood constituents in large amounts (38, 149), which is also supported by the fact, that thrombocytes are devoid one of the major S1P degrading enzyme S1P lyase (SPL) (149, 150). Furthermore, upon stimulation by thrombin (151), or shear stress (152), platelets release S1P in a PKC dependent manner (149, 151). In contrast with this, mice deficient in nuclear factor erythroid 2, a major transcriptional regulator in megakaryocyte development and platelet production (153), had normal plasma S1P levels, however had virtually no circulating thrombocytes (154). Pappu and colleagues demonstrated in 2007, that plasma (but not lymph) S1P predominantly derives from hematopoietic sources, primarily from red blood cells (RBCs) (154). Consistently with this, RBCs are reported to lack all intracellular S1P metabolizing enzymes, which allows the storage of S1P in

high concentrations (155). According circulating hypotheses, RBCs may release S1P on a constant basis, producing a basal S1P level, whereas platelets do it in an activation-dependent manner, generating high S1P concentrations in the local environment (49). Thereafter, studies revealed that every cell is capable of S1P production by sphingomyelin metabolism (156), and that endothelial cells also contribute significantly to plasma S1P levels (157).

Since plasma S1P is primarily produced intracellularly and is impermeable to the plasma membrane due to its polar head, S1P requires transporters to be able to act in the extracellular environment. Numerous members of the adenosine trisphosphate-binding cassette (ABC)-type transporter family have been proposed to be responsible for S1P release, including ABCC1 (158), ABCA1 (159), ABCG1 (160) and ABCA7 (161, 162). Despite supportive pharmacological results, these findings gained no confirmation in in vivo studies (163). Interestingly, Spinster2 (Spns2) was identified in zebrafish, was shown to be a transporter of S1P and analogue Fingolimod (FTY720). Thus far, it is the only S1P transporter molecule, which was confirmed in vitro as well as in vivo (164, 165).

Similarly to its synthesis, degradation of LPA can occur in three distinct pathways (33). Dephosphorylation of LPA by phosphatases leads to monoacyl-glycerol, while removal of the fatty acid chain by lysophospholipases results in the formation of glycerol 3-phosphate. LPA can be converted to PA by acyltransferases.

Phosphate headgroup of LPA can be hydrolyzed by lipid phosphate phosphohydrolases (LPPs) of which three isoforms have been described: LPP1 and its splice variant LPP1a, LPP2 and LPP3 (166). Since the crystal structure of LPPs has not been yet clarified, our information on its orientation, structure, and mechanism of action lay predominantly on the analysis of related enzymes chloroperoxidase and phosphatidyl-glycerophosphate phosphatase B from Escherichia coli. All members of the LPP family are integral membrane proteins with six transmembrane regions. Both the amino- and carboxy-termini are located intracellularly, whilst the three conserved catalytic domains face the extracellular space. Two of the three catalytic domains (C1 and C2) can be found in the second extracellular loop, while the remaining one (C3) on the third. C1 contributes to substrate recognition, at the same time the other two mediate the phosphotransferase reaction (33, 166). Although functional as monomers, LPPs tend

to form homo- and heterooligomers (166). Nevertheless, LPP1 hypomorph mice exhibit increased concentration and elongated half-life of plasma LPA, overexpression of the same enzyme did not alter LPA levels (166). LPP3 also binds to integrins, but in contrast with ATX, on its RGD motif. The integrins recognized by LPP3 are $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (166). Consistently with this, LPP1, which lacks RGD, showed no ability to integrin binding (166). Transgenic mice and in vitro studies indicate a significant role for LPPs in physiological and pathological functions as vascular development and permeability regulation, fur and hair growth, cell cycle modulation, and fertility (166, 167). Considering the actions of ATX in tumor biology, lower expressions of LPPs could be expected in various tumors. In accordance with that, LPP1 and LPP3 expressions are considerably reduced in breast, lung, and ovarian cancer. In contrast, LPP2 showed elevated expression in these types of tumors (166). A feasible explanation to that could be the role of LPP2 in cell cycle regulation, i.e. LPP2 facilitates premature S-phase entry thus enhances tumor growth (166).

LPA is hydrolyzed to glycerol 3-phosphate by lysophospholipases, of which LPA specific species has been isolated from rat brain (168).

The third pathway of LPA degradation is PA synthesis by acyl-transferases. These proteins belong to the 1-acylgylcerol 3-phosphate acyltransferase (1-AGPAT) or lysophosphatidic acid acyltransferase (LPAAT) family of five isoenzymes, labeled LPAATα-ε or 1-AGPAT 1-5 (33). Based on the facts, that LPAATα and LPAATβ have the highest catalytic activity, and LPAATα favors LPA as acyl acceptor the most, these two enzymes assumed to be responsible for the bulk of the intracellular LPA degrading process (33).

Metabolism of the alkyl-, alkenyl- and cyclic analogues of LPA is still poorly understood. A serine hydrolase, KIAA1363 (169) and diacylglycerol kinases (170) assumed to be involved in intracellular alkyl-LPA generation. CPA is synthesized in mammalian cells by phospholipase D2 (PLD2) (28) or ATX (16). However, the in vivo relevance of the latter is disputed (16). It is of note, that two recently discovered members of the glycerophosphodiester phosphodiesterase family (GP-PDEs or GDEs), GDE4 and GDE7 cleave 1-O-alkyl-sn-glycero-3-phosphocholine and LPC, thus generate alkyl- and acyl forms of LPA, respectively (171).

Extracellular degradation of S1P is also catalyzed by LPPs, which are discussed in detail in context with LPA. This reaction leads to dephosphorylation of S1P. Since LPPs act extracellularly, they have the most direct influence on plasma S1P levels and thus S1P signaling on GPCRs among S1P metabolizing pathways (166).

Intracellular dephosphorylation of S1P is mediated by S1P phosphatases (SPPs). SPP1 and SPP2 are sphingoid base-specific phosphatases located in the ER. SPP2 is expressed in embryonic mouse kidney, where it mediates S1P levels along with SKs, thus influences kidney development. SPP1 is proposed to have a role in formation of ceramide and other sphingolipids in the ER. Since SPP1 is located intracellularly, extracellular S1P requires transporters to reach the enzyme. ABC-type transporters are assumed to serve this function (56).

S1P can be irreversibly metabolized by the intracellular enzyme SPL (56). During the reaction, the sphingoid base is cleaved at position C2,3, resulting in formation of heaxadecenal and ethanolamine phosphate, which products can enter further lipid synthetic processes. Even though SPL is a ubiquitously expressed enzyme, there are reports of its role in ischemia, radiation and chemical injury of tissues (56). Besides, SPL has been shown to be regulated by PDGF signaling (56).

It is of note, that mice deficient in SPL die around weaning age, exhibiting anemia, myeloid cell hyperplasia, and pathological abnormalities of the lung, heart, urinary tract, and bone (56). Accumulation of long chain bases and ceramide in neurons and vital organs as well as cytotoxic concentrations of S1P may be involved in these phenotypic alterations. Moreover, high plasma triglyceride levels and absence of adipose tissue found in SPL KO mice surmise abruptions in global lipid metabolism (56). This is supported by reports, describing SPL product ethanolamine phosphate being essential for functioning of sterol regulatory element-binding proteins and that SPL KO mice showed changes in expression of PPARγ target genes, which contribute to general lipid metabolism (56).

As seen above, multiple pathways are involved in either synthesis or degradation of lysophospholipids; however, these processes are well organized and strictly regulated, through which the concentration of these mediators remains in a narrow range under physiological circumstances.

2.3. Lysophospholipid receptors

Both LPA and S1P have extracellular as well as intracellular targets (16, 56). Although, LPA after its discovery quickly emerged as an extracellular bioactive lysophospholipid, its mechanisms of action remained elusive. Several early hypotheses and theories emerged regarding these mechanisms including membrane perturbation (172), calcium chelation (173), and signal transductional pathways involving intracellular- and plasma membrane receptors (174-176). The cloning and identification of the first LPA GPCR in 1996 set a new era in the lysophospholipid field (9). During the past two decades, since the recognition of LPA_1, five other LPA receptors have been reported (LPA_{2-6}); moreover intracellular targets of LPA also have been confirmed (10). In contrast, S1P was already an established intracellular mediator, regulating cell growth (11) and suppressing apoptosis (35), when it has been demonstrated that S1P acts on cell surface receptors ($S1P_{1-5}$). All of these receptors are rhodopsin-like GPCR with seven transmembrane regions (TMs).

2.3.1. G protein-coupled lysophospholipid receptors

Extracellularly LPA can bind to six (LPA_{1-6}), whilst S1P to five ($S1P_{1-5}$) subtypes of GPCRs, specific to the corresponding mediator. Recently, new types lysophospholipid receptors have been reported attributed to lysophosphatidyl-inositol (LPI_1) and lysophosphatidyl-serine ($LysoPS_{1-3}$) (177). However, this thesis focuses only on LPA and S1P GPCR, thus those other receptors will not be discussed here. Three of the six LPA receptors (LPA_{1-3}) and all reported S1P GPCRs belong to the EDG family, while the remaining three LPA receptors (LPA_{4-6}) are part of the P2Y purinergic cluster (16, 56, 178). The LPA and S1P GPCRs with their respective signal transduction are depicted in figure 6 and 7, respectively.

During the last five years the crystal structures of three lysophospholipid receptors were solved ($S1P_1$, LPA_1 and LPA_6) (179-181), which provided new aspects to our former knowledge of ligand binding and structure-activity properties of these receptors, based on homology studies primarily with rhodopsin and β-adrenergic receptors (182-184). Description of the crystal structure of the first lysophospholipid receptor $S1P_1$ also gave a boost to homology studies (179), as $S1P_1$ shared a greater homology with the other EDG-like receptors as rhodopsin or the β receptors, which made possible to

analyze the receptor-agonist/antagonist interaction of the remaining LPA and S1P GPCRs more precisely (185). Although these former experiments lead to the recognition of multiple interactions between receptor and ligand, they have limitations, and thus results gained this way are to be treated with cautiousness (G. Tigyi personal communication).

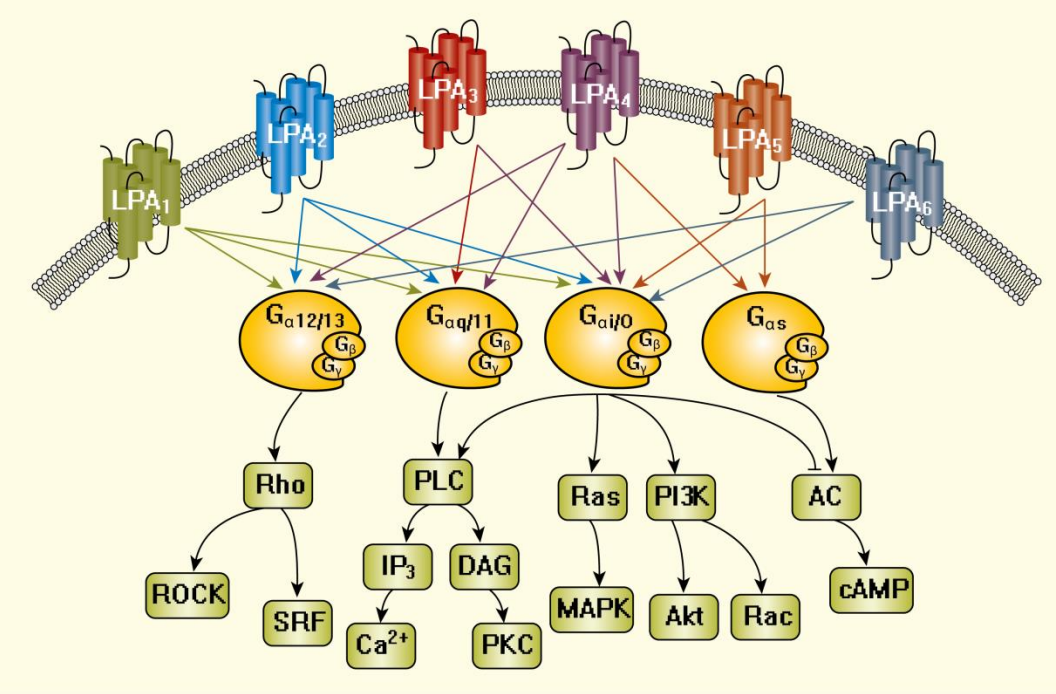

Figure 6. The six confirmed GPCRs of LPA and the signaling pathways they activate; ROCK: Rho kinase, SRF: Serum response factor, PLC: Phospholipase C, IP_3: Inositol trisphosphate, DAG: Diacylglycerol, PKC: Protein kinase C, MAPK: Mitogen-activated protein kinase, PI3K: Phosphoinositide 3-kinase, AC: Adenylyl cyclase, cAMP: Cyclic adenosine monophosphate; after H. Mirendil, M. E. Lin und J. Chun (186)

2.3.1.1. EDG receptors

$S1P_1$ (formerly EDG1) was the first recognized member of the EDG family by Hla and colleagues in 1990, though at this time unaware of S1P being its cognate ligand, thus being an S1P receptor (13). Hecht and colleagues described LPA_1 (EDG2) as the first lysophospholipid receptor in 1996 (9). Since these early discoveries, all eight members of the EDG gene cluster have been reported and validated to be a receptor specific to either LPA or S1P.

In case of EDG family LPA receptors, three key interactions of ligand binding have been revealed. Cationic amino acids (Arg and Lys) of TM3 and TM7 form ion-pairs with the phosphate group of LPA, while a Gln of TM3 establishes a hydrogen bond with the sn-2 hydroxyl group (33, 183). Further experiments deciphered, that the Arg residue of TM3 is conserved in the whole EDG family and also required for S1P binding of S1P receptors. Based on more in depth investigations of $S1P_1$, S1P binding also depends on three amino acid residues; two Arginines, which make an ion-pair with the phosphate group and a Glu of TM3, which corresponds the aforementioned Gln in LPA receptors, and interacts the ammonium moiety of S1P (182). Wang and colleagues reported that the Gln/Glu residue in TM3 determines LPA/S1P specificity respectively. In a range of single-mutation experiments, they showed, if the Gln in LPA_1 is mutated to Glu, LPA_1 is able to bind S1P, on the other hand if Glu of $S1P_1$ is shifted to Gln, $S1P_1$ binds only LPA and unable to be activated by its own ligand S1P (183). The defined crystal structures of LPA_1 and $S1P_1$ also highlighted some intriguing details of ligand docking abilities of both receptors. While the extracellular loops (ECLs) and TMs of LPA_1 are organized in a way that LPA_1 accepts ligands from the extracellular space, in case of $S1P_1$ the N-terminal with ECL1 and ECL2 forms a cap, which appears to block the entry of ligands, approaching this way. On the other hand, TM1 and TM2 are closer to TM3 than in other GPCRs, which leaves a gap between TM1 and TM7 making it possible for ligands to enter laterally from the outer leaflet of the plasma membrane (179, 180).

LPA_1 (EDG2) is the first identified (9) and most thoroughly studied lysophospholipid receptor. In mammalian cells the LPAR1 gene encodes a protein of 364 amino acids, of which one variant has been reported with an 18 amino acid deletion (187). LPA_1 has a broad expression profile, in humans it has been detected in brain, heart, placenta, spleen, kidney, colon, small intestine, prostate, testis, ovary, pancreas, skeletal muscle and thymus (188), while it is highly abundant in murine brain, heart, lung, stomach, small intestine, spleen, thymus, testis and skeletal muscle (189). The murine Lpar1 gene or formerly ventricular zone gene-1 is highly expressed in the neocortical region called ventricular zone of the developing brain (9). The ventricular zone disappears before birth but Lpar1 expression persists mainly in cells forming the white matter tracts and seems to play a role in myelination (190). In support of this,

expression of Lpar1 has been detected in oligodendrocytes and Schwann cells (SC), the myelinating cells of the central and peripheral nervous systems respectively (190, 191).

LPA_1 KO mice exhibit about 50% perinatal lethality, attributed to abnormal suckling behavior, which may be a consequence of impaired olfaction. Besides, KO mice have reduced body and brain sizes, craniofacial dysmorphism with blunted snouts and wide-spaced eyes, and increased apoptosis in sciatic nerve SCs. 2.5% percent of LPA_1 null embryos showed frontal cephalic hematomas (192). It is of interest, that LPA_1 KO mice are significantly protected against bleomycin induced pulmonary fibrosis (193). During breeding of this KO strain a spontaneous variant emerged named MálagaLPA_1 (maLPA_1) named after the place of its discovery (194). Despite negligible perinatal lethality, maLPA_1 mice show more severe defects in the brain than ordinary LPA_1 KO mice (194) and exhibit multiple behavioral abnormalities including inhibition of fear extinction (195) and aggravation of chronic stress-induced impairment to hippocampal neurogenesis (196).

LPA_1 couples to $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, which can activate a wide range of downstream signaling pathways through phospholipase C (PLC), mitogen-activated protein kinase (MAPK), Akt, Ca^{2+} mobilization, Rho and Rho kinase (ROCK). LPA_1 can elicit multiple cellular responses including cell proliferation, migration, survival as well as cytoskeletal changes, and establishment of intercellular connections (189, 197, 198). Uniquely among LPA receptors, LPA_1 is trafficked to early endosomes, which is mediated by C-terminal binding of the GAIP interacting protein (199). LPA has also been implicated to regulate the Hippo-Yes-associated protein pathway, however, the LPA receptor(s) involved in this process remain(s) unidentified (200, 201).

Sequence homology investigations of LPA_1 led to the identification of **LPA_2** (EDG4), a 351/348 (human/murine) amino acid protein coded by the genes LPAR2 and lpar2 in human and mouse respectively, which shows ~60% amino acid identity to LPA_1 (202). Contrary to LPAR1, expression of LPAR2 is quite restricted, showing the highest abundance in testis and leukocytes and lower in the prostate, spleen, thymus, and pancreas (188). In mice, lpar2 is highly expressed in the kidney, uterus, and testis, while moderate levels of mRNA are detected in the lung, stomach, spleen, thymus, brain, and heart (203). During development, lpar2 expression has been shown in the limb buds, the craniofacial region, Rathke's pouch, and the embryonic brain (204-207).

LPA_2 KO mice appear lean with no phenotypic alterations (202). LPA_2 null mice were however, protected in a colitis-associated tumor model compared to WT mice (208), while bronchoalveolar lavage fluid of lpar2 heterozygotes contained reduced number of eosinophil granulocytes and lower levels of prostaglandin (PG) E_2 (209). LPA_2 mice show increased sensitivity to genotoxic stress induced by ionizing radiation and chemotherapeutics (210) and display delayed resolutions of DNA double breaks indicative of impaired DNA damage repair (211).

LPA_1/LPA_2 double KO mice have also been generated and showed the same phenotype as LPA_1 KOs with an increased incidence of frontal hematomas (26% vs 2.5% for LPA_1/LPA_2 double KO vs LPA_1 KO respectively) (202). These mice, however highlighted the opposing effects of LPA_1 and LPA_2 on primary VSMCs and injury-induced neointimal hyperplasia, LPA_1 being a negative, whilst LPA_2 a positive regulator of VSMC migration (212).

LPA_2, similarly to LPA_1, couples to $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, through which it can initiate the activation of Ras, Rac, phosphoinositide 3-kinase (PI3K), MAPK, PLC, diacylglycerol, and Rho pathways (197). LPA_2 regulates cell survival and migration. Ligands stimulating LPA_2 provide protection against exposure to genotoxic stressors and protect Lgr5 marker positive intestinal stem cells and hematopoietic progenitor cell in the bone marrow (213, 214). The LPA_2 PDZ-domain binding motif is unique among LPA receptors and also regulates Na^+/H^+ exchange regulator factor 2 (NHERF2), which activates PLCβ3 and Akt/ERK signaling and inhibits the cystic fibrosis transmembrane conductance regulator (215). Mechanistically, LPA_2 makes physical interaction with the cystic fibrosis transmembrane regulator Cl^- channel and due to its coupling to the heterotrimeric G_i, protein inhibits cAMP production in the apical compartment of the epithelial cell membrane leading to inhibition of Cl^- secretion into the lumen (216). This mechanism plays an important role and offer therapeutic intervention in the treatment of secretory diarrhea caused by activation of this regulator protein (217). LPA_2 via its C-terminal PDZ protein interaction motif and another LIM-protein binding motif forms a ligand activation-dependent ternary complex with NHERF2 and the thyroid receptor-interacting protein 6 (TRIP6). This ternary complex is required for the anti-apoptotic effect of LPA_2 that is linked to a robust and long-lasting activation of the PI3K-NF-κB and ERK1/2 pro-survival pathways (218, 219). Cell migration is presumed to be

initiated by the interaction of the receptor C-terminal with TRIP6 (220, 221) and other PDZ-domain and zinc-finger proteins (215). The fact, that LPA_2 signaling is reported to be able to suppress EGF-induced migration and invasion of pancreatic cancer cells, raises the possibility of transactivation/cross-regulation between LPA GPCRs and tyrosine kinase receptors (222, 223).

LPA_3 (EDG7) was identified by two research groups independently, conducting homology studies with LPA_1 (224, 225). LPAR3/Lpar3 encodes a 353/354 (human/murine) amino acid protein with ~54% and ~49% homology to LPA_1 and LPA_2 respectively (224). Highest abundance of LPA_3 mRNA was found in human heart, testis, prostate, and pancreas (224, 225) and murine lung, kidney, uterus, and testis (189). Somewhat lower levels were detected in human lung, ovary, and brain (224, 225) as well as in murine small intestine, brain, heart, stomach, placenta, spleen, and thymus (189). Lpar3 is also expressed in heart, mesonephros, and in three spots in the otic vesicle during development (204).

LPA_3 KO mice appear normal; however, KO females show delayed embryo implantation, altered embryo spacing, and reduced litter size (226).

LPA_3 couples with $G\alpha_{i/o}$ and $G\alpha_{q/11}$ through which mediates Ca^{2+} mobilization, adenylyl cyclase (AC) inhibition and activation of PLC, and MAPK (227). Uniquely, LPA_3 has been reported to show marked preference for sn2 isoforms of LPA and for those containing unsaturated fatty acids (24, 224).

LPA_3 seems to play a role in determining vertebrate left-right patterning. Downregulation or inhibition of LPA_3 or ATX resulted in disruption of asymmetric gene expression and organ asymmetry in zebrafish (88).

Although **$S1P_1$** (EDG1) was the first identified member of the EDG family by Hla and Maciag in 1990, it was designated as an orphan GPCR until 1998, when two research groups independently confirmed S1P as its specific agonist (13-15). The human S1P1R gene encodes a 381 amino acid GPCR (13). High amount of $S1P_1$ mRNA was detectable in murine brain, heart, lung, liver, and spleen, while lower levels were found in kidney, thymus, and muscle specimens. Murine testis, stomach, and small intestine express $S1P_1$ in negligible amounts (228, 229). It is of note, that $S1P_1$ is highly expressed in developing central nervous-, cardiovascular-, and skeletal structures (228, 230).

Classical $S1P_1$ KO mice show a striking phenotype, as they die in utero between embryonic days 12.5 and 14.5 due to massive intraembryonic hemorrhage and edema throughout the body and limbs. These mice exhibited abnormal vascular maturation despite of normal angio- and vasculogenesis, which can be attributed to a disruption in VSMC and perycyte migration resulting in inadequate ensheatment of endothelial cells in nascent blood vessels (230). Generation of endothelium-specific $S1P_1$ null mice applying the Tie2 Cre-loxP system highlighted the fact, that the severe alterations seen in classical KOs are caused by the lack of $S1P_1$ in endothelial rather than VSM cells (231, 232).

Investigation of other tissue-specific KO mice lead to the recognition of the role of $S1P_1$ in lymphocyte trafficking. Studies of T-cell specific $S1P_1$ null mice showed, that $S1P_1$ is crucial for mature T-cells to egress from the thymus, moreover hematopoietic deletion of the receptor caused the same defect of T- as well as B-cell egress (233-235). It is of note, that $S1P_1$ is the sole lysophospholipid receptor, targeted by an already FDA-administered drug (236).

$S1P_1$ exclusively couples with $G\alpha_{i/o}$ and can activate ERK, PLC, and can cause Ca^{2+} mobilization and inhibit AC (237). Besides $S1P_1$-elicited PI3K/Akt and Rac activation have been shown to mediate cell proliferation, survival, migration, and changes in cytoskeletal structure (36, 238, 239). Studies with mouse embryonic fibroblast cells implicated cross-talk between $S1P_1$ and PDGF signaling, with the latter being upstream, which is also supported by the fact, that PDGF receptor KO mice recapitulate the phenotype of classic $S1P_1$ KO (240, 241).

$S1P_2$ (EDG5) was first isolated from rat cardiovascular and nervous systems, later confirmed by multiple groups being specific for S1P. The murine $S1pr_2$ gene encodes a 352 amino acid GPCR (242-244). $S1P_2$ is ubiquitously expressed, including murine heart, lung, thymus, brain, liver, kidney, spleen, and adipose tissue (229, 245). In the brain, $S1P_2$ expression is the highest at embryonic age and decreases throughout development, reaching an almost undetectable level at adulthood (229, 243, 246-248).

$S1P_2$ KO mice exhibit no obvious phenotypical abnormality, however show a slight yet significant decrease in litter size, which was augmented in $S1P_2$/$S1P_3$ double KO animals (249). Studies with these mice revealed progressive vestibule-cochlear loss with aging, including deafness, which proved to be a result of vascular abnormalities in the

inner ear and sensing hair cell loss in the organ of Corti (250). Besides KO mice showed seizure activity (247), disruption in wound healing (251), and in vascular function (252, 253), as well as reduction in inflammatory cell infiltration and pathological neovascularization in ischemia-induced retinopathy (253). Further investigation of $S1P_2$ KO mice on $Apoe^{-/-}$ background demonstrated that $S1P_2$ signaling is pro-atherogenic (254). Furthermore, the zebrafish homologue of the mammalian $S1pr_2$ gene proved to be essential for cardiac development, however this phenotype was not observed in mice (249, 255).

$S1P_2$ couples with $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$ through which induces serum response element, ERK, c-Jun N-terminal kinase (JNK), P38, PLC, Rho, and PIP3 phosphatase (PTEN) activation and mediates cell survival, rounding, and proliferation (256). It is noteworthy, that $S1P_2$ inhibits cell migration through the activation of PTEN, which is in contrast with $S1P_1$ action (257).

$S1P_3$ (EDG3) was first cloned as a 378 amino acid orphan human GPCR, later proved to be (like $S1P_2$) a high affinity S1P and a low-affinity SPC receptor (197, 258). Expression of $S1P_3$ was detected in murine spleen, heart, lung, thymus, kidney, testis, brain, and skeletal muscle (229, 245) as well as in human heart, placenta, kidney, liver, pancreas, skeletal muscle, and brain (258).

$S1P_3$ KO mice appear lean, with a small but significant drop in litter size (245). However, deletion of $S1P_3$ disrupts a certain amount of S1P actions in the cardiovascular system like negative chronotropic and hypertensive effects as well as vasoconstriction in basilar artery and nitric oxide (NO)-dependent vasodilation (245, 259). $S1P_3$ deficiency also prevented HDL-elicited vasodilation, highlighting the role of $S1P_3$ in the regulation of the vascular tone (260). Besides, S1P and HDL proved to be protective in myocardial ischemia/reperfusion injury through the activation of both $S1P_2$ and $S1P_3$ (261).

Although $S1P_3$ shows greater homology with $S1P_1$, its signaling resembles to that of $S1P_2$, as $S1P_3$ couples with $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ and activates ERK, serum response element, Rho, and Rac through which mediates cell proliferation, survival, migration and rounding (198, 262). It is noteworthy, that in mouse embryonic fibroblast cells $S1P_3$ is not involved in Rho activation (245).

It is of note that $S1P_3$ can act as a downstream effector of the protease-activated receptor 1 (PAR1). In this manner, $S1P_3$ signaling is involved in LPS-induced IL-1β and tissue factor production, which is an essential component in the pathogenesis of sepsis (263).

$S1P_4$ (EDG6) was first isolated from in vitro differentiated human and murine dendritic cells (264). The 384/386 amino acid (human/murine respectively) orphan GPCR later proved to be specific for S1P (265, 266). Expression of $S1P_4$ is restricted to hematopoietic and lymphatic tissues (264).

$S1P_4$ KO mice appear without any phenotypical abnormality. However, a significant amount of megakaryocytes in these animals showed aberrant, non-grained cytoplasm with vacuoles. Furthermore, they exhibited delayed recovery after monoclonal antibody-induced thrombocytopenia, without any reactive thrombocytosis compared with wild-type mice. Nonetheless, megakaryocyte count in bone marrow, platelet count in peripheral blood, plasma thrombopoietin level, and bleeding time were normal in $S1P_4$ null animals, implying a role for $S1P_4$ in the later phase of megakaryocyte maturation (267). Further studies with these animals suggested a role for $S1P_4$ in neutrophil trafficking, and pro-inflammatory cytokine release (268) as well as in $CD4^+$ T cell signaling (269).

$S1P_4$ couples with $G\alpha_{i/o}$, $G\alpha_{12/13}$ and possibly $G\alpha_s$, and can activate ERK, PLC, AC, Rho and small Rho family GTPase Cdc42. $S1P_4$ influences cell stress fiber formation and migration as well (198, 262).

$S1P_5$ (EDG8) was isolated from rat pheocromocytoma (PC12) cells and identified as an S1P receptor at the beginning of the second millennium (270, 271). The rat variant encodes a 400 amino acid GPCR (270). Expression of $S1P_5$ is restricted to the brain, spleen, and peripheral leukocytes in humans and the brain, skin, and spleen in mice and rats (245, 270, 271). In rat brain, $S1P_5$ is localized predominantly to the white matter tracts and cells of oligodendrocyte lineage, suggesting a role in proper myelination (271, 272).

$S1P_5$ KO mice were generated and appear lean without any phenotypic difference (272). In vivo studies however highlighted the role of $S1P_5$ in natural killer cell (NK cell) functions. NK cells show high levels of $S1P_5$ and show abnormal tissue

distribution upon $S1P_5$ ablation (273). Besides, $S1P_5$ expression has been shown to be co-mediated with NK cell maturation (274).

$S1P_5$ couples with $G\alpha_{i/o}$, and $G\alpha_{12/13}$ through which mediates AC inhibition, Ca^{2+} mobilization and, in contrast with other S1P receptors, ERK and cell migration inhibition (245, 271, 275, 276).

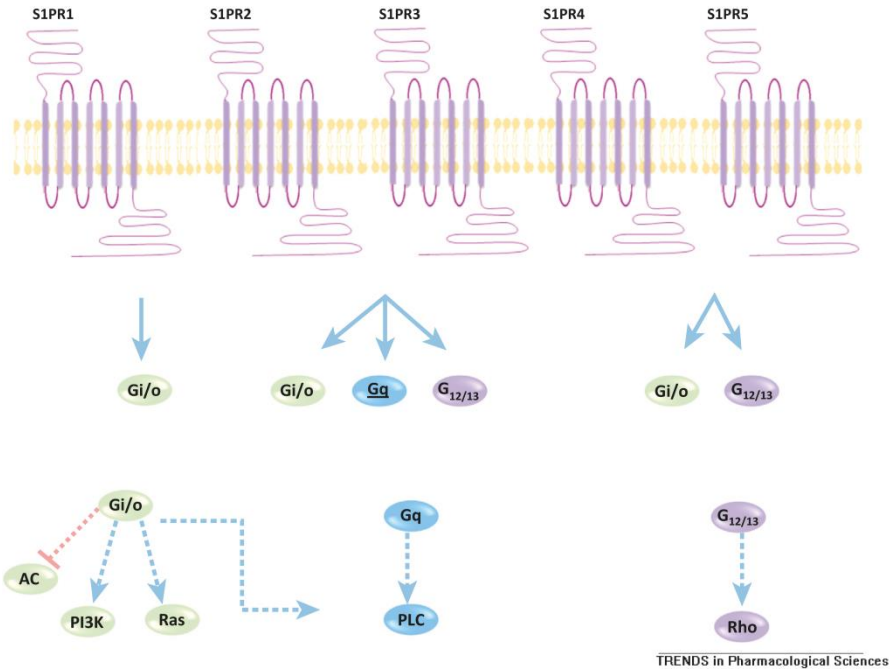

Figure 7. S1P GPCRs and activated signaling pathways; AC: Adenylyl cyclase, PI3K: Phosphoinositide 3-kinase, PLC: Phospholipase C; after C. O'Sullivan and K. K. Dev (277)

2.3.1.2. Non-EDG family LPA receptors

Although description of the EDG LPA receptors clarified a large amount of the extracellular actions of LPA, there are several responses, including platelet aggregation and certain aspects of vascular development that could not be fully explained by the three EDG family LPA GPCRs (278, 279). In 2003, Noguchi and colleagues, using ligand screening by Ca^{2+}-mobilization assay, identified a previously known GPCR, $P2Y_9$ of the purinergic cluster, as an LPA receptor (280). This observation gave new momentum to the lysophospholipid field, and till 2009 two other GPCRs were found to be specific to LPA (281-284). These receptors (LPA_4, LPA_5 and LPA_6 in order of confirmation) all belong to the P2Y purinergic family, however none of them responds to nucleotide ligands. A recent study in 2017 deciphered the crystal structure and ligand

binding properties of LPA_6 (181). During their research, Taniguchi and colleagues found that LPA_6 possesses a gap between TM4 and TM5, which forms vertical cleft open towards the plasma membrane. Besides, the presence of several hydrophobic amino acids in the cleft indicates its role as a lipid-binding site. They speculate that LPA_6, in contrast with LPA_1, can be accessible for its ligands from the extracellular environment as well as laterally from the plasma membrane. Considering the fact, that the ligand-binding pocket of LPA_6, formed by TM3, TM4 and TM5 is highly conserved in the P2Y family, binding properties of LPA_6 may provide insight into these functions of the other two non-EDG LPA receptors as well (181).

LPA_4 (formerly $P2Y_9$ and GPR23) was the first LPA receptor identified, that does not belong to the EDG family (280). LPAR4 encodes a 370 amino acid GPCR and is located on chromosome X (178). LPA_4 shows high abundance in human ovary, lower abundance in thymus, pancreas, brain, heart, small intestine, testis, prostate, colon, spleen, and platelets (66, 285). Among murine tissues, LPA_4 is present in heart, ovary, skin, thymus, and bone marrow (66). During development, LPA_4 is found in brain, maxillary process, branchial arches, limb buds, liver, and somites (204).

LPA_4 KO mice do not exhibit any obvious phenotypical alteration (286). However, approximately 30% of the embryos do not survive gestation, which can be attributed to hemorrhage and abnormal, dilated blood vessels with abrupt VSM and perycyte recruitment (279). Besides, LPA_4 is also assumed to have a role in lymphatic vessel development, as null mice show dilated lymphatic vessels and lymph sacs (279). Furthermore, LPA_4 KO mice display increased trabecular bone volume, number, and thickness, which are in contrast with what has been seen in LPA_1 KO, suggesting counteracting roles for LPA_1 and LPA_4 in bone formation (287).

LPA_4 couples with $G\alpha_{12/13}$, $G\alpha_{q/11}$, $G\alpha_{i/o}$, and uniquely among LPA receptors $G\alpha_s$ as well (288). Through these G proteins, LPA_4 can trigger Rho/ROCK activation, intracellular cyclic adenosine monophosphate (cAMP) accumulation, and ERK and PI3K activation (288-290). LPA_4 facilitates cell adhesion via N-cadherin and, in contrast with other LPA GPCRs, e.g. LPA_1, inhibits cell migration (286, 289).

LPA_5 (formerly GPR92) was first identified as an LPA receptor in 2006 (281, 282). LPAR5 encodes a 372 amino acid GPCR, which is highly expressed in human spleen, and mast cells while in a lesser extent in heart, small intestine, placenta, colon, liver,

and platelets (281, 285, 291). In mice LPA_5 mRNA shoes high abundance in small intestine, whilst moderately high in lung, heart, stomach, colon, spleen, thymus, skin, liver, platelets, mast cells, gastrointestinal lymphocytes, and dorsal root ganglia (281, 282). LPA_5 expression is high in the early embryonic forebrain, rostral midbrain, and hindbrain; however later on (from embryonic days 9.5-12.5) the expressional pattern becomes more diffuse throughout the whole brain (204).

LPA_5 KO mice appear lean; however, they seem to be protected against neuropathic pain, caused by partial sciatic nerve ligation (PSNL) (292).

LPA_5 couples with $G\alpha_{12/13}$, and $G\alpha_{q/11}$, through which elicits neurite retraction, stress fiber formation, receptor internalization and intracellular Ca^{2+} mobilization respectively (282). LPA_5 has been reported to increase intracellular cAMP levels in a presumably $G\alpha_s$-independent manner (281, 282). It is of note, that LPA_5 shows a marked preference for alkyl-LPA analogues (25, 26). Besides LPA_5 can interact with NHERF2, through which it enhances the recruitment of the Na^+/H^+ exchanger 3 to the microvilli of the colon, and facilitates Na^+-dependent water resorption (293).

LPA_6 ($P2Y_5$) is the most recently identified LPA receptor described by Pasternack et colleagues in 2008 (283). Expression of LPA_6 was reported in human intestinal mucosa cells, scalp hair follicles and skin (283, 294). Currently LPA_6 is the only non-EDG LPA receptor with an available crystal structure (181), which was briefly covered in the introduction of this section.

LPA_6 KO mice have been recently reported. Although these mice exhibited normal blood pressure and heart rate, they showed decreased vascular responses to adrenergic stimuli by phenylephrine or noradrenalin. Furthermore, LPA_6 KO mice showed abruption in postnatal retinal vessel formation, indicating that LPA_6 signaling is essential for the development of the normal vasculature (295).

LPA_6 signaling is still obscure, however evidences suggest, that LPA_6 couples with $G\alpha_{12/13}$ and $G\alpha_{i/o}$ and can activate Rho, ROCK, PLC, Ras, and PI3K on the other side inhibits AC (284, 294, 296). LPA_6 has been shown to have marked preference for sn2 regioisomers of LPA (294).

At the time of its characterization, LPA_6 was reported to be a genetic risk factor for an autosomal recessive form for hypotrichosis simplex and woolly hair (283, 297). Besides, mutations of the lipase member H, the human orthologue of PA-PLA_1 enzyme,

that produces sn-2 LPA also proved to cause the same condition (298, 299). These results hint a possible future role for LPA_6 signaling in the therapy of human hair loss. Homozygous inactivation of LPA_6 has been reported in bladder cancer (300).

2.3.2. Intracellular lysophospholipid targets

Besides the GPCRs detailed above, both LPA and S1P can act on intracellular targets (16, 17). Since S1P is generated inside the cells, the biosynthetic pathways of S1P acting intra- and extracellularly do not differ. On the contrary, the bulk of plasma LPA is produced extracellularly primarily by ATX as has been covered in previous sections thus, and since the majority of circulating LPA is albumin-bound (16), and transmembrane migration of albumin-bound LPA is minimal. Thus, intracellular LPA is generated in a different manner compared with extracellular, mainly through the action of Glycerol-3-phosphate acyltransferases (16).

2.3.2.1. Intracellular actions of LPA

McIntyre and colleagues reported in 2003, that LPA activates **PPARγ**, an essential regulator of lipid and glucose homeostasis (10). There are three different PPAR isoforms, labelled as α,β/δ and γ (301). PPARγ itself has two isoforms $PPAR\gamma_1$ and $PPAR\gamma_2$ (302). $PPAR\gamma_1$ is ubiquitously expressed, whilst $PPAR\gamma_2$ is restricted to adipose tissue (303). Deletion of the γ_1 isoform causes embryonic lethality (304), while that of γ_2 results in minor alterations in lipid metabolism (305). In case of its activation, PPARγ forms a heterodimer with the retinoid X receptor α (RXRα) and together they bind to the peroxisome proliferator response element in the promoter region of the target genes, through which regulate their transcription. In the absence of an agonist, the nuclear receptor co-repressor 1 and silencing mediator of retinoic acid and thyroid hormone receptor bind the heterodimer and repress its action (306-309).

Unlike LPA GPCRs, PPARγ is stereoselective and can only be activated by S-isomers carrying unsaturated acyl chains. Besides, alkyl-LPA analogues are more potent activators of PPARγ than acyl ones, a feature shared with LPA_5 (16, 310, 311).

LPA, activating PPARγ, increases the transcription of enzymes involved in lipogenesis, lipid storage, and adipocyte differentiation. It means, that the accumulation of this intermediate shifts the lipid metabolism of the given cell towards lipogenesis and

storage, instead of β-oxidation. Under pathophysiologic circumstances, this regulation may have a role in the development of non-alcoholic fatty liver disease (16, 312, 313).

It is of note, that synthetic agonists of PPARγ, the thiazolidinediones are applied in the therapy of type 2 diabetes.

CPA, a naturally occurring LPA analogue, on the other side is an antagonist of PPARγ (28).

Yoshida and colleagues described first, that LPA induces neointima formation in a non-injury infusion model of rat carotid artery (314). LPA was injected through the external carotid artery into a segment of the common carotid artery that was previously ligated, rinsed free of blood and maintained at near physiologic pressure. A 1 h exposure to LPA induced neointima formation. This process occurred only if unsaturated species of LPA was applied (314). Others concluded that the effect was PPARγ-dependent, as GW9662, a specific inhibitor of PPARγ, abolished the neointima formation (310).

PPARγ enhances the transcription of the CD36 scavenger receptor as well, which facilitates oxidized LDL uptake of the vessel walls (10).

PPARγ, along with LPA_1 and LPA_3, also has a role in mast cell and dendritic cell differentiation. In these cells, LPA (and cardiolipin), through the regulation of CD1 expression, can influence antigen presentation, however this hypothesis needs more evidence to be confirmed (315, 316).

Apart from PPARγ, LPA can attach to multiple actin-binding proteins, like **gelsolin**, **formin**, **adseverin**, and **villin**. These interactions can have a role in the regulation of the cytoskeleton (16).

2.3.2.2. Intracellular actions of S1P

Since its discovery, S1P has been known to promote cell survival and inhibit apoptosis (11, 35). Furthermore, studies showed that S1P elicits Ca^{2+}-release from the ER (17). However, the mechanism of these actions remained elusive. Recently, several intracellular targets of S1P have been identified, which highlighted the importance of S1P signaling not only through GPCRs, but via intracellular targets as well.

As discussed formerly, SK2 has nuclear localization and export signals and can be translocated to the nucleus (133, 135). Interestingly, it has been revealed, that SK2 localized in the nucleus forms a repressor complex with the histone H3-histone

deacetylase (**HDAC**) **1/2** bound to the promoter of certain genes. Moreover, S1P generated by SK2 binds to HDAC1/2 and prevents histone deacetylation, thus enhances transcription of genes including the cyclin-dependent kinase inhibitor p21 and the transcriptional master regulator c-Fos (317).

Furthermore, in fibroblasts, nuclear S1P can stabilize the human telomerase reverse transcriptase (**hTERT**), which is the catalytic subunit of the telomerase complex and maintains telomeres, that is often seen in transformed cancer cells. S1P binding of hTERT blocks its interaction with the makorin ring finger protein 1, which itself is an E3 ubiquitin ligase, thus S1P interaction with hTERT prevents the proteasomal degradation of this transcriptase, thus maintaining telomerase activity. Although the exact molecular explanation is still lacking, it appears, that S1P binding of hTERT mimics its phosphorylation at Asp 684 (318).

Intracellular S1P, produced by SK1 can directly target the **TRAF2** (112). This protein is an essential adaptor for the regulatory ubiquitination of receptor interacting protein, which is critical in activation of NF-κB in response to TNFα. It was previously shown, that TNFα stimulates the association of SK1 and TRAF2, which increases the activity of the former (319). Besides, ligase activity of TRAF2 was detectable only in presence of S1P but not that of dihydro-S1P. These findings may also explain the fact, that dihydro-S1P although is equally potent on S1P GPCRs, in contrast with S1P it has no cytoprotective effect (112).

S1P, produced in mitochondria by SK2, can bind to **prohibitin 2**, a protein necessary for mitochondrial assembly and function (320). The importance of this interaction is emphasized by the fact, that mitochondrial respiration is reduced in SK2 KO mice, due to the presence of an abnormal form of cytochrome c oxidase with low activity (320).

In neurons, S1P was shown to modulate the activity of **β-site amyloid precursor protein cleaving enzyme-1**, which is the rate-limiting step in amyloid-β peptide (Aβ) production. SK1 inhibition or downregulation, as well as overexpression of S1P-degrading enzymes decreased the activity of the aforementioned enzyme. Besides, Alzheimer's disease (AD) patients exhibited upregulation of SK2, hinting a possible involvement of S1P in pathogenesis of AD (321).

2.4. Roles of lysophospholipids in physiological and pathological responses

2.4.1. Lysophospholipids in the nervous system

Lysophospholipids have a pivotal role in the nervous system. This is well demonstrated by the fact, that the firs identified lysophospholipid receptor, LPA_1, was isolated from the ventricular zone of the murine brain (9). Since that, both LPA and S1P became well-established regulators in development, physiological function, and pathologies of the nervous system (322-324).

2.4.1.1. Neural development and function

Neural progenitor cells (NPCs) are located in the ventricular zone of the developing brain and undergo several steps including proliferation, differentiation, maturation, and migration to form nascent neurons (322, 323). LPA_1, LPA_2, and LPA_4 show marked expression in NPCs (206). In vitro and ex vivo studies implicate, that LPA_1 signaling controls cell proliferation and differentiation, playing a key role in development of the cortex of the brain. LPA_1 KO NPCs lack migration, proliferation, differentiation, and morphological changes essential to neurogenesis (192, 325). Ex vivo studies show that LPA_2 also has a role in LPA-induced survival and differentiation (326). The importance of LPA_1 is well emphasized by the phenotype of LPA_1 KO and the more severe developmental defects of maLPA_1 KO mice, described in detail in the previous chapter.

Our knowledge of S1P in neurodevelopment is much scanter. Although all S1P receptors are expressed in NPCs (248), only $S1P_1$ KO mice exhibit abruption in neurogenesis with increased cell death and decreased proliferation (101).

In post-mitotic neurons, LPA mediates neurite retraction, and growth cone collapse, however the receptor dependency is still obscure (46). On the other hand, Nerve growth factor can activate S1P production via SK1, which then elicits neurite extension in an $S1P_1$-dependent manner; however, overexpression of $S1P_2$ or $S1P_5$ inhibited this effect (115, 327). Besides, both LPA and S1P signaling have multiple roles in synaptic transmission, reviewed extensively (322, 324).

Astrocytes express $LPA_{1\text{-}5}$ and $S1P_{1,3,4,5}$ (46). It is of interest, that the injection of either LPA or S1P causes astrogliosis in vivo (46). Oligodendrocytes and Schwann cells (SCs) are the myelinating cells of the central and peripheral nervous systems respectively. Oligodendrocytes express LPA_1, LPA_3, and low levels of LPA_2 (46). It is

of interest that LPA_1 expression correlates to oligodendrocyte maturation stage. In addition, LPA increases myelin basic protein mRNA levels and myelin formation in vitro (46). On the other hand, S1P, through $S1P_5$ mediates process retraction, inhibits migration, and promotes survival of mature oligodendrocytes. In SCs, LPA is a well-established survival factor, acting on LPA_1 coupled to $G\alpha_{i/o}$, PI3K, and Akt. Besides, the same receptor regulates actin cytoskeletal rearrangements and enhances cell-to-cell adhesion via Rho and ROCK. LPA_1 signaling was also demonstrated in SCs in vivo, as LPA_1 KO mice exhibited increased apoptosis of the sciatic nerve SCs (46).

Microglia are the resident macrophages of neural tissues, which, upon stimulation, activate neuro-inflammatory processes. Microglia express LPA_{1-3} and $S1P_{1,2,3,5}$, which may alter cell activation. Besides, both S1P and LPA are able to mediate a multitude of cellular responses in microglia, reviewed elsewhere (46, 322).

2.4.1.2. Role of lysophospholipids in neural pathologies

Both LPA and S1P are involved in multiple processes in neural pathophysiology; however, this thesis does not focus on these responses, hence this chapter highlights only some aspects of lysophospholipid actions in the neural diseases, which are either well-documented or raise the possibility to use lysophospholipid analogs as future drugs.

2.4.1.2.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune neurodegenerative disease characterized by chronic inflammation, demyelination and axonal loss, as well as damage of the blood-brain-barrier leading to infiltration of lymphocytes and other immune cells (328). Efficacy of FTY720 (Fingolimod) in MS patients shed light on the role of S1P signaling in this disease (329). FTY720 is a sphingosine analogue, that is phosphorylated by SK2 (100, 330) and so is modulator of four of five S1P receptors, $S1P_{1,3,4,5}$ (331, 332). Mechanism of Fingolimod action is attributed to its effect on T-lymphocyte $S1P_1$, functioning as an antagonist and causing receptor internalization, through which lymphocytes fail to egress from secondary lymphoid organs (333-335). However, compelling amount of data has been presented, which implicates, that lymphocyte-$S1P_1$ signaling is not the only site of action of FTY720 (336). Fingolimod did not alleviate MS symptoms in astrocyte line $S1P_1$ KO mice in the widely used MS-model,

experimental autoimmune encephalitis, notwithstanding, the decrease in peripheral leukocyte count was present, showing, that FTY720 had an effect on T-lymphocytes (336). Besides, microglia get activated in experimental autoimmune encephalitis, which was absent in central nervous system-specific $S1P_1$ KO mice on application of FTY720 (336). It is also assumed, that Fingolimod improves blood-brain-barrier function through a yet unknown mechanism (322, 329). FTY720, under the name Gilenya has been approved in the relapsing-remitting form of MS in 2010 in Europe, US, and several other countries and is still the sole approved drug, targeting a lysophospholipid receptor (337). Unfortunately, it failed in trials with the primary progressive form of MS, which require alternative forms of therapy (329).

2.4.1.2.2. Ischemia

It is of note, that in case of ischemic stroke, S1P levels in the infarct area, as well as LPA levels in plasma increase (59, 338, 339). SK2 is upregulated in ischemic brains, however not in transient ischemia, where no changes of SK1/2 expression were shown (340-342). Furthermore, a subsequent study established SK2 as a protective factor (342). Interestingly, LPA_1, and LPA_2 expression is upregulated during retinal ischemia-reperfusion injury (343). FTY720, detailed in the previous part, on the other hand, proved to reduce brain damage in certain models (322). These data highlight the importance of lysophospholipid signaling in ischemic lesions of the central nervous system, however also show, that our knowledge is still quite rudimentary in this area.

2.4.1.2.3. Neuropsychiatric disorders

The role of lysophospholipids occurred early, as LPA_1 KO mice exhibited multiple traits as olfaction problems, craniofacial dysmorphism, failure in pre-pulse inhibition of the startle reflex, and learning, as well as memory deficiencies, commonly seen in autism and schizophrenia (344). Furthermore, maLPA_1 KOs show several behavioral abnormalities as general anhedonia, anxiety, and stress hypersensitivity (196, 345). Consistent with this, LPA_1 is downregulated in blood lymphocyte of human schizophrenia patients (346).

LPA signaling has been assumed to have a role in several molecular and neurotransmitter pathways, linked to neuropsychiatric functions, including glutamate and serotonin metabolism, regulation of glutamate receptors, and Ca^{2+}/calmodulin-

dependent protein kinase II activity (323). Notwithstanding, a study showed, that LPA exposure to the cortex elicited anhedonia, anxiety, and depression-associated immobility in adult mice, traits also seen in LPA_1 KOs (347). Moreover, cortical administration of LPA in high concentrations to embryos in the period of neurogenesis leads to fetal hydrocephalus (see in a later part in detail) (348).

On the other hand, much less is known about S1P in neuropsychiatry. However, $S1P_2$ KO mice showed anxiety and disruption in spatial memory (349).

2.4.1.2.4. Alzheimer's disease

AD is a neurodegenerative illness associated with dementia that affects millions of mostly elderly people all around the world (350). AD is a progressive disorder with a loss of specific neuron population and is characterized by senile plaques composed of β-amyloid (Aβ) and aggregated tau protein (350).

Enzymes of LPA and S1P production ATX and SK2 respectively, have been reported to be upregulated in brain specimens of AD patients (321, 351).

On the contrary, S1P was reported to reduce the activity if BACE, the enzyme responsible for the accumulation of abnormal Aβ in AD (321, 352). In AD patients, decreased levels of S1P and Aβ-induced downregulation of SK1 action were reported (353), and in consistence, SK1-dependent reduction of Aβ-induced cell death (354).

These results imply the role of S1P in AD is far away from being established, further studies hopefully clarify its protective or neurotoxic nature, pointing to possible drug targets in S1P signaling pathways.

2.4.1.2.5. Fetal hydrocephalus

Fetal hydrocephalus is a common neurological disorder of newborns with prenatal hemorrhagic events, and is associated with dilated ventricles, enlarged head, and third ventricle and aqueduct occlusion (355).

Yung and colleagues reported in 2011, that injection of either blood or LPA into the lateral ventricle of fetal brains induced hydrocephalus, with the clinical characteristics of fetal hydrocephalus in humans (348). Inhibition of $LPA_{1/3}$ by Ki16425 or lack of $LPA_{1\&2}$ prevented the formation of hydrocephalus, which showed the putative role of LPA_1 in this disease (348). The study not only pointed out the potential involvement of

LPA_1 and LPA signaling in fetal hydrocephalus but proposed a new model of the disorder as well.

2.4.1.2.6. Neuropathic pain

Neuropathic pain is a condition, commonly caused by nerve lesion or inflammation, and is often associated with lowered nociceptive threshold and consequent allodynia and hyperalgesia (356). Hypo/dysesthesia is also frequently seen in neuropathic pain (356). Additionally, demyelination can be detected in almost all cases, which presumed to have pathologic role in this disorder (356). Clinically, neuropathic pain can accompany chronic diseases like diabetes or MS; however can occur as a side effect of chemotherapeutical drugs (357).

LPA signaling is involved in multiple levels in the initiation and maintenance of neuropathic pain. This issue has been broadly reviewed (356-358); hereby it is intended to highlight the main aspects of LPA and its receptors in this process.

There are multiple animal models addressing neuropathic pain, including PSNL, intrathecal LPA injection, UVB irradiation, and ischemia induced pain (358). Interestingly, mice lacking LPA_1 or LPA_5 are protected against neuropathic pain induced by PSNL (292, 359). These studies shed light on the role of LPA summarized by the hypothesis of Ueda and colleagues (356, 357). As a result of nerve injury, large amounts of glutamate and substance P (SP) are produced and released by primary afferent neurons, which act on N-methyl-D-aspartate (NMDA) and neurokinin 1 receptors respectively. Activation of neurokinin 1 leads to Ca^{2+} mobilization via the conventional $G\alpha_{q/11}$ pathway, whilst NMDA promotes Ca^{2+} influx. The elevated intracellular Ca^{2+} concentration directly activates cPLA2 and indirectly, through calcium influx factor (CIF) iPLA2, which produce LPC from PC in the plasma membrane. As ATX is abundant in the cerebrospinal fluid, the LPC, produced via the aforementioned mechanism, is rapidly converted to LPA (356, 357).

LPA, through LPA_1 is involved in demyelination, as LPA_1 KO exhibit no demyelination in the PSNL model (359). Further studies deciphered, that LPA_1 signaling has multiple roles in this phenomenon. In SC, LPA_1 activates $G\alpha_{q/11}$ and enhances intracellular Ca^{2+} concentrations. Calpain is a Ca^{2+}-dependent protease, that is activated upon LPA_1 agonism and induces the proteolysis of myelin-associated glycoprotein, which is a key component of SC-neuron interactions. Conjointly, LPA_1,

activating the $G\alpha_{12/13}$-Rho-ROCK pathway, elicits JNK activation, which on one hand through PTEN activity, inhibits Akt-mediated myelination, on the other hand, enhances the transcription of c-jun, which downregulates the expression of Egr2 and downstream myelin protein myelin-associated protein, myelin basic protein, myelin protein zero, and peripheral myelin protein 22. LPA-mediated demyelination subsequently leads to induction of axonal sprouting, as axonal surfaces become free of inhibition of the myelin sheath. Sprouting of these axons establish connections between nociceptive and sensory fibers, which may be the pathophysiological basis of the sensory symptoms in neuropathic pain (356, 357).

In dorsal root ganglion neurons, LPA_1 upregulates the levels of several proteins, involved in molecular regulation of pathologic pain, like α2-δ unit of N-type Ca^{2+} channels, and ephrinB1 (356). EphrinB1, acting on its receptor Eph, mediates function and trafficking of NMDA receptors, influencing synaptic plasticity (356).

LPA, via LPA_3, also activates astrocytes and microglia; these two cell types seem to be involved in the initial process of neuropathic pain. LPA generation induces further elevation of LPA levels in a feed-forward manner, activating microglia LPA_3 receptors and possibly PLA2 enzymes (356, 357).

It is noteworthy, that LPA_5 KO mice do not exhibit neuropathic pain in the PSNL model (292). Interestingly, there was no difference in demyelination or other protein levels mediated by LPA1, however cAMP response element binding protein showed decreased phosphorylation (292). Further studies will reveal the significance of LPA_5 in this disorder.

As seen from the above, the presence of LPA signaling is well established in the pathogenesis and pathophysiology of neuropathic pain. These studies point out several points of action as possible future drug targets; however, many questions remain to be answered to gain a better understanding of this field.

2.4.2. Lysophospholipids in immune function

Both LPA and S1P perform several roles in immune cell processes, which are reviewed extensively divided by cell type elsewhere (46, 269, 360). As immunological actions of lysophospholipids are not in the focus of the present thesis, a few well-described roles of S1P and LPA will be listed in the physiological and pathological mechanisms of the immune system.

2.4.2.1. Lysophospholipids in immune cell trafficking

As already mentioned previously, S1P through $S1P_1$ signaling plays a key role in lymphocyte (both T and B) egress from the thymus and secondary lymphoid organs (269). The main driving force behind this is the S1P gradient between plasma, lymph, and the tissues. Plasma S1P is mainly of erythroid origin and is the highest, while the lymph contains S1P in lower concentrations originating almost exclusively from lymphatic endothelial cells. Tissues have usually the lowest levels of S1P, due to high S1PL activity. The gradient is a result of coordinated and strictly regulated function of S1P synthesizing, degrading, and transport proteins, discussed in detail previously (269).

In case of T and B cells, as well as NK T cells, expression of S1P is also required for egress. Therefore, S1P expression is thoroughly regulated in these cells. Krüppel-like factor 2 (KLF2) is a transcriptional factor, that regulates $S1P_1$ expression, driving its upregulation (269). During T cell maturation, driven by KLF2 $S1P_1$ expression increases, whilst after T cell activation $S1P_1$ mRNA levels decrease, which is assumed to be the result of ubiquitination and degradation of KLF2 (269). S1P expression is also regulated through agonist-induced downregulation. High concentrations of S1P elicit internalization and degradation of $S1P_1$. It is of note, that Fingolimod is much more effective in inducing this phenomenon than the natural agonist (269). Underlying mechanisms involve the action of GPCR kinase 2, which phosphorylates the receptor at a serine-rich region of the C-terminal and initiates its internalization (361, 362). Modulation of $S1P_1$ expression also happens through protein-protein interaction. CD69 is a C-type lectin, expressed on lymphoid cells right after their activation. If T cells are activated by type I interferon stimulation, the increase in CD69 expression downregulates $S1P_1$ (269).

Two hypotheses exist regarding the control of immune cell egress (269). The first one is the so-called lymphocyte-centered hypothesis, which implies, that lymphocytes sense the S1P gradient via $S1P_1$ expression, which drives them out of the lymph nodes. CCR7 –chemokine receptor-mediated retention signals are counteracting forces against S1P-$S1P_1$ signaling (269). The other hypothesis emphasizes the role of endothelial $S1P_1$. In this model, lymphocyte egress happens constitutively under physiological concentrations of S1P and is blocked by $S1P_1$ agonism on endothelial cells, closing sites

of egress (269). The evidence in support of both hypotheses is somewhat controversial. $S1P_1$ agonism-induced block (e.g. FTY720) and the lack of antagonism-induced egress block are in favor of the endothelium-centered hypothesis (363-365). However, the facts, that lymphocytes lacking $S1P_1$ could not egress from lymph nodes, and decreased $S1P_1$ expression results in decreased rate of egress, as well as the lack of S1P production through conditional deletion of SK1 and SK2 blocks egress, support the lymphocyte-centered view (154, 233, 235, 366). Further experiments are needed with decisive evidence in favor of one or the other hypothesis, or a new integrated hypothesis should be developed that resolves the above described controversies. It is noteworthy, that S1P also mediates NK cell mobilization, although in an $S1P_5$-dependent manner (273).

In contrast to S1P, LPA is assumed to interfere in T cell homing in high endothelial venules (HEVs) (360). Constitutive expression and secretion of LPA-producing enzyme ATX has been confirmed in HEV endothelial cells in 2008 (92, 367). Furthermore, ATX can bind to the plasma membrane of lymphocytes in either an integrin-dependent or independent manner, where it converts membrane LPC to LPA. It is however disputed, if the locally accumulated LPA drives lymphocytes to the lymph nodes or the LPA generated this way acts on the endothelial cells (360). Considering, that HEV endothelium expresses LPA_1 and LPA_4, LPA induces cytoskeletal redistribution, and inhibition of ATX disrupts extravasation but not lymphocyte accumulation in HEV, the second option seems more plausible (367-369).

Apart from these effects on immune cell trafficking, it is noteworthy, that both mediators have been established in various immunological pathologies as rheumatoid arthritis, psoriasis, systemic lupus erythematosus and inflammatory bowel disease. For more in depth reviews on this issue see: (178, 361).

2.4.3. Lysophospholipids in the reproductive system

2.4.3.1. Female reproduction

Involvement of LPA in reproductive functions has been known for almost 30 years (16). Studies in the early 90's identified LPA-mediated oscillatory Cl^- currents in Xenopus oocytes (5, 7). Since then, LPA signaling has been described in almost every organ and tissue of the reproductive tract mediating processes that include oocyte

maturation, acrosome reaction, oviduct contraction, early embryo development, and uterine smooth muscle contraction (370). Besides, LPA concentration in follicular fluid in healthy females is approximately double of that in serum (371). LPA-production is also elevated during pregnancy (32).

The most thoroughly investigated aspect of LPA in reproduction is though the role of LPA_3 in implantation. LPA_3 KO mice have delayed embryo implantation, embryo crowding, and reduced litter size. The fact, that transfer of WT embryos into LPA_3 KO dams reproduced this phenotype argued in favor of a maternal role for LPA_3 (226). Interestingly, mice deficient in COX2 showed the same defects. Moreover, LPA_3 KO mice exhibited low expression of COX2 and suppressed levels of prostaglandin end-products PGE_2 and PGI_2 in embryonic day (E3.5) which is generally the day of preimplantation (226). Exogenously applied PGE_2 and PGI_2 could rescue the delayed implantation and reduced litter size but not the defects in embryo spacing, suggesting the role of different prostaglandins or non-prostaglandin mediators in the process (372). Besides, expression of LPA_3 in the murine uterus was restricted to the luminal epithelium and showed a peak at the preimplantation time point, in contrast to other lysophospholipid receptors, which are constitutively expressed in all layers of the uterus (373). In agreement with the murine data, expression of LPA_3 in human uterus changes with the menstrual cycle showing highest mRNA levels during early and late secretory phase (374). Surprisingly, expression of LPA_3 is upregulated by progesterone and suppressed by estrogen (16, 226, 372). Conversely, deletion of LPA_3 leads to elevated ratio of progesterone signaling/estrogen signaling (375). It is also of interest, that LPA_3 KO mice showed delayed collagen clearance and decreased expression of matrix-degrading metallo- and serine-proteinases in the uterus at E3.5 compared with WT animals. These results demonstrate the involvement of LPA_3 in the dynamic remodeling of uterine extracellular matrix in the peri-implantation period (376).

As described above, disrupted LPA_3 signaling may have a role in the pathogenesis of diseases like non-receptive endometrium, placenta previa, placenta accrete, and certain types of infertilities (16, 370).

Our knowledge of the role of S1P in female reproduction remains incomplete in comparison with that of LPA. S1P was suggested to mediate acrosomal reaction,

ovulation, early embryo development through anti-apoptotic effects, and regulating placental trophoblast differentiation and vascular tone (370).

2.4.3.2. Male reproduction

Mice deficient in $LPA_{1/2/3}$, which are highly expressed in the murine testis (189), exhibited testosterone-independent decreased germ cell survival, mating activity, and higher prevalence of azoospermia in aging mice (377). As for S1P, it has been reported to be a survival factor for germ cells (378). Besides, it contributes to erectile function, as S1P has been demonstrated to potentiate acetylcholine (Ach)-mediated relaxation in human corpus cavernosum strips, in a Ca^{2+}-independent manner, through the Akt-eNOS pathway (379).

As previously demonstrated, both LPA and S1P have well-established roles in reproduction, however this field still requires intensive investigation, in order to assign new points of intervention for managing certain reproductive disorders.

2.4.4. Lysophospholipids in tumor biology

Both LPA and S1P were implicated in oncology, as the precursor of LPA, LPC was detected in elevated levels in the serum of ovary carcinoma patients (66), and anti-apoptotic functions of S1P were described shortly after its discovery (17).

2.4.4.1. Cell proliferation, tumorigenesis

Ovarian cancer patients not only exhibited elevated levels of LPC in serum, but markedly increased concentrations of LPA in ascites samples (16). Furthermore, a role of LPA in tumor biology is also implicated by the fact, that aberrantly increased expression of ATX was detected in several tumor types including breast cancer, glioblastoma, renal cell carcinoma, and hepatocellular carcinoma, responsible for the formation of high LPA concentrations locally in the tumor microenvironment (380). In addition, ATX itself was identified first as a motogen factor for melanoma cells in 1992; however, that time its Lyso-PLD activity was unknown (74).

LPA acting on its GPCRs can promote cell proliferation through $G\alpha_i$-RAS-MAPK, and $G\alpha_{12}$-JNK pathways and LPA signaling was associated with cyclin D1, c-Myc and β-catenin activation (380). In addition, LPA via PI3K-Akt signaling enhances cell survival, whilst via $G\alpha_{12/13}$-Rho-ROCK pathway plays a role in cell motility and

migration (380). It is of interest, that in certain Burkitt lymphoma and melanoma cell lines LPA_2 was implied to mediate cell survival via $G\alpha_s$ (380). On the other hand, LPA receptor signaling was shown to inhibit major tumor suppressor p53 (380).

Among LPA GPCRs, LPA_2 is assumed to have a relevant role in tumorigenesis/tumor-progression, as LPA_2 KO mice seem to be protected in DSS-model of colon cancer (208). Wnt/β-catenin is thought to be of grave importance in this type of tumors, in which Wnt-signaling prevents the degradation of β-catenin by the protein complex, formed by adenomatous polyposis coli, axins, casein kinase 1α, and glycogen synthase kinase 3. β-catenin, if remains intact, can interact with its partner proteins in the nucleus and promote the transcription of genes encoding c-Myc and cyclin D1, thus aiding cell proliferation. LPA_2 takes part in this process by inhibiting glycogen synthase kinase 3, via $G\alpha_q$-PLC-Ca^{2+}-PKC (381). Further evidence supporting the role of LPA_2 in colon cancer is provided by the study, in which deletion of LPA_2 in $APC^{min/+}$ mice, a genetic model of human familial adenomatous polyposis, significantly attenuated the initiation and progression of colon cancer (382).

LPA_2 activation also increases the concentration of VEGF locally, which is essential for novel vessel-formation and thus propagation of tumors. VEGF in return induces ATX expression, leading to LPA production, establishing a potential feed-forward loop involving LPA_2, VEGF, and possibly LPA_4 signaling (16).

LPA_2 was also implicated in regulation of urokinase and MMP expression, enzymes, which are key players in the invasiveness of primary tumors (16).

As already discussed in previous chapters, the effect of S1P on cell proliferation and motility cannot be taken out of context, as the ratio of pro-apoptotic ceramide and anti-apoptotic S1P determines the influence of the sphingolipid rheostat on cell-survival. Alterations of sphingolipid producing and metabolizing enzymes in multiple types of cancer have been widely reviewed elsewhere (383). Here, some direct connections between S1P signal transduction and certain oncological diseases will be highlighted.

Pro-oncogenic S1P signaling happens dependently as well as independently of S1P receptors. The role of $S1P_1$ occurred in colitis-associated colon cancer, via the NF-κB-IL-6-STAT3 pathway (384). The same receptor acting on PI3K and Rac can have pro-migratory, pro-invasive functions in Wilms' tumor (384). In fibrosarcoma cells, $S1P_1$ enhances the activity of membrane-type MMP, while in glioblastoma that of urokinase;

thus, it increases invasiveness of these tumors (384). Furthermore, $S1P_1$ has been associated with neovascularization, as $S1P_1$ KO mice die in utero between embryonic days 12.5 and 14.5 due to severe vascular malformations (230).

$S1P_2$ is the only receptor, which has pro- as well as anti-oncogenic roles (384). $S1P_2$ signaling enhances the transcription of master transcription factors c-Jun and c-Fos, arguing for its proliferative role (384). On the other hand, $S1P_2$ KO mice show frequent, spontaneous development of diffuse B-cell lymphomas with age (384). The signaling responsible for these pathologies is however still unknown.

$S1P_3$ is the most abundant of the five S1P receptors in human breast cancer cell lines. Furthermore, it activates Notch to expand the aldehyde dehydrogenase positive cancer stem cell population, which is assumed to be crucial in tumorigenesis (384).

$S1P_4$ and $S1P_5$ have less well-established roles in oncology. Although, $S1P_5$ can activate PI3K-Akt-Polo-like kinase 1, which is generally considered to be pro-oncogenic, inhibitory functions of $S1P_5$ also occurred (384).

Independently of its receptors, S1P activates intracellularly HDAC1 and 2 and hTERT (17). The relevance of these enzymes has been addressed in the chapter discussing intracellular S1P actions.

2.4.4.2. Metastasis

Both LPA and S1P have been implicated in metastasis.

Bone metastases are dreaded complication of many types of tumors including breast, prostate, kidney, thyroid, and lung cancers. Bone metastases are categorized as osteolytic and osteoblastic lesions, corresponding for sites with excessive loss or formation of bone tissue respectively (385). Latest evidence suggests that tumor cells do not directly evoke bone loss or formation, but influence the functioning of osteoclasts, osteoblasts, and osteocytes. Several studies pointed out the possible contribution of LPA to osteolytic lesions and some to osteoblastic ones. The knowledge available is excellently reviewed elsewhere (386). Here the hypothesis, based on the results concerning the role of LPA in bone metastasis, will be briefly summarized. LPA, present in a high concentration at the location of metastatic sites, due to platelet activation and/or high ATX expression of metastatic cancer cells, acts on the cancer cells, increasing the production of factors that influence osteoblast and osteoclast activity. Endothelin-1 has been described as a mediator of osteoblastic metastases,

released upon LPA stimuli. It inhibits osteoclasts and simultaneously stimulates osteoblasts. In case of osteolytic lesions, LPA stimulates the production of Dikkopf 1, IL-6, IL-8, GM-CSF, monocyte chemoattractant protein 1 (MCP1) (also known as chemokine ligand 2 CCL2), and Groα by metastatic cells, which increase phagocytic activity and maturation of osteoclast and inhibit osteoblast activity (386).

LPA has a direct effect on bone cells, promoting osteoclast differentiation, survival, and bone resorption. $LPA_{1/3}$ receptors may be involved in this process, as the $LPA_{1/3}$ antagonists Ki16425, and VPC32183 inhibited formation and propagation of osteolytic metastases in vitro and in vivo as well (386). Simultaneous inhibition of LPA GPCRs and ATX by BrP-LPA is also a promising approach for future therapies of these types of metastases (386).

The involvement of S1P in metastasis formation is highlighted by the fact that the deletion of Spns2, the ubiquitous S1P transporter reduced the lung metastases of various types of cancer cell lines injected in murine tail vein (387). Deletion of SK1 decreased the occurrence bladder cancer and melanoma metastases. $S1P_2$ was shown to repress breast cancer metastasis suppressor 1 a suppressing factor of metastases, which was reactivated upon application of sonepcizumab, an anti-S1P antibody. Furthermore, S1P activates the ezrin-radixin-moesin proteins, through which increases motility and invasion, features of high importance for metastases. $S1P_3$ was also shown to promote metastasis in lung cancer, influencing the TGFβ-SMAD pathway (383).

2.4.4.3. Resistance against chemo- and radiotherapy

LPA_2 does not only play a role in tumorigenesis of ovary carcinoma, but studies suggest it may also contribute to its resistance against chemotherapeutical drugs. LPA_2, via its C-terminal, promotes certain anti- and inhibits other pro-apoptotic processes. The LPA_2 C-terminal can bind TRIP6 as well as NHERF2 and these proteins bind each other as well. Additionally, NHERF2 forms a homodimer with another NHERF2 molecule. This complex enhances and prolongs the activation of ERK1/2 and Akt, mediators of cell survival and proliferation. Furthermore, LPA_2 binds the Siva-1 transcription factor through its C-terminal. Siva-1 is a pro-apoptotic factor, downstream of p53 which, when bound to LPA_2 in a ligand-activated mechanism gets polyubiquitinated and thus degraded in the proteasome. By increased impairment of

Siva-1, LPA_2 impedes the pro-apoptotic answer of the cell, triggered by p53, upon DNA-damage, for instance in case of cytotoxic chemotherapeutical agents (16).

Additionally to LPA, S1P may also contribute to the development of chemo- and radioresistance. In case of colorectal cancer, overexpression of SK1 was correlated with intrinsic or acquired cetuximab-resistance. Conversely, inhibition of S1P GPCRs, by FTY720, sensitized this type of cancer to cetuximab (388). Excessive expression of SK1 was also associated with imatinib- and nilotinib-resistance in chronic myeloid leukemia, via inhibition of protein phosphatase 2A by $S1P_2$. Interestingly, application of Fingolimod, but not that of Fingolimod-phosphate, restored imatinib-sensitivity in chronic myeloid leukemia (389). The aforementioned phenomenon could be the result of a direct interaction between FTY720 and protein phosphatase 2A, as $S1P_2$ is the only S1P receptor on which FTY720 has minimal or no effect (337). Likewise, silencing SPP1, an S1P metabolizing enzyme, by miR-95, enhanced resistance against radiation in breast and prostate cancer cells (390). Furthermore, high expression of $S1P_3$ was correlated with tamoxifen-resistance in human breast cancer cells (391).

This short summary highlights the potential of both lysophospholipid mediators to point out future drug targets, however further intensive research of the field is inevitable to gain a clear view of the roles of certain receptors and their signal transductional pathways.

2.4.5. Lysophospholipids in the cardiovascular system

The early reports by Tokumura and colleagues in the late seventies already established a role of importance for LPA in the cardiovascular system (3, 4, 392). Since then, LPA emerged as a key player in multiple aspects of vascular pathology. The other lysophospholipid S1P and its receptors also proved to have physiologically relevant interactions with the cells of the circulatory system.

2.4.5.1. Vascular development

The roles of both mediators in developmental steps of the vasculature have been addressed widely earlier in the chapter discussing ATX and the lysophospholipid receptors.

2.4.5.2. Atherosclerosis and atherothrombotic events

The first investigation that associated LPA with atherosclerosis derives from Siess and colleagues back in 1999, when they described the accumulation of LPA in human atherosclerotic plaques (67). Since that time, LPA has been described to affect almost every cell type involved in this process (393).

LPA acts on the endothelium, and so enhances cell migration, and upregulates the expression of adhesion molecules, such as intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) (393, 394). Secretion of chemokines like CXCL1 and CCL2 also increases (393). LPA is hypothesized to have a biphasic role, as it promotes the secretion as well as transcription of CXCL1 in an $LPA_{1/3}$- and NF-κB-dependent manner, respectively (395). Furthermore, LPA promotes endothelial permeability in an LPA_1-dependent manner, as mice deficient in LPA_1 showed decreased vascular leakage in a bleomycin-induced lung injury model (193). However, other studies supported the role of LPA in stabilizing the endothelial barrier function (393).

Early stages of atherosclerosis include the migration and dedifferentiation of VSMCs (396). LPA has been shown to promote migration as well as this shift from contractile to pro-inflammatory, secretory phenotype and to enhance SMC proliferation via LPA_1, including $G\alpha_i$, $G\alpha_q$, PKC, ERK1/2, PI3K/Akt and MAPK cascades (393). LPA_1, $G\alpha_q$, $G\alpha_i$ and MAPK also mediate the migratory effect of LPA (393). At the same time, the activation of LPA_1 upregulates the expression of pro-inflammatory cytokines IL-6, CCL2 and facilitates the production of NADPH-oxidase-dependent reactive oxygen species (ROS) (393). Besides, downregulation of the contractile proteins occur, in an LPA_3-depednent pathway (397, 398). On the other hand, activation of the intracellular LPA receptor PPARγ attenuates neointima formation after vascular injury (393), whilst inhibition of LPA_3 by Ki16425 diminished neointimal hyperplasia after carotid wire injury. Unsaturated LPA species mobilized smooth muscle progenitor cells from bone marrow in a pathway linked to CXCL12. This process could be interrupted by silencing either LPA_1 or LPA_3 (399).

LPA also influences monocytes, recruitment of which into the vessel wall is a crucial step in plaque formation (400). It enhances mox-LDL-uptake of monocytes/macrophages, and expression of pro-atherogenic IL-1β in murine

macrophages. Upregulation of the scavenger receptor A via $LPA_{1/3}$ intensifies lipid accumulation in these cells. Activation of PPARγ in monocytes increases the expression of other scavenger receptor CD36 (393). While deletion of PPARγ in macrophages promoted atherosclerosis. LPA on one hand evokes monocyte migration, and inhibits reverse transmigration, which results in an entrapment of monocytes in the plaque (393).

It is noteworthy, that LPA may also have a role in acute atherothrombosis on basis of ruptured plaques. Activated platelets bind ATX via β3 integrins and thus facilitate LPA production from LPC as discussed previously. Although thrombocyte activation has been implicated to be a major source of local LPA production, the exact mechanism is still obscure.

Former studies suggested a multistep process, in which intracellular- and secreted PLA enzymes produce the precursor for ATX (mainly LPC), which binds to the platelets and generates LPA; however, the exact PLA isoenzyme was still lacking (393). Bolen and colleagues described a new PLA, secreted from activated platelets. The enzyme, acyl-protein thioesterase 1, also known as lysophospholipase A-I has PLA_1 activity, thus produces sn2 lysophospholipids, which then undergo acyl-migration as previously mentioned. The sn1 lysophospholipids generated this way are well-known substrates of ATX (401) and explain the dominance of 18:2 and 20:4 molecular species of LPA in serum as these fatty acids are in the sn2 position of phospholipids.

However, the role of two other PLA enzymes also occurred. Group II $sPLA_2$ and lipoprotein-associated PLA_2 are enzymes implicated in chronic inflammation and produce LPC. Varespladib, an inhibitor of the former as well as darapladib, which inhibits the latter, reduced atherosclerosis in mice (402). These enzymes, however, seem to play a role in the chronic process of plaque building and not in acute thrombotic events.

Furthermore, LPA was not only associated with platelet activation as a product, but itself was also assumed to elicit thrombocyte activation. In accordance, human and cat platelets are activated by LPA, while that of rodents are not (403, 404). Moreover, murine platelets are inhibited by LPA (54). In support of this hypothesis, ATX overexpression in adult mice evokes hemorrhages, whereas mice heterozygote for ATX, that have a plasma concentration of LPA approximately 50% of that in WT, develop

thromboses more often (54). Furthermore, the thrombocytes of 20% of the healthy human population failed to respond upon LPA-stimulation (405). Further studies found, that LPA-induced platelet aggregation was ADP-dependent, which displayed, that LPA itself does not cause thrombocyte activation, but plays a role in the shape-change of platelets in an ADP- or other platelet activator-dependent manner (406). In support of this, LPA has been shown not to act on $G\alpha_i$ in thrombocytes, which is the initial step in their activation (393). LPA-induced shape-change has been described to be $G\alpha_{12/13}$-dependent, through which LPA activates Rho, ROCK, and the actomyosin system on the one hand and the LIM-kinase-1 cascade on the other hand (393). As for the receptors involved, human platelets express all known LPA GPCRs with LPA_4 and LPA_5 in the highest amount (285, 407, 408). First, only indirect evidence suggested a role for either of these receptor, as alkyl-analogues of LPA were more potent than acyl ones, a feature typical for LPA_5 (393). A further investigation by Kandoga and colleagues showed, that knockdown of LPA_5, but not that of LPA_{1-4} or LPA_6 inhibited LPA-mediated shape-change in human megakaryocytic cell lines (278). As for LPA_4, it is hypothesized, that it would be responsible for the LPA-mediated inhibition in rodents and in that 20% of the human population, whose platelets do not respond to LPA (408).

There are two studies of human subjects from the same group, which bind LPA directly to acute coronary syndrome (ACS), one of the fatal consequences of atherosclerosis (409, 410). In the former paper it was reposted, that circulating plasma LPA levels increase in patients with ACS compared to patients with stabile angina pectoris or angiographically normal coronary arteries (409). In the latter publication, a higher LPA level was found at sites in culprit coronary arteries than in the peripheral circulation of patient with ACS (410). Although, these experiments exhibit a potential biomarker role for LPA in ACS, they are difficult to interpret, because no precautions were taken to inhibit in vitro LPA generation during sample handling, and LPA levels in healthy subjects were higher than previously reported by others (393).

The putative role of S1P in atherogenesis was identified early, as plasma S1P is largely bound to HDL, a well-known atheroprotective factor. Although S1P has been extensively studied in this context, it could not be established as either a pro- or an anti-atherogenic mediator till today. S1P also affects nearly all cell types involved in plaque formation (17).

In endothelial cells, S1P was found to suppress IL-8 and CCL2. Furthermore, S1P inhibited VCAM-1, a key adhesion molecule, mediating monocyte invasion into the vessel wall. In contrast, additional studies described enhanced VCAM-1 and E-selectin expression upon S1P-treatment. It is noteworthy, that opposite effects occurred upon application of different concentrations of S1P. While micromolar concentrations increased, nanomolar ones lowered the expression of the aforementioned adhesion molecules (411). Early studies reported, that S1P improves endothelial barrier function by facilitating adherens junction formation via activation of $S1P_1$ (412). The possible role of $S1P_3$ has been proposed, however, this issue is still under debate (412). In contrast, $S1P_2$ proved to increase endothelial permeability, acting on Rho-ROCK and PTEN (411). Nonetheless, the net effect of S1P on vascular permeability is rather an enhancement of its barrier function, as mice deficient in plasma S1P exhibited vascular leakage, a feature could be restored by either transfusion of WT type red blood cells or application of an $S1P_1$ agonist. Besides, SK1 global KO mice also suffer from vascular leakage, however in a less extent (411). Furthermore, barrier-enhancing functions of activated protein C proved to be at least partially S1P-dependent, and an $S1P_1$ agonist can rescue mice from PAF-evoked general vascular leaking (411).

Three independent studies investigated the therapeutic potential of FTY720 in atherosclerosis, two of which concluded attenuation in plaque-formation in two distinct established mouse models of the disease (413, 414) and in a third one, Fingolimod had no effect, although evoked hypercholesterinemia (415). The interpretation of these studies is however difficult, considering the wide range of effects FTY720 has on S1P receptors and producing enzymes, addressed previously in detail.

Experiments conducted with S1P receptor KO mice, however, contradict with the former results. $S1P_2$ KO animals on ApoE KO background showed clearly reduced plaque burden, decreased macrophage density and increased VSMC content of the plaques. Bone-marrow transplantation studies pointed out, that $S1P_2$ receptor located on macrophages are responsible for the aforementioned effect. Absence of $S1P_3$ had no direct influence on atherosclerosis, though it alleviated monocyte/macrophage content of the lesions (411).

Finally, S1P also has an ambiguous effect on cytokine production, with $S1P_1$ on the inhibitory and $S1P_2$ on the promoting side (411).

As seen from the observations listed above, while LPA actions can be concluded pro-atherogenic, with $LPA_{1/3}$ signaling in plaque formation and LPA_5 activation in thrombocyte shape-change, the role of S1P in atherogenesis is far from being clear, as $S1P_1$ signaling seems to be anti-atherogenic, and $S1P_2$ is pro-atherogenic on the other hand.

2.4.5.3. Cardiac functions

In contrast with LPA, the role of S1P in functions of the heart has been extensively studied, hence its effect on this organ should be addressed separated and in more detail than that of LPA. S1P influences heart development, highlighted by the fact, that cardiomyocyte-specific $S1P_1$ KO mice exhibit ventricular septal defects (416). Besides, S1P was found to be involved in ischemia-reperfusion injury, in which it would be a protective factor. In a porcine ischemia-reperfusion injury model, treatment with FTY720 increased myocardial salvage and reduced adverse post-injury remodeling. Furthermore, cardiomyocytes lacking SK1 proved to be more sensitive to hypoxia than WT ones, manifested in a greater extent of cell-death. Treatment with exogenous S1P could however, increase the survival of KO as well as WT cardiomyocytes. Moreover, hearts of SPL KO mice exhibited higher S1P levels and smaller infarct sizes following myocardial ischemia (417).

Multiple studies point to a cross-talk mechanism between $S1P_1$ and β_1-adrenergic signaling, in which $S1P_1$ agonism counteracts β_1-activation, decreasing contractility. At first sight, this effect may seem to be deleterious for the heart in a post-ischemic condition. The level of circulating catecholamines is increased in this set up though, and that would lead to an excessive activation and rapid desensitization of β_1 receptors. Hence, this opposing effect of S1P can be beneficial under these circumstances. Moreover, $S1P_1$ action was reported to be modulated by β_3 activation in cardiomyocytes, a mechanism of interest, which may have a role in preventing post-ischemic heart failure (417).

In addition, $S1P_1$, $S1P_2$, and $S1P_3$ may also contribute to the protection of the heart. Although $S1P_2$ and $S1P_3$ KO animals showed similar sizes of infarcts upon ischemia to WT ones, $S1P_{2/3}$ double KO mice exhibited infarct sizes increased more than 50% (261).

Furthermore, the role of S1P also occurred in the regulation of the hearth rate. Studies conducted by Bünemann and colleagues in the mid 90's revealed that in isolated guinea pig atrial myocytes S1P and SPC activate the G protein-coupled inwardly rectifying potassium channel in a GPCR-dependent manner, the channel also targeted by the M_2 ACh receptors upon parasympathetic stimuli. This effect of S1P and SPC is mediated by $G\alpha_i$, as it can be suppressed by PTX. S1P and SPC showed homologue desensitization, implicating that the two mediators act on the same receptor, which is a distinct one of M_2, as carbachol could not desensitize their effect (418, 419). In addition, Liliom and colleagues found that guinea pig atrial myocytes express $S1P_{1\text{-}3}$, and $S1P_5$, and that SPC has a negative chronotropic effect on perfused guinea pig hearts (147). Moreover, bradycardia is a recognized adverse effect of Fingolimod, applied in multiple sclerosis. This effect was lacking in mice deficient in $S1P_3$ (420). However, data acquired in rats (421) and humans (422) supported a participation of $S1P_1$ in this process.

Lastly, changes in circulating S1P levels were also investigated in coronary artery disease and acute myocardial infarction. Reduction in S1P levels were reported in humans with acute myocardial infarction (423). The same was observed in case of post-ischemic heart failure in rodents. The bulk of S1P in plasma, is however, bound to HDL, the level of which is also altered in these conditions (417). Algraves and colleagues found that circulating S1P, dihydro-S1P and C24:1-ceramide levels in HDL were inversely correlated with the incidence of ischemic heart disease (424).

2.4.5.4. Regulation of the vascular tone

The initial report on LPA by Tokumura described, that intravenous administration of LPA elicited hypertension in rats and guinea pigs but the same molecular species proved to be hypotensive in cats and rabbits (4). This hypotensive effect in cats was attributed to a decreased cardiac output, which was a result of pulmonary vasoconstriction upon platelet aggregation (404). Intravenous application of LPA elevated the mean arterial pressure in LPA_1 KO, LPA_2 KO and $LPA_{1/2}$ double KO mice as well as in LPA_4 KO and smooth muscle specific PPARγ KO animals. The same effect was also observed in LPA_3 KO animals (425). A recent report by Kano and colleagues described a hypertensive response upon intravenous LPA application in mice. This effect was alleviated in absence of LPA_4 or LPA_6 or upon the application of

the ROCK inhibitor Y-27632. However, LPA_6 KO mice also showed attenuated vasoactive responses upon adrenergic stimuli and exhibited abrupt vessel formation (295).

LPA applied on the extraluminal surface of porcine pial arteries in a cranial window set-up elicited a dose-dependent vasoconstriction (426). This effect was $G\alpha_i$ dependent, because it could be inhibited by PTX. Because LPA receptors were undiscovered that time, the receptor responsible for the effect remained obscure. Furthermore, intrathecal application of autologous blood or Endothelin-1, as a model of subarachnoidal hemorrhage, raised the concentration of LPA in the cerebrospinal fluid into the vasoactive range (427).

The endothelium and eNOS play key roles in regulation of the vascular tone. LPA was shown to activate eNOS and thus elicit NO generation in endothelial cell cultures (428-430), however 24 h incubation of porcine coronary endothel cells with LPA downregulated the transcription of eNOS (431). In bovine aortic endothelial cell (BAEC) culture, LPA-induced eNOS activation was mediated by PI3K (429), nonetheless, in an other study, conducted also on BAEC, inhibition of PI3K had no effect on eNOS activity (430). These investigations, however, should be treated with caution as endothelial cell properties may vary on experimental setup (432, 433). Expressional profile of BAEC changes in a great extent under cultured conditions (434). A report also pointed out the lack of glycocalyx in cultured HUVECs; however, it mediates important physiological interactions (435). Endothelium-dependent flow-stimulated NO production is a key regulator of the tone of resistance arterioles in mammals (436). A recent study reported, that in adipose tissue arterioles of human subjects with coronary artery disease, LPA elicits a shift in the mediator of flow-induced dilation from NO to mitochondria-derived H_2O_2 in an LPA_1-dependent manner (437). LPA, on the other hand, was also shown to induce endothelium-dependent vasoconstriction if shear stress was present (438).

The effects of S1P on the vascular tone have been studied broadly. An early report described S1P as a constrictor in basilar artery of dogs in a Rho-ROCK-dependent manner (439). In vivo, injected into the cysterna magna, S1P elicited a contraction of duration lasting for approximately 2 days. Since then, this effect has been confirmed in multiple species in various vascular beds. However, in larger species S1P proved to be

potent only in smaller arteries (cerebral, mesenteric) with moderate (coronary) to no effect (aorta, femoralis) in larger ones (440). In mice, the efficacy was in negative correlation with size (440). Further, in mice S1P caused $S1P_3$-dependent contraction of the basilar artery (441). A study, conducted in human chorionic plate arteries, found, that the outcome also depends on the experimental setup (isometric vs. isobar mounting) (442). Pharmacological investigations confirmed the S1P-elicited vasoconstriction to be $S1P_3$-dependent in basilar and coronary arteries (411, 440).

Thorough investigations of $S1P_2$ KO mice revealed interesting results though. These animals exhibited a decreased vascular tone; however, this had no influence on blood pressure. Furthermore, blood flow of the renal and mesenteric areas was elevated with consequent smaller vascular resistance. Additionally, responses of the vessels to α-adrenergic stimuli were also decreased (252). The subjects also showed a more severe drop in blood pressure in an anaphylactic test (443).

Receptor-independent constrictive actions of S1P were also reported (411). Vessels deficient in SK1 showed reduced contractile responses to KCl, S1P, or the thromboxane receptor agonist U46619 compared to WT, $S1P_2$ KO or $S1P_3$ KO arteries (411). Besides, S1P can activate store-operated calcium entry through receptor-dependent and independent pathways (444).

Interestingly, S1P was found to activate eNOS and thus promote NO release in rodent aortic rings, which proved to be mediated via $S1P_3$ at least in mice (445, 446). A possible contribution of $S1P_1$ also occurred, however the lack of highly selective antagonists makes these results disputable (440). On the other hand, S1P elicited eNOS activation in COS-7 cells, transfected with eNOS and $S1P_1$ in an $S1P_1$-dependent manner (447). Furthermore, S1P elicited eNOS activation in BAEC (448). Moreover, VEGF was shown to increase $S1P_1$ expression in aortic endothelial cells, and pretreatment of isolated vessels with VEGF enhanced S1P-dependent vasodilation (449). In rat mesenteric arterioles, S1P-induced dilatation was inhibited by PTX, and the PI3K inhibitor wortmannin. Likewise, S1P can activate eNOS via AMP-activated protein kinase. Nonetheless, the $S1P_1$ agonist SEW2871 failed to induce any relaxation in basilar, femoral or mesenteric arteries of rats (440).

In summary, LPA as well as S1P have a multitude of actions in the cardiovascular system, which may provide potential drug targets and/or biomarkers for the future,

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however in both cases, the receptor-dependency is mostly unsure, which require further investigations of this topic, by which millions of patients are affected worldwide.

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3. Aims of the study

The brief summary of the last chapter showed that both LPA and S1P play several, physiologically significant roles in the cardiovascular system. As for the regulation of the vascular tone, a large amount of data is available for both mediators. Constrictor and dilator responses have been reported in case of both lysophospholipids. Nonetheless, these results and especially the receptor-dependency of the described effects are unclear and often inconsequent.

Accordingly, in the present study we addressed the following questions:

1. Are there any EDG LPA receptor-mediated vasoactive actions?
2. If yes, are they endothelium-dependent or independent?
3. Which receptors and underlying signaling pathways may be involved?
4. Does the activation of the S1P GPCRs mediate any vasoactive processes?
5. If yes, are they dependent of the endothelium?
6. Which receptors and downstream pathways participate?

4. Materials and Methods

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the National Scientific Ethical Committee on Animal Experimentation (PEI/001/2706-13/2014).

4.1. Animals

C57BL/6 and eNOS KO mice were obtained from Charles River Laboratories (Isaszeg, Hungary). C57BL/6 mice are referred to as WT in the text and figures. All transgenic mouse lines were on C57BL/6 genetic background. Mice deficient in LPA_1 or LPA_2 receptors (LPA_1 KO and LPA_2 KO, respectively) were generated as previously described (192, 202, 326, 450). Cyclooxygenase-1 KO (COX1 KO) mice were from Dr. Ingvar Bjarnason (Department of Medicine, Guy's, King's College, and St. Thomas' School of Medical Education, London, UK). Thromboxane prostanoid receptor-deficient (TP KO) mice were kindly provided by Dr. Shuh Narumiya (Kyoto University, Kyoto, Japan). The smooth muscle-specific $G\alpha_{q/11}$ and $G\alpha_{12/13}$ deficient mice ($G\alpha_{q/11}$ KO and $G\alpha_{12/13}$ KO respectively) and their respective controls ($G\alpha_{q/11}$ CTRL and $G\alpha_{12/13}$ CTRL), were generated as described (451). Mice deficient in $S1P_2$- and $S1P_3$ receptors ($S1P_2$ KO, $S1P_3$ KO) and their controls were kindly provided by Dr. Richard L. Proia (National Institute of Diabetes and Digestive and Kidney Disease, NIH, Bethesda, USA). In experiments performed with LPA_1 KO, LPA_2 KO or COX1 KO mice, wild-type animals from the same strain served as controls and are referred to as LPA_1 CTRL, LPA_2 CTRL and COX1 CTRL, respectively. Because the TP mice have been maintained in our animal facility with KO x KO mating, WT C57BL/6 mice served as controls (TP CTRL). PTX was administered intraperitoneally in some of the animals for 5 days prior to the experiments in a dose of 50 μg/kg body weight in order to inhibit G_i proteins (452, 453).

4.2. Preparation of Vessels

Adult male animals were perfused transcardially with 10 mL heparinized (10 IU/mL) Krebs solution under deep ether anesthesia as described previously (454). The aorta was removed and cleaned of fat and connective tissue under a dissection microscope (M3Z, Wild Heerbrugg AG; Gais, Switzerland) and immersed in a Krebs solution of the

following composition (mM): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2 \cdot 2\ H_2O$, 1.2 $MgSO_4 \cdot 7\ H_2O$, 20 $NaHCO_3$, 0.03 EDTA, and 10 glucose at room temperature and pH 7.4. Abdominal and thoracic aortae were cut into ~3 mm-long segments and mounted on stainless steel vessel holders (200 μm in diameter) in a myograph (610 M multiwire myograph system; Danish Myo Technology A/S; Aarhus, Denmark). In certain experiments special care was taken to preserve the endothelium of the segments, in all other cases the endothelium was removed intentionally by gently rotating the segments on the holder pins and mechanical ablation with surgical thread. Integrity or absence of the endothelium was confirmed by the presence or lack of ACh-induced vasorelaxation respectively. Thoracic aortae were also cut into segments and subjected, with the endothelium preserved, to thromboxane B_2 ELISA as described below in detail.

4.3. Myography

Chambers of the myographs were filled with 6 mL gassed (95% O_2–5% CO_2) Krebs solution. The vessels were allowed a 30-min resting period, during which the bath solution was warmed up to 37 ^oC and the passive tension was adjusted to 10 mN in case of abdominal and to 15 mN in case of thoracic segments, which was determined to be optional in a previous study (454). Subsequently, the tissues were exposed to 124 mM K^+ Krebs solution (made by isoosmolar replacement of Na^+ by K^+) for 1 min, followed by several washes with normal Krebs solution. A contraction evoked by 10 μM phenylephrine (PE) followed by administration of 0.1 μM ACh served as a test of the reactivity of the smooth muscle and the endothelium, respectively. After repeated washing, during which the vascular tension returned to the resting level, the segments were exposed to 124 mM K^+ Krebs solution for 3 min in order to elicit a reference contraction. Subsequently after a 30-min resting period, increasing concentrations of PE (0.1 nM to 10 μM) and ACh (1 nM to 10 μM) were administered to determine the reactivity of the vessel and to verify the integrity or the proper denudation of the endothelium. We proceeded with a 30-min resting period. Thereafter, we followed three distinct protocols, depending on the aim and setup of the given experiment.

4.3.1. Protocol for testing vasoactive effects in precontracted vessels

Thoracic vessels were precontracted to 70–90% of the reference contraction by an appropriate concentration of PE, and after reaching a stabile plateau, the effect of either

the LPA_{1-3} agonist VPC31143 (455) in a concentration of 10 μM or that of S1P in 5 μM was determined in vessels of different genetic background. Vasoconstrictions were normalized to the reference contraction induced by 124 mM K^+, whereas vasorelaxations were expressed as percentage of the precontraction produced by PE

4.3.2. Protocol for testing vasoactive effects on resting tone

In this type of protocols, the vessels were exposed to either 10 μM VPC31143 or different concentrations of the LPA_3 agonist T13 (456) or 5 μM S1P at the resting tone. In some experiments, the $LPA_{1\&3}$ receptor antagonist Ki16425 (457) or the selective LPA_3 antagonist diacylglycerol pyrophosphate (DGPP) (458) was applied to the bath chambers at a concentration of 10 μM, 30 min prior to the administration of VPC31143. Vasoconstrictions are expressed as percentage of the reference contraction induced by 124 mM K^+.

4.3.3. Protocol for testing the long-term vasoactive effects of S1P

In these experiments, we investigated the potentiating effect of S1P on an α_1 agonist-induced contraction in thoracic vessels. Vasoconstrictions were elicited in every 20 minutes by repeated administration of PE. Mean of the first three contractions served as reference and was considered as 100%. After the third administration of PE, we incubated the vessel with either S1P in a concentration of 5 μM, or its vehicle 0.3 N sodium hydroxide (NaOH). Subsequently, PE was applied every 20 minutes for three hours after the incubation. Vasoconstrictions are expressed as percentage of the mean of the three contractions, evoked before the incubation.

4.4. Quantification of Vascular Thromboxane A_2 Release

Thoracic aortae were cut into 5 segments and allowed a 2-h resting period. In some of the experiments, 3 μg/mL PTX was applied for 2 h in order to inhibit G_i (459). Thereafter, the vessels were incubated in 200 μL Krebs solution at 37°C for 2 min to obtain a baseline level of TXA_2 release. After the incubation, the supernatant was replaced with 200 μL of Krebs solution containing 10 μM VPC31143 and incubated for 2 min. Supernatants of the resting and the VPC31143-stimulated vessels were snap-frozen and stored at -80°C until the measurement of thromboxane levels. Concentrations of thromboxane B_2 (TXB_2), a non-enzymatically produced stable metabolite of TXA_2,

were determined using a TXB_2 EIA kit, purchased from Cayman Chemical Co. (Ann Arbor, MI, USA; Cat. No.: 501020). TXB_2 production was calculated as pg/min. Vessels with a baseline production of TXB_2 higher than 20 pg/min were considered pre-activated and were excluded from the experiment.

4.5. Expression Analysis of LPA and S1P Receptors in VSM

Endothelium-denuded thoracic and abdominal aortae were isolated, and the adventitia of the vessels was carefully removed under a dissection microscope. Thereafter the vessels were fast-frozen and stored at -80°C until PCR analysis. RNA was isolated from VSM with the RNeasy Micro kit (Qiagen, Valencia, CA, USA; Cat. No. 74004), and RNA concentration and quality were assessed with Nanodrop (Thermo Fischer Scientific; Waltham, MA, USA). Up to 500 ng total RNA was converted to cDNA using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen; Carlsbad, CA, USA; Cat. No.: 11754050).

Assessment of mRNA expression was performed by quantitative real-time PCR using cDNA corresponding to 20 ng RNA template. PCR reactions were carried out in triplicate with 300 nmol of each primer in a final volume of 25 μL of 2 x Maxima SYBR Green/ROX qPCR master mix (Thermo Fischer Scientific; Cat. No. K0223). Amplification was performed after one initial step of 10 min at 95°C for 40 cycles at 94°C /15 s and 60°C /60 s with a StepOnePlus real-time PCR system (Applied Biosystems; Carlsbad, CA, USA). Relative gene expression of each mRNA to GAPDH was determined using the dCt method. The primer sequences of LPA GPCR are listed in Table 1. The primers used for expression analysis of S1P receptors were manufacturer designed TaqMan probes (Thermo Fischer Scientific).

Table 1. Primers used for quantitative real-time PCR, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, A: Adenine, T: Thymine, G: Guanine, C: Cytosine, LPA_{1-6}: LPA_{1-6} Receptors

	Primers, 5'-3'	
Gene	**Forward**	**Reverse**
GAPDH	CTGCACCACCAACTGCTTAG	GGGCCATCCACAGTCTTCT
LPA_1	CACCATGATGAGCCTTCTGA	GCAGCACACATCCAGCAATA
LPA_2	CCAGCCTGCTTGTCTTCCTA	GTGTCCAGCACACCACAAAT
LPA_3	AGGGCTCCCATGAAGCTAAT	TGCACGTTACACTGCTTGC
LPA_4	ACAGTGCCTCCCTGTTTGTC	AAATCAGAGAGGGCCAGGTT
LPA_5	TCATCATCTTCCTGCTGTGC	ATCGCGGTCCTGAATACTGT
LPA_6	TCGCTCATGAGGACACAGAC	CAAAGCAGCAGTTGGAAACA

4.6. Reagents

LPA (18:1) and VPC31143 were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and dissolved in saline immediately before administration. DGPP was purchased from Avanti Polar Lipids and dissolved in methanol. Ki16425 was purchased from Cayman and dissolved in DMSO to make a 100-fold concentrated stock solution. In these experiments, vehicle treatment served as control. PTX was purchased from List Biological Laboratories, Inc. (Campbell, CA, USA) and dissolved in glycerol. T13 was synthesized as described previously (456) and was dissolved in PBS containing 0.1 % fatty acid free bovine serum albumin. Sphingosine 1-phosphate was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) and dissolved in 0.3 N NaOH before administration. All other drugs and chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA). In myography experiments, all concentrations are expressed as the final concentration in the organ bath.

4.7. Data Analysis

An MP100 system and AcqKnowledge 3.72 software from Biopac System Inc. (Goleta, CA, USA) were used to record and analyze changes in the vascular tone. All data are presented as mean ± SE, and *n* indicates either the number of vessels tested in myography experiments or the number of animals tested in the case of TXB_2 EIA or qPCR. Statistical analysis was performed using the GraphPad Prism software v.6.07 from GraphPad Software Inc. (La Jolla, CA, USA). Student's unpaired *t* test was applied when comparing two variables, whereas all other comparisons between the different experimental groups were made by ANOVA followed by either Tukey's or Bonferroni's post hoc test. A *p* value of less than 0.05 was considered statistically significant.

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5. Results

5.1. Activation of EDG-like LPA receptors induce endothelium-dependent and -independent changes of the vascular tension

In order to evaluate the potential vascular effects of LPA EDG-like GPCRs, the LPA_{1-3} agonist VPC31143 was administered to WT thoracic aortic segments after PE-induced precontraction. VPC31143 elicited a marked vasorelaxation (Figures 8A and 9A). Dose-response relationship of this effect is shown in Figure 9B. The agonist-induced vasorelaxation has an EC_{50} of 15 nM and an E_{max} of 51.9% expressed as percentage of the precontraction.

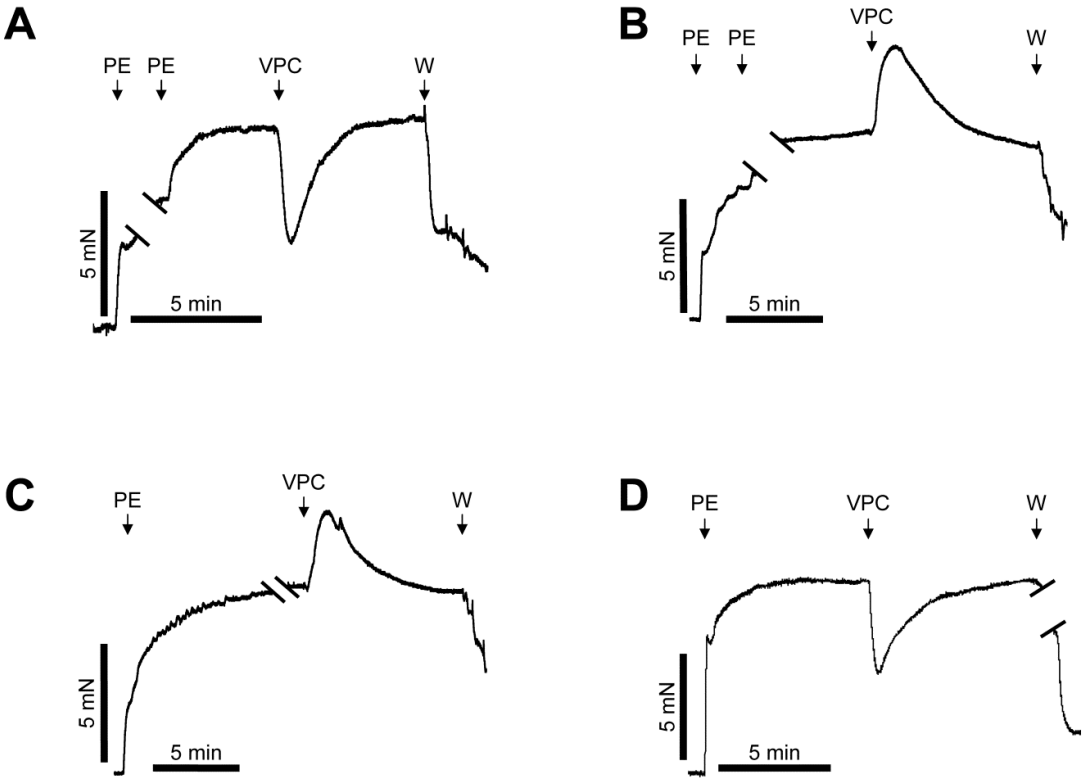

Figure 8. Effect of VPC31143 on the tone of precontracted thoracic aortae. Representative recordings of vessels prepared from wild type (WT) mice with intact (A) or denuded (B) endothelium as well as vessels from endothelial nitric oxide synthase (eNOS) KO (C) and cyclooxygenase-1 (COX1) KO (D) mice. VPC31143 was applied at 10 μM after phenylephrine (PE)-induced contraction reached a stable plateau. Horizontal and vertical bars indicate 5 min and 5 mN, respectively. PE and VPC denote the application of phenylephrine and VPC31143 respectively, while W stands for washing of the organ chamber with fresh Krebs solution.

In order to analyze the mechanism of VPC31143-evoked relaxation the possible role of endothelium-derived relaxing agents was assessed first. To do so, we tested WT

vessels, mechanically denuded of endothelium, in which the VPC31143-elicited relaxation was not only abolished but was converted to vasoconstriction (Figure 8B) indicating a major role of endothelium-derived vasoactive mediators in the vasoactive effect of VPC31143. In order to identify the mediator(s) involved, vessels of either COX1 KO or eNOS KO mice were tested. Whereas the absence of COX1 did not alter the dilator effect of VPC31143 (Figure 8D), the lack of eNOS, similarly to that of the endothelium, prevented the VPC31143-elicited relaxation and turned it to contraction (Figure 8C). Taken together, these results indicate that the vasorelaxant effect of EDG-like LPA receptor activation is mediated by endothelial NO whereas COX1-derived prostanoid mediators are not involved (Figure 9A).

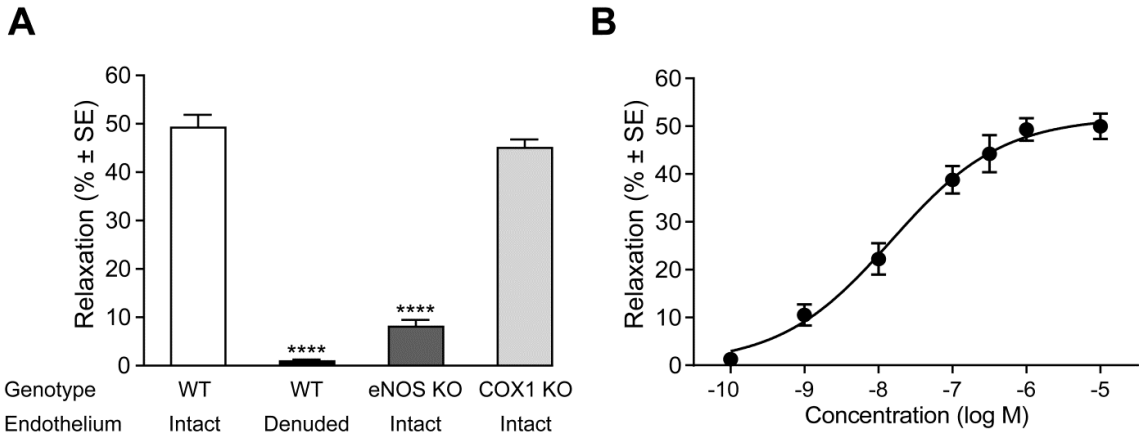

Figure 9. A) Endothelium-derived NO mediates the vasorelaxation induced by VPC31143. Effects of 10 μM VPC31143 in wild type (WT) vessels with intact (open bar) or denuded endothelium (solid bar) as well as in vessels of endothelial nitric oxide synthase (eNOS) KO (dark grey bar) and cyclooxygenase-1 (COX1) KO (light gray bar) mice. Absence of endothelium or eNOS but not that of COX1 abolished VPC31143 induced vasorelaxation. $^{****}P<0.0001$ vs. WT with intact endothelium; One-way ANOVA with Tukey's post hoc test; *n* = 11-28 B) Dose-response relationship of vasorelaxation induced by VPC31143 in WT thoracic aortic vessels with intact endothelium. Each dose has been tested in independent aortic segments in order to avoid receptor desensitization. E_{max} and EC_{50} values are 51.9% and 15 nM, respectively; *n*=7-29.

In the next phase of our study, we sought an in-depth analysis of the VPC31143-evoked vasoconstriction. In order to analyze the potential regional differences between the different parts of the aorta the agonist was applied to WT thoracic and abdominal aortic segments (TA and AA, respectively) at resting tension. In vessels with intact endothelium VPC31143 in a dose of 10 μM elicited moderate vasoconstriction compared to the reference contraction induced by 124 mM K^+ (Figures 10A, 10C and 11A).

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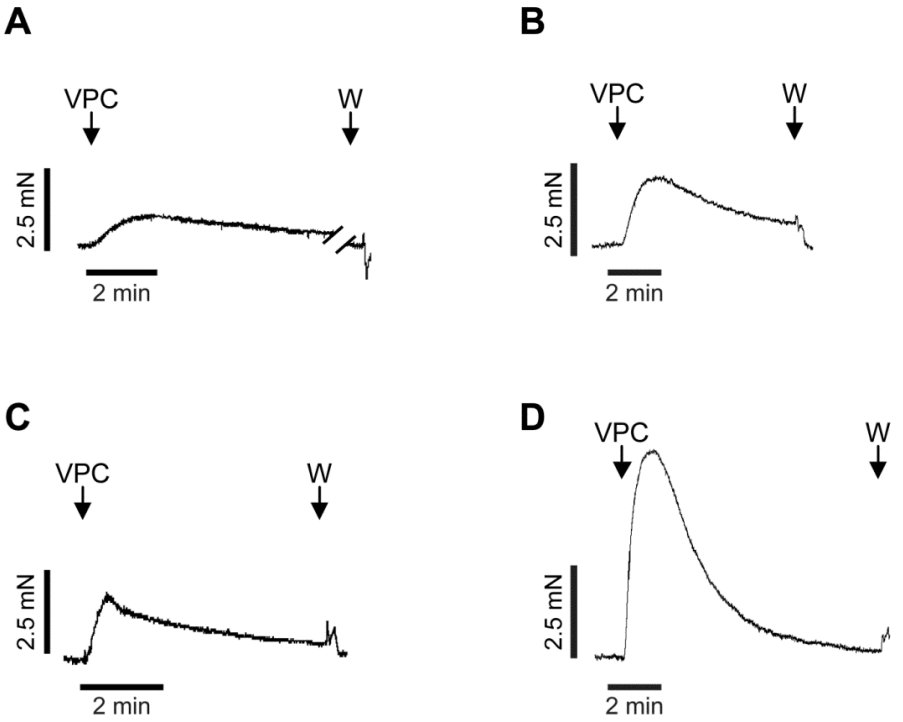

Figure 10. Representative recordings depicting the effect of VPC31143 in murine thoracic (TA) and abdominal aorta (AA) with intact endothelium (A and C respectively) and in TA and AA after endothelium-denudation (B and D, respectively). VPC denotes the application of VPC31143, while W stands for washing of the organ chamber with fresh Krebs solution.

However, removal of the endothelium unmasked the constrictor effect of VPC31143 resulting in an approximately three-fold increase in the AA (Figures 10B, 10D and 11A). Figures 11B shows the dose-response relationship of the contractile effect of VPC31143 in AA. The vasoconstriction has an EC_{50} of 4.1 μM and an E_{max} of 87.4% as compared to the reference contraction.

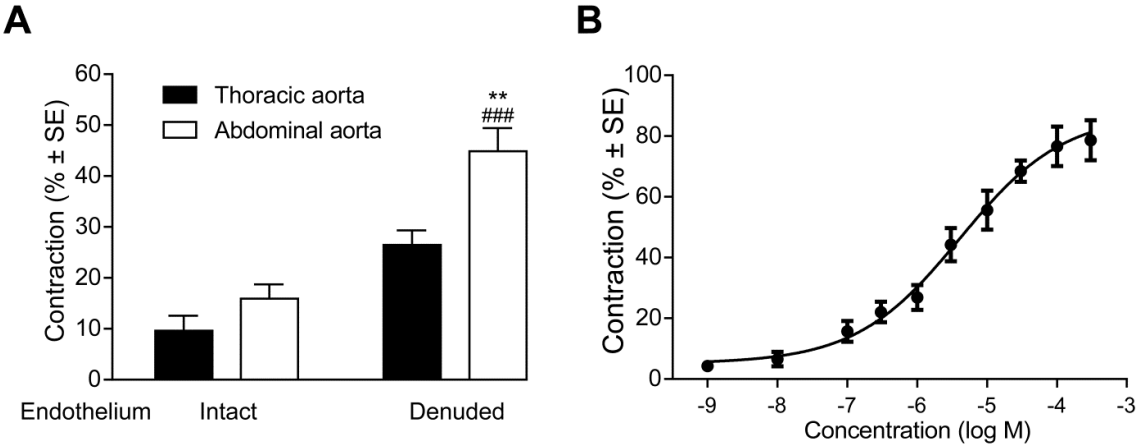

Figure 11. A) Removal of the endothelium enhances the VPC31143-elicited vasoconstriction in the abdominal aorta (AA). Effect of 10 μM VPC31143 in wild type (WT) thoracic aorta (TA) (solid bars) and AA (open bars) $^{**}P<0.01$ vs. TA with denuded endothelium; $^{###}P<0.001$ vs. AA with intact endothelium; Two-way ANOVA with Tukey's post hoc test *n*=8-37. B) Dose-response relationship of vasoconstriction

elicited by VPC31143 in WT AA vessels after the removal of the endothelium. E_{max} and EC_{50} values are 87.4% and 4.1 μM, respectively; *n*=4-30.

5.2. Identification of the LPA receptor(s) mediating VPC31143-induced vasoconstriction

Since removal of the endothelium augmented the vasoconstrictor effect of VPC31143, our attention turned to LPA GPCRs expressed in the vascular smooth muscle (VSM). First, the gene expression profile of LPA receptors was determined in isolated tunica media of the murine TA and AA. LPA_1, LPA_2, LPA_4, and LPA_6 mRNA were most abundantly detectable, with a slightly higher expression of LPA_4 and LPA_6 in the AA as compared to the TA (Figure 12). LPA_3 transcripts had the lowest abundance, nonetheless they found to be expressed in a higher amount in TA than AA. Taken together, these results identified LPA_1 and LPA_2 as likely candidates for mediating EDG-like LPA receptor related vasoconstriction.

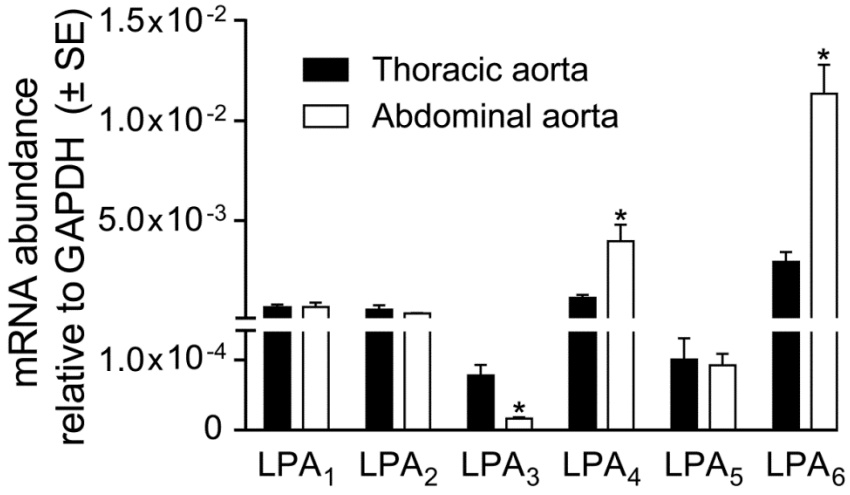

Figure 12. Expression profile of LPA receptors in freshly isolated tunica media of wild type murine thoracic (TA) and abdominal aortic (AA) segments, determined by quantitative PCR. Murine aortic vascular smooth muscle cells predominantly express LPA_1, LPA_2, LPA_4, and LPA_6. LPA_4 and LPA_6 proved to be more abundant in the AA whereas LPA_3, the least abundant subtype, showed higher expression in the TA. $^*P<0.05$ vs. TA; Student's unpaired *t* test; *n*=3-9. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Thereafter, we implemented pharmacological and genetic methodologies to determine the LPA receptor responsible for the vasoconstrictor effect (Figures 13 and 14). Whereas the $LPA_{1/3}$ antagonist Ki16425 revoked the 10μM VPC31143-elicited vasoconstriction, the selective LPA_3 antagonist DGPP failed to influence it as compared to vehicle treatment in the AA of WT mice. Aortic rings isolated from LPA_1 KO mice

failed to contract upon VPC31143 application, while the vessels of LPA_2 KO animals showed similar responses to those of WT, indicating that the vasoconstriction is mediated by LPA_1 (Figure 13).

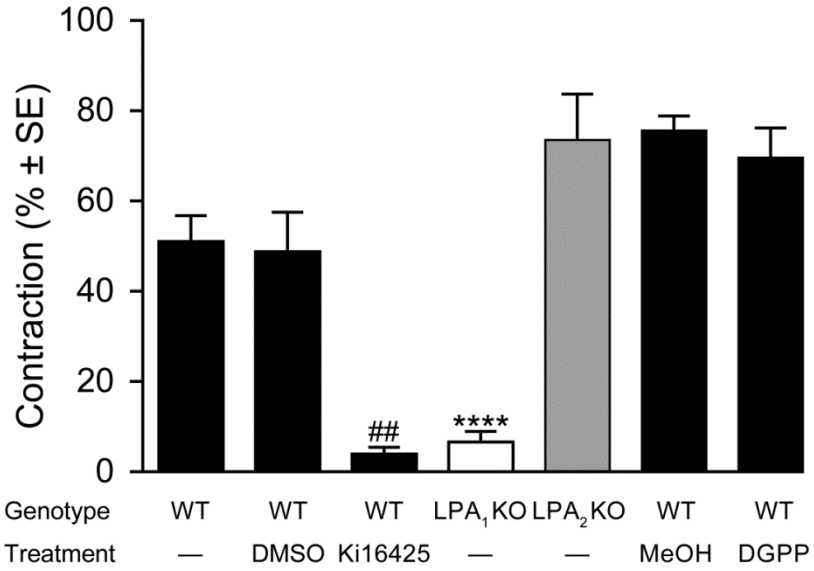

Figure 13. LPA_1 receptors mediate VPC31143-elicited vasoconstriction. Ki16425, an $LPA_{1/3}$ antagonist, and the lack of LPA_1 but not that of LPA_2 receptors nor the selective LPA_3 antagonist DGPP abolish VPC31143-evoked contraction. Segments of C57Bl/6, LPA_1 control (CTRL), and LPA_2 CTRL exhibited identical responses and are therefore pooled and referred to as WT in the figure. Both Ki16425 and DGPP were applied at 10 μM for 30 min before the administration of VPC31143. Vehicle-treated control vessels were exposed to 1% Dimethyl sulfoxide (DMSO) or methanol (MeOH). $^{****}P<0.0001$ vs. WT; $^{\#\#}P<0.01$ v. WT with DMSO treatment; One-way ANOVA with Tukey's post hoc test; *n*=5-23.

To obtain a more detailed evaluation of the possible involvement of LPA_3 in the vasoconstrictor response, the vasoactive effects of T13 were investigated, which had been implied to activate LPA_3 selectively and with high efficiency at 10 nM, however at higher concentrations, it stimulates other LPA GPCRs as well (456). T13, applied in 10 nM concentration, failed to induce vasoconstriction in AA segments of LPA_1 CTRL animals, however at higher concentrations a dose-dependent contractile response developed (Figure 14). This effect was absent in vessels isolated from LPA_1 KO animals, which is consistent with our hypothesis, that the vasoconstrictor response is solely mediated by LPA_1.

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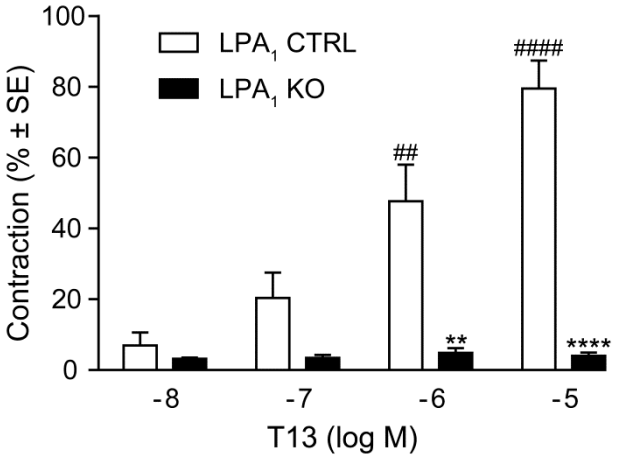

Figure 14. LPA_3 has no role in VPC31143-mediated contraction. The selective LPA_3 agonist T13 failed to evoke vasoconstriction in LPA_1 control (CTRL) vessels at 10 nM where it selectively activates LPA_3. The application of higher concentrations of T13, which are reported to stimulate LPA_1, resulted in dose-dependent contraction that was completely abolished by the lack of LPA_1. $^{**}P<0.01$, $^{****}P<0.0001$ vs. LPA_1 CTRL; $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$ vs. 10 nm LPA_1 CTRL; Two-way ANOVA with Tukey's post hoc test; *n*= 16-22.

5.3. Identification of the signal transduction pathways of LPA_1-mediated vasoconstriction

LPA signaling has been implicated to interact with the prostanoid system. COX1-mediated effects of LPA (460) and the fact that LPA-evoked contractions of the longitudinal smooth muscle layer of guinea-pig ileum were shown to be indomethacin-sensitive (461), raised our hypothesis, that TXA_2, a potent vasoconstrictor (462), might have a role in the contractile effect mediated by LPA_1. In order to investigate this possibility, vessels of WT, COX1 KO and TP KO mice were exposed to 10 μM VPC31143. The absence COX1 and TP markedly decreased the agonist-induced contractions (Fig. 15), implying that COX1-derived TXA_2 could be the mediator, that activates TP, thus elicits (or at least contributes to) vasoconstriction.

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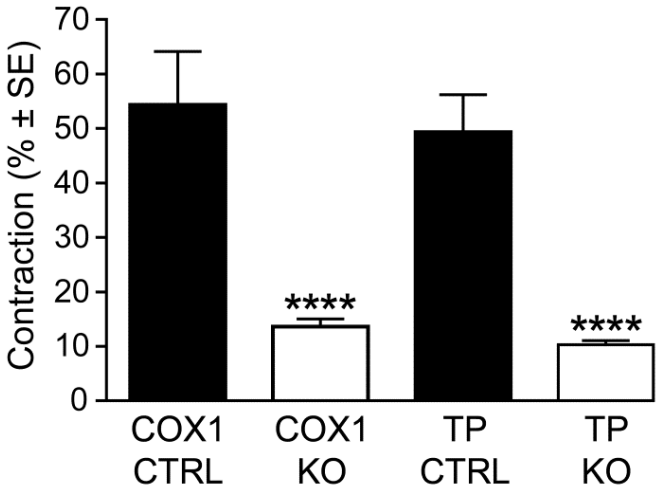

Figure 15. Prostanoids are involved in VPC31143-mediated contraction. The vasoconstriction evoked by VPC31143 at 10 μM was markedly reduced in the abdominal aortic segments of cyclooxygenase-1 (COX1) KO and Thromboxane prostanoid receptor (TP) KO mice as compared with control (CTRL) animals. ****P<0.0001 vs. the corresponding CTRL; One-way ANOVA with Tukey's post hoc test; *n*=8-23.

To verify the presence of the constrictor agent TXA_2, levels of TXB_2, a metabolite of TXA_2 with a longer life span, were measured from the supernatants of vessels exposed to VPC31143 for 2 min (Figures 16 and 17). VPC31143 treatment enhanced the TXB_2 production of WT specimens more than two-fold, which was also the situation in case of LPA_2 KO aortae. However, the agonist failed to alter the amount of TXB_2, released from LPA_1 KO vessels (Figure 16).

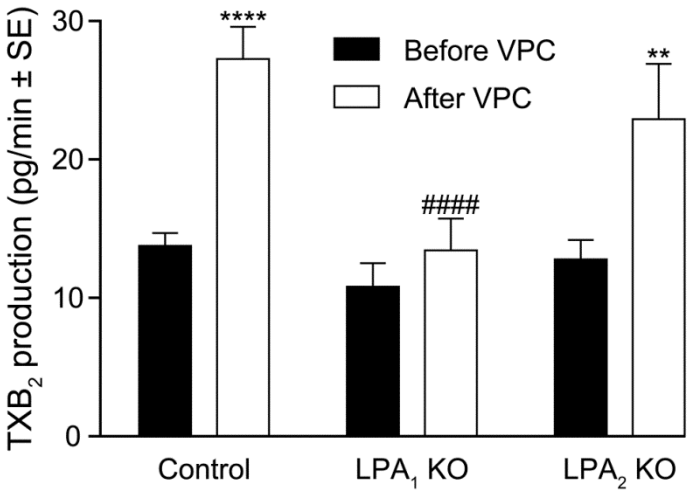

Figure 16. LPA_1 is responsible for VPC31143 mediated vascular Thromboxane A_2 (TXA_2) release. The lack of LPA_1 but not that of LPA_2 abolished the VPC31143-elicited increase in TXA_2 production in TA segments. LPA_1- and LPA_2 control mice showed identical responses and are therefore pooled and referred to as control. **P<0.01, ****P<0.0001 vs. before VPC; $^{####}$P<0.0001 vs. control after VPC; Two-way ANOVA with Bonferroni's post hoc test, *n*=6-16.

Aortae of COX1 KO mice showed a diminished basal rate of TXB_2 production, which remained unaltered upon VPC31143 application. Nonetheless, absence of the TP receptors had no such effect on either resting or stimulated release of TXB_2 (Figure 17). The above mentioned results are consistent with our hypothesis of LPA_1-mediated COX1 activation and TXA_2 production, leading to TP activation and consequent contraction of VSM.

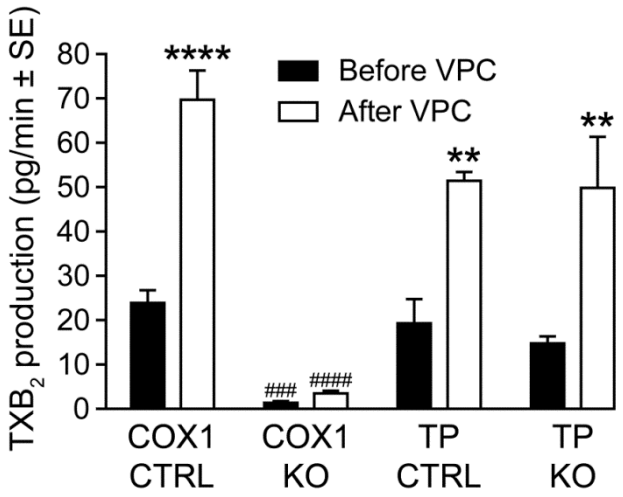

Figure 17. VPC31143 treatment leads to increased Thromboxane A_2 (TXA_2) release in cyclooxygenase (COX1) control (CTRL) thoracic aortic segments, whereas COX1 KO vessels exhibited diminished basal rate of TXA_2 production that did not increase upon VPC31143 administration. On the contrary, basal and VPC31143-stimulated TXA_2 release from thromboxane prostanoid receptor (TP) KO vessels showed no difference compared with TP CTRL segments. $^{**}P<0.01$, $^{****}P<0.0001$ vs. before VPC; $^{###}P<0.001$, $^{####}P<0.0001$ vs. corresponding COX1 CTRL; Two-way ANOVA with Bonferroni's post hoc test, *n*=3-7.

As LPA_1 is often coupled to $G\alpha_{i/o}$ (66), and $G\alpha_{i/o}$ may activate PLA_2 and TXA_2 production (463-465), we sought evidence of its possible role in LPA_1-mediated vasoconstriction. In consistence with our hypothesis, PTX pretreatment of WT vessels abolished the VPC31143-induced increase in TXB_2 production (Figure 18A). Moreover, aortic segments of PTX-pretreated WT animals showed diminished vasoconstriction upon VPC31143 administration (Figure 18B).

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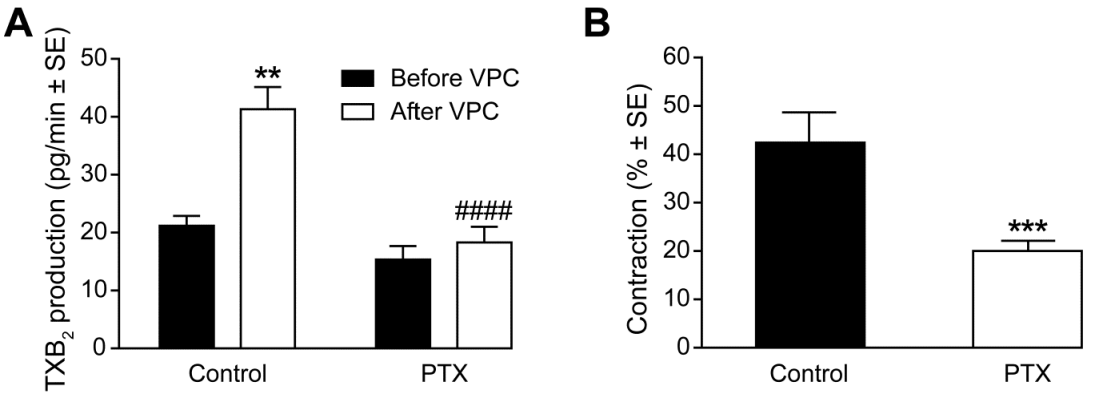

Figure 18. $G\alpha_i$ mediates VPC31143-mediated thromboxane A_2 (TXA_2) production and vasoconstriction. A) Pertussis toxin (PTX) pretreatment abolished VPC31143-induced elevation in TXA_2 production in wild type vessels. $^{**}P<0.01$ vs. before VPC; $^{\#\#\#\#}P<0.0001$ vs. control after VPC; Two-way ANOVA with Bonferroni's post hoc test; *n*=5-8. B) PTX treatment markedly decreased VPC31143-elicited contraction in wild type vessels. $^{***}P<0.001$ vs. control; Student's unpaired *t* test; *n*=14-19.

We also investigated, if the well-established regulators of smooth muscle contraction, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, could play a role in the VPC31143-induced vasoconstriction. The smooth muscle-specific absence of $G\alpha_{q/11}$ (Figure 19A) as well as that of $G\alpha_{12/13}$ (Figure 19B) decreased the contraction, evoked by 10 μM VPC31143, however both failed to completely abolish it.

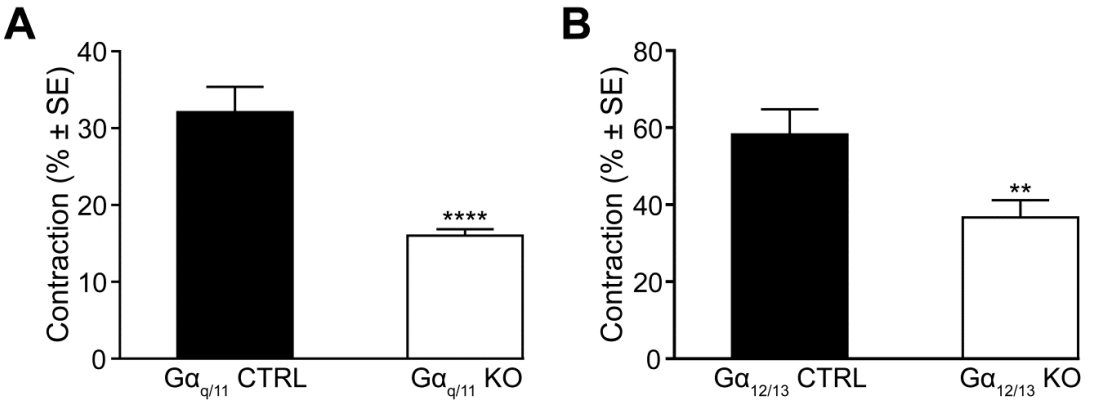

Figure 19. Involvement of $G\alpha_{q/11}$ and $G\alpha_{12/13}$ in VPC31143-induced vasoconstriction. A) Vessels of smooth muscle-specific $G\alpha_{q/11}$ KO mice showed diminished contraction upon VPC31143-administration as compared with control (CTRL) segments. $^{****}P<0.0001$ vs. CTRL; Student's unpaired *t* test; *n*=48-50. B) The lack of $G\alpha_{12/13}$ in the smooth muscle caused a reduction in VPC31143-evoked vasoconstriction as compared to CTRL vessels. $^{**}P<0.01$ vs. CTRL; Student's unpaired *t* test; *n*=16-18.

5.4. Vasoactive effects of S1P depend on the presence of other constrictors

In the next part of our experiments, we focused on the vasoactive effects of the other thoroughly investigated lipid mediator, S1P. S1P, administered on the basal tone, had negligible effect in either thoracic or abdominal aortic segments of WT animals (Figures 20A, 20B and 21). Removal of the endothelium did not influence this minor effect significantly (Figures 20C, 20D and 21). Nonetheless, after PE-induced precontraction of endothelium-denuded WT vessels S1P elicited a marked vasoconstriction (Figures 20E, 20F and 21). Although, S1P applied alone failed to significantly influence the basal tone, in presence of an other constrictor, elicited vasoconstriction. Considering the fact, that under physiological conditions, several mediators, (e.g. constrictors, dilators) are present in the circulation and actively regulate the tension of the vessels, the above-mentioned effect could play major roles in cardiovascular regulation in vivo.

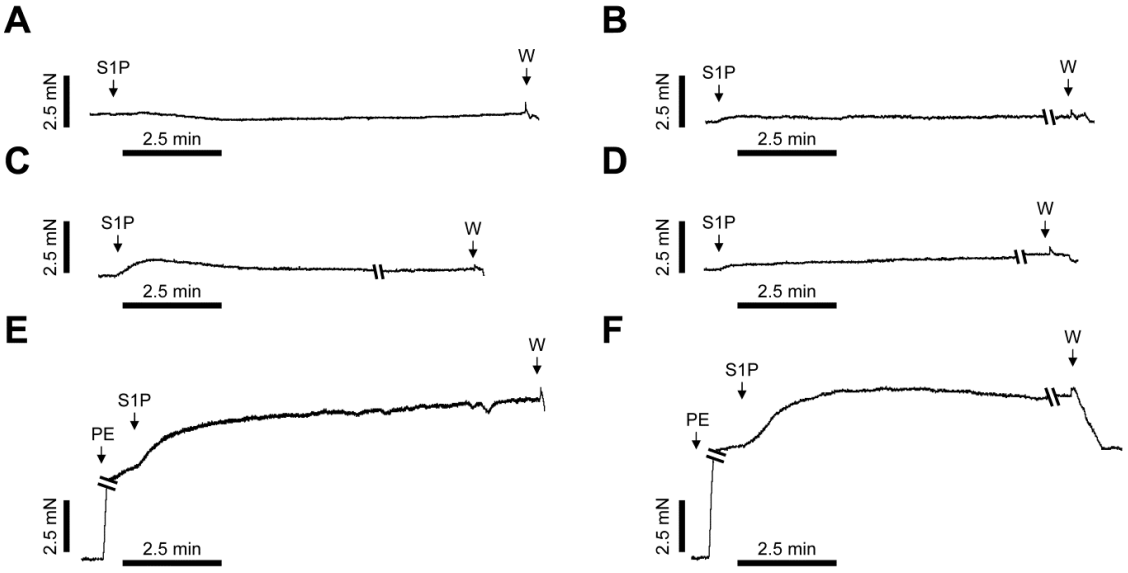

Figure 20. Effect of S1P applied either at the basal tone or after precontraction. Representative recordings of thoracic (A, C and E) or abdominal aortic segments (B, D and F) prepared from wild type mice with intact (A, B) or denuded (C-F) endothelium. S1P was applied at 5 μM either at the basal tone (A-D) or after phenylephrine (PE)-induced contraction reached a stable plateau (E and F). Horizontal and vertical bars indicate 2.5 min and 2.5 mN respectively. S1P and PE denote the application of the respective agents, while W stands for washing of the organ chamber with fresh Krebs solution.

In recognition of the imperceptible effect of S1P, applied solely, we investigated if this mediator influences the contractile effect of other constrictors. For this purpose, vasoconstrictions elicited by the α_1 adrenergic receptor agonist PE at 100 nM in WT endothelium-intact TAs were investigated and recorded before and after the administration of S1P.

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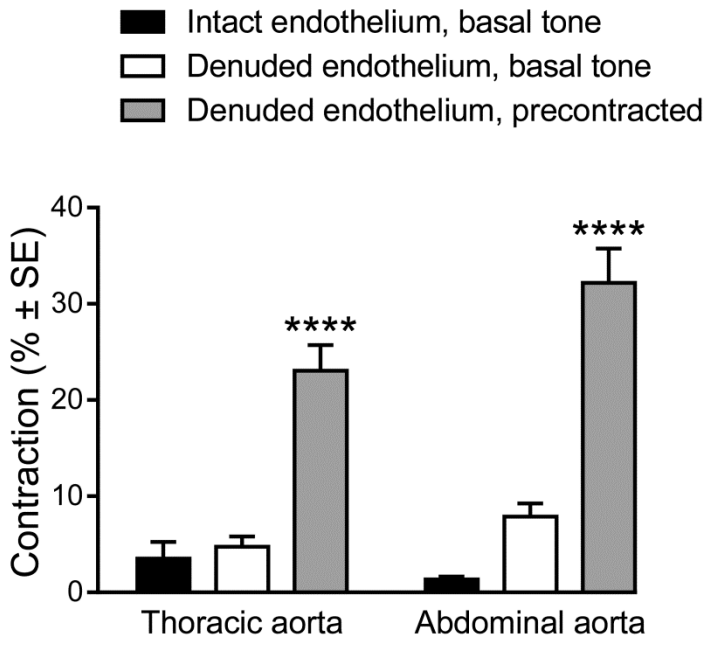

Figure 21. S1P applied at 10μM at the basal tone had negligible effect on the vascular tone in case of intact as well as denuded endothelium. However, after precontraction S1P elicited a pronounced vasoconstriction. ****P<0.0001 vs. intact endothelium, basal tone of the corresponding type of vessel; Two-way ANOVA with Tukey's post hoc test; *n*=4-6.

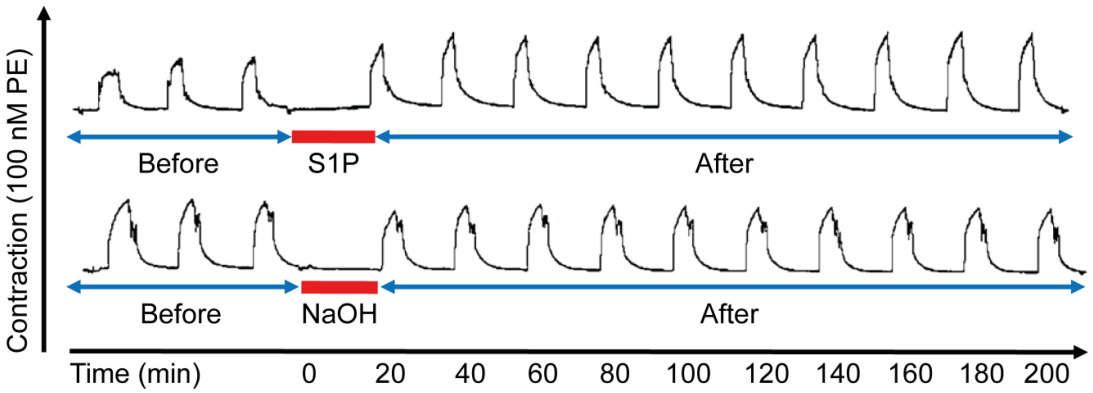

Figure 22. The effect of S1P on the constrictor effect of the α_1 agonist phenylephrine (PE). Representative recordings show, whilst incubation with S1P for 20 min significantly enhanced PE-induced contraction, the vehicle sodium hydroxide (NaOH) failed to do so. The effect was present even 180 min after the incubation. PE was applied three times before the incubation and the mean of these contractions was considered 100% and served as reference for the evaluation of later effects. S1P and PE were applied at 5 μM and 0.1 μM respectively.

Incubation with 5 μM S1P for 20 min, increased PE-mediated contractions almost 1.5-fold after 40 min and surprisingly the contractions remained enhanced even 180 min

after the incubation (Figures 22 and 23). In contrast, 0.3 N NaOH, the vehicle of S1P, had no effect on PE-elicited vasoconstrictions (Figures 22 and 23).

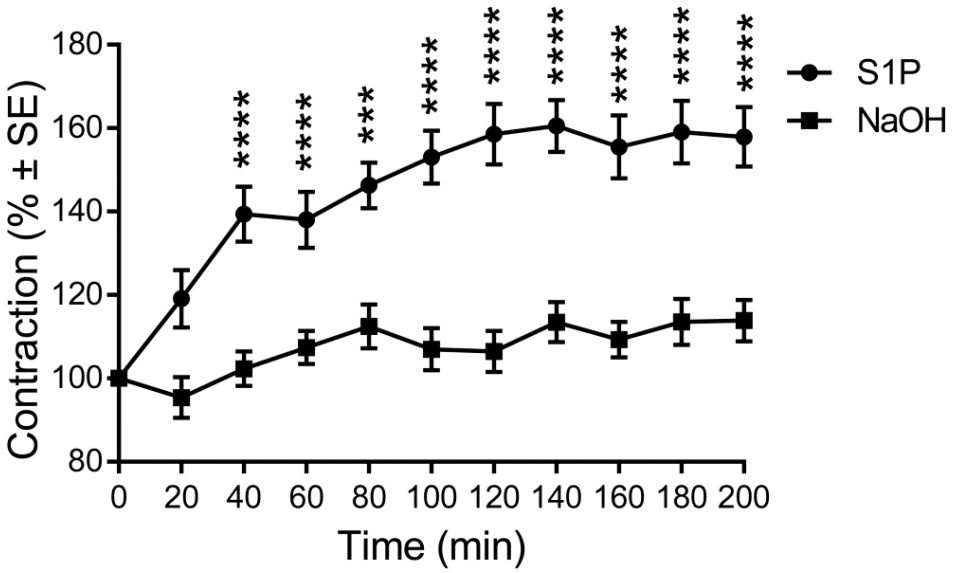

Figure 23. Incubation with S1P for 20 min markedly enhanced phenylephrine (PE)-mediated contractions, whereas the vehicle sodium hydroxide (NaOH) had no effect on it. The potentiation lasted at least for three hours. S1P and PE were applied at 5 μM and 0.1 μM respectively. $^{***}P<0.001$, $^{****}P<0.0001$ vs. 0 min; Two-way ANOVA with Tukey's post hoc test; *n*=15-18.

5.5. Identification of the S1P receptors, mediating the potentiating effect of S1P

Thereafter, we aimed to identify the S1P receptor responsible for the S1P-mediated augmentation of PE-induced contractions. For this purpose, we first determined the expression profile of S1P GPCRs in VSM of the murine thoracic aorta. In WT specimens, mRNAs of $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ were detectable, with $S1P_1$ as the most abundant S1P GPCR (Figure 24).

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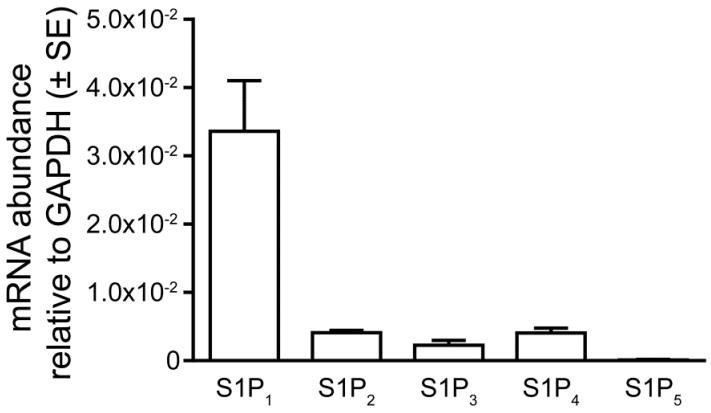

Figure 24. Expression profile of S1P receptors in freshly isolated tunica media of wild type murine thoracic aorta, determined by quantitative PCR. Murine thoracic aortic vascular smooth muscle cells predominantly express $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$. $S1P_1$ showed the highest abundance, whereas $S1P_5$ was barely detectable in our specimens.

In the next phase, we applied a genetic approach, thus we tested vessels prepared from $S1P_2$ KO and $S1P_3$ KO mice two of the GPCRs which were detectable in the isolated tunica media of murine thoracic aorta. In segments of $S1P_3$ KO animals, the S1P-induced potentiation was not different from that observed in WT vessels. Nevertheless, aortae of $S1P_2$ KO mice failed to develop the above mentioned phenomenon after 20 min incubation with S1P, highlighting the role of this receptor in S1P-induced potentiation of PE-mediated contractions (Figures 25).

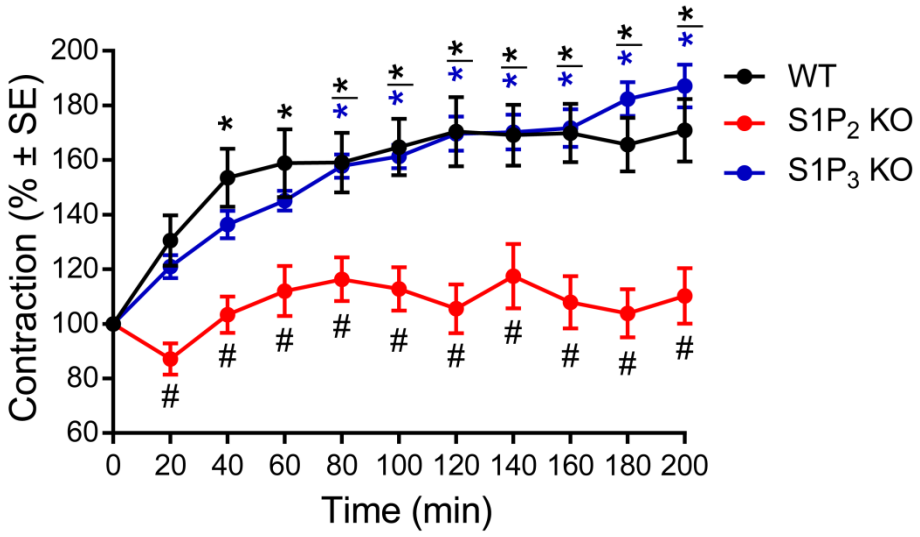

Figure 25. $S1P_2$ is responsible for the potentiating effect of S1P. The lack of $S1P_2$ but not that of $S1P_3$ abolished the S1P-induced increase in phenylephrine (PE)-mediated vasoconstrictions. S1P and PE were applied at 5 μM and 0.1 μM respectively. $^{*}P<0.05$ vs. 0 min; $^{\#}P<0.05$ vs. wild type (WT); Two-way ANOVA with Tukey's post hoc test; *n*=15-28.

6. Discussion

The EDG family of GPCRs contains three of the six confirmed LPA GPCRs (LPA_{1-3}) and all of the known S1P receptors ($S1P_{1-5}$). In the present study, we investigated their roles in the regulation of the vascular tone with pharmacological and genetic approaches.

6.1. Role of LPA receptors in the regulation of the vascular tone

In our studies, activation of the EDG-like LPA receptors had ambiguous effects in the murine aorta. In precontracted intact vessels, the LPA_{1-3} agonist VPC31143 elicited vasorelaxation in an endothelium- and eNOS-dependent manner, moreover, we could rule out the possible role of dilator prostaglandins. Interestingly, mechanical removal of the endothelium did not only abolish the dilator effect, but turned it to vasoconstriction. We presented first, that this contraction is mediated by LPA_1 receptors. The absence of vasoconstriction in LPA_1 KO mice, the inhibitory impact of the $LPA_{1/3}$ antagonist Ki16425 but not of the LPA_3 inhibitor DGPP or the lack of LPA_2 receptors emphasized the role of LPA_1 in this process. Furthermore, our former study conducted with the natural ligand LPA concluded, that LPA_1 is also responsible for the eNOS-dependent vasodilation via activation of PLC enzymes (466).

Vasoactive actions of the naturally occurring agonist LPA were described early in the initial reports of Tokumura and colleagues. The effect however, seemed species-dependent, as in vivo administration of LPA elicited hypertension in rats and guinea pigs, whereas hypotension in cats and rabbits (4). Schumacher and colleagues made clear, that the hypotensive effect in cats was a result of excessive pulmonary vasoconstriction upon platelet aggregation and a consequent drop of cardiac output (404). A recent report of Kano and colleagues showed that intravenous application of LPA elicited a hypertensive response in anesthetized mice in an LPA_4- and Rho-ROCK-dependent manner (295). These in vivo studies were certainly unable to differentiate the role of VSM and endothelium in mediating the responses. It is noteworthy, that LPA had an enhanced pressor impact in spontaneously hypertensive rats compared with Wistar-Kyoto rats (467), which implies an increased effect in case of dysfunctional endothelium. Studies conducted by Tigyi and colleagues in the 90's described an LPA-dependent vasoconstriction in pial arteries of piglets (426, 468). These results are

consistent with ours, because in the cranial window setup they used LPA was applied to the extraluminal surface of pial vessels, in which case the mediator reaches the VSM primarily.

Our quantitative PCR results, obtained from freshly isolated murine thoracic and abdominal aortic VSMC, showed a rank order of LPA receptors subtype transcripts as 6>4>1≥2>5>3. Our former expression analysis in murine aortic endothelial cells also confirmed the expression of LPA_{1-5} as well as ATX (466). Others showed that LPA_6 is expressed in human pulmonary arterial- and microvascular endothelial cells (469). These observations indicate that LPA may be involved in both endothelium-dependent and –independent regulation of the vascular tone.

Although LPA_1 was first described in the developing brain (9), since that time it has been implicated in a multitude of physiological and pathological processes as described in detail in the introduction of the present thesis. Cardiovascular functions can be found among these roles, which are highlighted by the fact, that 2.5% of LPA_1 KO mice exhibit frontal hematomas (192). Moreover, LPA_1 has a role on atherogenesis and platelet activation; this latter action is however disputed (470).

By seeking to clarify the signal transduction, downstream of LPA_1 in the constrictor effect, we hypothesized the possible involvement of the constrictor prostanoid TXA_2. Our results are in support of this hypothesis, as we found, that the application of the EDG-agonist VPC31143 elicited increased TXA_2 production in isolated vessels, in an LPA_1- and COX1- but not LPA_2- dependent manner. Besides, VPC31143-evoked contractions were alleviated in vessels of mice deficient in either COX1 or TP, which is in favor of this mechanism, in which the LPA_1-dependent activation of COX1 leads to the release of TXA_2. Moreover, pretreatment with PTX abolished TXA_2 generation as well as vasoconstriction induced by VPC31143, implying the role of G_i in COX1 activation. Our further results indicate the involvement of $G\alpha_{q/11}$ and $G\alpha_{12/13}$ in the process. Because the treatment with PTX abolished the VPC31143-elicited elevation in TXA_2 production, we conclude that LPA_1 agonism leads to COX1 activation via G_i and not $G\alpha_{q/11}$ or $G\alpha_{12/13}$. Both $G\alpha_{q/11}$ and $G\alpha_{12/13}$ were however associated with TP signaling (471). Therefore, we hypothesize that $G\alpha_{q/11}$ and $G\alpha_{12/13}$ are downstream of TP in this mechanism. Although, it must be taken into consideration, that on one hand, absence of TP and COX1 did not totally abolished the VPC31143-induced contraction,

on the other hand, LPA_1 was found to be linked to PLC in our former study (466), which is associated with $G\alpha_{q/11}$. In conclusion, the possibility of a direct link between LPA_1 and these G proteins cannot be ruled out, which would result in an LPA_1-mediated direct VSMC contraction. This signaling may participate in our system, however, considering the remaining contraction in the absence of COX1, TP, or after PTX pretreatment, COX1-dependent TXA_2 production seems to be dominant in this process.

Interaction of the LPA and the COX/TXA_2 pathway has been already described, however only in a shear stress-dependent context. Ohata and colleagues reported, that LPA stimulate Ca^{2+}-influx under shear stress in BAEC and murine aortic endothelial cells via mechanosensitive cation channels (472, 473). Furthermore, LPA elicited increased PE-induced vasoconstriction and alleviated ACh-evoked relaxation in rat mesenteric arteries in the presence of shear stress in an endothelium-dependent manner, which was abolished by the non-selective COX inhibitor indomethacin and the TP antagonist SQ29548 (474). Moreover, LPA caused elevation of intracellular Ca^{2+}-concentration in VSMCs and contraction of the murine aorta in an endothelial shear stress-dependent way. This latter effect could be prevented by application of the COX blocker aspirin, the TXA_2-synthase inhibitor OKY-046, or the TP antagonist SQ29548 (438).

Even though the effects discussed above show similarities with our results, it should be emphasized, that in those cases, the process was endothelium- and shear stress-dependent, whereas our experiments have been performed in vessels denuded of endothelium and in absence of shear stress, which indicate a completely different mechanism of action. It is noteworthy however, that under pathological conditions, which are associated with endothelial damage, e.g. hypertension, the amount of shear stress also increases. In such cases, the two mechanisms could be present together. In this scenario, LPA would induce TXA_2 release from the endothelium or VSMCs, leading to vasoconstriction. Upon reduced vascular diameter, shear stress and endothelial damage may escalate further, establishing a vicious cycle.

Further literary data available on the potential interaction of LPA- and prostanoid signaling are scant and controversial. LPA-induced contraction in guinea pig ileum was reported to be blocked by indomethacin (461); however, the same group found that

inhibition of COX signaling had no effect on LPA-induced contraction of the rat colon (475). Ohata et al. described that LPA enhances Ca^{2+}-influx upon mechanical stimulation in cultured smooth muscle cells, a similar process they reported earlier in endothelial cells, however this study did not investigate the possible contribution of prostanoids (476, 477). Besides, LPA regulated COX2 expression in the uterus, this effect was mediated by LPA_3 though, and resulted in production of prostaglandins E_2 and I_2 (226). Our findings however imply a direct link between the LPA and thromboxane signaling, as activation of the LPA_1 receptors on VSMC elicits TXA_2 production and consequently induces a vasoconstriction.

The results presented here indicate an ambiguous effect upon activation of EDG-like LPA GPCRs in the vasculature. To evaluate the potential pathophysiological relevance of this process, it must be considered that the production of the natural ligand LPA is linked to activation of the thrombocytes. In this context, upon vascular injury, where platelets activate and interact directly with VSMCs, the LPA produced locally activates LPA_1 on VSMCs that leads to TXA_2 release via G_i and COX1. TXA_2 on one hand constricts VSM, on the other hand acts on its receptor on platelets, eliciting further activation and aggregation. This interaction between the LPA-LPA_1 and the TXA_2-TP signaling may initiate a vicious circle, in which production of LPA leads to further production of TXA_2, which in return promotes further LPA release/production from platelets, and the elevated levels of these mediators promote thrombus growth and sustained vasoconstriction. If the thrombus reaches an intact part of the vessel wall, covered by functional endothelium, activation of the endothelial EDG-like LPA receptors occurs. In this case, NO-production will follow upon LPA GPCR-dependent activation of eNOS as our results illustrate. The NO released prevents further platelet activation and acting on VSM elicits vasorelaxation. Our former study revealed a role of LPA_1 and endothelial PLCs in LPA-dependent eNOS activation (466) (Figure 26).

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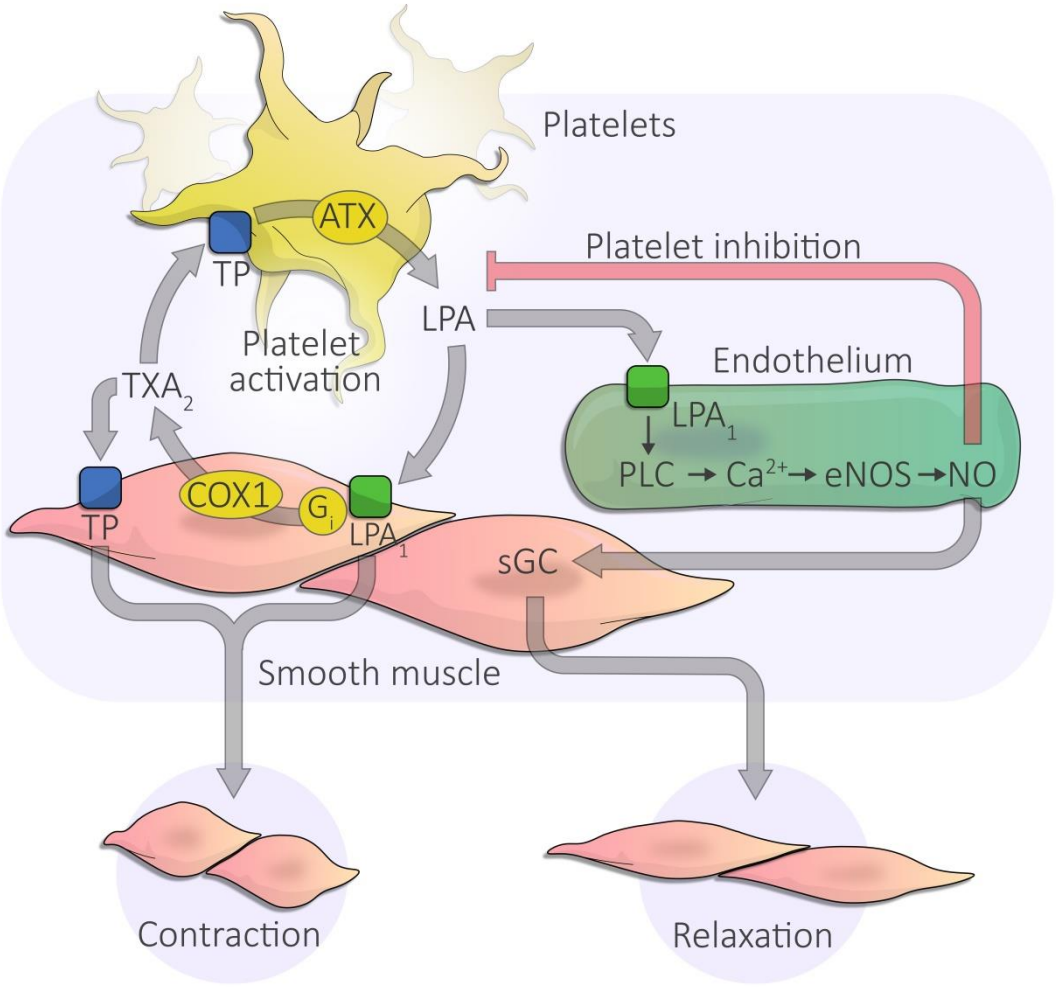

Figure 26. Integrated hypothesis of LPA_1-mediated vasoactive effects in intact vessels versus damaged endothelium. Under physiological conditions LPA stimulates endothelial nitric oxide production in an LPA_1/phospholipase C-dependent manner (466), resulting in vasorelaxation and inhibition of platelets. In absence of the endothelium, however, platelet activation initiates LPA production, which in turn acts on LPA_1 in vascular smooth muscle cells and induces thromboxane A_2 (TXA_2) production. TXA_2 on one hand elicits contraction via the activation of the thromboxane prostanoid receptor (TP) in VSMC, and on the other hand promotes further platelet activation acting on TP in platelets. TP-mediated activation of platelets results in additional LPA production. This mechanism represents a potential positive feed-back loop in which platelet activation promotes contraction and further platelet activation via a vicious circle involving LPA/LPA_1 and TXA_2/TP receptors resulting in a pathophysiological vasoconstriction or even vasospasm. ATX: Autotaxin, COX1: cyclooxygenase-1, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, PLC: phospholipase C, sGC: soluble guanylate cyclase

LPA accumulation in atherosclerotic plaques has been reported (67). Taking into consideration, that atherosclerosis is associated with endothelial dysfunction, in case of plaque rupture a large amount of LPA can be released into the local circulation, which acts on VSM and launches the aforementioned process. Moreover, accumulation of LPA has been demonstrated, systemically as well as locally in patient with ACS (409, 410). Therefore, this phenomenon may have a role in pathological vasospasm in a post-

ischemic phase. Potentially fatal consequences of this mechanism in cerebral or coronary vessels need not to be emphasized.

Vasoconstrictor effects of LPA_1-activation may be of importance after hemorrhage, where the natural agonist LPA in blood can also directly contact VSMCs without being engaged by functional endothelium. Studies conducted by Tigyi and colleagues in the 90's showed, that LPA, applied to the subarachnoid space of piglets elicited vasospasm (426, 468). Moreover, in a model of subarachnoid hemorrhage, 4 days after the injection of autologous blood or Endothelin-1, elevated levels of an LPA-like mediator could be detected in the cerebrospinal fluid (426). These results are in consistence with the actions found by the Chun-group in posthemorrhagic hydrocephalus, which was also mediated by LPA_1 (348). Besides, with the same latency (i.e. 3-4 days after subarachnoid hemorrhage), as the LPA-like mediator was detected (426), inhibition of TXA_2 synthesis alleviated the development of postsubarachnoidal vasospasm (478, 479). Our results, together with the aforementioned studies provide a potential mechanism of action in case of vasospasm, a life-threatening complication after subarachnoid hemorrhage. Nonetheless, to verify this process and to point out potential intervening drug targets, further in vivo and clinical studies are essential.

6.2. Role of S1P receptors in the regulation of the vascular tone

In the next phase of our experiments, we focused on the vasoactive actions of S1P and its receptors. S1P applied on the resting tone had no effect on intact murine aortae. Furthermore, mechanical removal of the endothelium did not alter this outcome. However, if the endothelium-denuded vessels were precontracted with PE, S1P elicited marked vasoconstriction.

The literature on this issue is also controversial. Although previous reports described a strong and long-lasting vasoconstriction, induced by S1P in canine basilar artery, in other species S1P proved to be less potent (439, 440). Furthermore, potency of S1P in other species was diverse in different vascular beds (440).

S1P was also found to activate eNOS in rodent aortic rings, mesenteric arteries and various types of cell cultures (440). In these experiments, eNOS-activation was mediated by $S1P_3$ in mice (441) and $S1P_1$ in COS-7 cells (447). Involvement of the G_i-PI3K and AMP-activated-kinase occurred as downstream signaling (440). Although, this appears to be contradictory with our results, it must be emphasized that the eNOS

activation was partly reported in experiments conducted in endothelial cell cultures, which may not fully represent the functionality of the tissues as in vivo and ex vivo studies do. Furthermore, as formerly mentioned the S1P-induced effect may alter upon species and vascular beds (440).

In our further experiments, we investigated the effect of S1P on PE-induced vasoconstriction. We found that 20 min incubation with S1P enhances PE-evoked contractions and this effect was maintained for at least 3 hours.

Afterwards, we determined the expressional profiles of S1P GPCRs in freshly isolated murine thoracic aortic VSMCs. Our results indicate that $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ are expressed with $S1P_1$ as most abundant.

Thereafter we repeated the former experiments in vessels prepared from mice deficient in $S1P_2$, or $S1P_3$. Although, absence of $S1P_3$ had no effect on S1P-induced potentiation of PE-induced vasoconstriction, the effect was abolished in $S1P_2$ KO vessels. These results indicate that S1P acting on $S1P_2$ interferes with α_1-adrenergic signaling and mediates a long-lasting elevation in α_1-mediated contractions.

Interactions of $S1P_2$ and α-adrenergic signaling have been emerged early at the time of generation of $S1P_2$ KO mice. These animals showed decreased vascular tone, elevated blood-flow and consequently decreased resistance in the renal and mesenteric areas. Although, vessels of these mice exhibited decreased contractile responsiveness to α-adrenergic stimulation, this phenotype had no effect on the blood pressure of the animals (252). These results are in consistence with ours, however it must be pointed out, that in our case $S1P_2$ signaling was not necessary to maintain the basal responsiveness to α-adrenergic stimuli but elicited further potentiation of it. Considering the fact, that S1P can be released from activated platelets (38, 149), the aforementioned mechanism can contribute to pathological states associated with blood coagulation, such as ischemic stroke or acute myocardial infarction. Hereby, it should be also noted, that the bulk of plasma S1P derives from the erythroid cell line as discussed in the introduction chapter, and that patients with acute myocardial infarction had lower circulating S1P levels than healthy controls (423). However, we hypothesize the effect to be mediated by locally accumulating S1P, which may not change plasma levels significantly.

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7. Conclusions

In our experiments we sought to answer the question, how the EDG-like lysophospholipid receptors influence the vascular tone. According to our results, we can conclude that:

- Activation of EDG-like lysophosphatidic acid (LPA) receptors elicits dose-dependent vasorelaxation in the intact murine aorta, which is an endothelium dependent process and independent of prostanoids.

- In the absence of endothelium, the EDG-like LPA agonist VPC31143 evoked dose-dependent vasoconstriction, which was more pronounced in the abdominal than in the thoracic aorta. This effect is mediated by LPA_1, which is relatively highly expressed in murine vascular smooth muscle cells. VPC31143 elicits TXA_2 release in a G_i- and COX1-dependent manner, which, acting on its receptor (TP), causes vasoconstriction. The G proteins $G\alpha_{q/11}$ and $G\alpha_{12/13}$ are also involved in the signal transduction, probably downstream of the TP receptor.

- Exposition to sphingosine 1-phosphate (S1P) potentiates α_1-dependent vasoconstriction in murine aorta, in a long-lasting manner. This impact is mediated by $S1P_2$ receptors, which are expressed in murine vascular smooth muscle.

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8. Summary

The endothelial differentiation gene (EDG) family is a group of G protein-coupled receptors (GPCR), which contains the first confirmed lysophospholipid receptors, specific for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), (LPA_{1-3}, $S1P_{1-5}$). Both mediators and their receptors have a multitude of regulatory functions in the cardiovascular system, including angiogenesis, platelet shape-change, and ischemia-reperfusion injury. The available literary data is however controversial and sparse on their role in vasoregulation. The aim of this study was to investigate, how the EDG-like lysophospholipid receptors influence the vascular tone.

We showed that activation of the EDG-like LPA GPCRs has an ambiguous role in vasoregulation. In the intact precontracted murine aorta, the LPA_{1-3} agonist VPC31143 elicited a dose-dependent vasorelaxation that was dependent of endothelial nitrogen-oxide synthase activation and independent of the prostanoid system. In absence of the endothelium, the relaxation turned into vasoconstriction. Our qPCR results indicate that freshly isolated murine aortic smooth muscle cells (VSMC) express every known LPA GPCR. The vasoconstriction is mediated by LPA_1, which elicits cyclooxygenase-1 activation and thromboxane A_2 (TXA_2) release via G_i. TXA_2 then acts on its receptor TP on VSMCs causing vasoconstriction. $G\alpha_{q/11}$ and $G\alpha_{12/13}$ are also involved in the process; however, the exact role should be further investigated. We hypothesize, that the Janus-faced activity of the EDG-like LPA GPCR may have a role in pathological processes associated with thrombus formation like ischemic stroke or myocardial infarction.

Activation of S1P GPCRs has only negligible influence on the basal tone of the intact murine aorta, and also after removal of the endothelium. In precontracted aortae, S1P evoked vasoconstriction. Furthermore, incubation with S1P potentiated phenylephrine-induced contractions and the effect lasted at least for three hours. Our results indicate that freshly isolated murine aortic VSMCs express $S1P_{1-4}$. Furthermore, we described, that activation of $S1P_2$ is responsible for the potentiating effect of S1P. As S1P can also be released upon platelet activation, hence we conclude, that this potentiating effect of S1P may have a role under pathological conditions associated with blood coagulation and increased level of naturally occurring vasoconstrictors e.g. ischemic stroke and myocardial infarction.

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9. Összefoglalás

Az endotheliális differenciációs gén család (EDG) a G protein-kapcsolt receptorok egy csoportja, mely magába foglalja az elsőként leírt lizofoszfolipid receptorokat (LPA_{1-3} és $S1P_{1-5}$), melyek természetes ligandjai a lizofoszfatidsav (LPA) és a szfingozin 1-foszfát (S1P). A kardiovaszkuláris rendszer működésében mindkét mediátor és receptoraik széleskörű szereppel bírnak, résztvevői olyan folyamatoknak, mint az angiogenezis, a thrombocyták alakváltozása vagy az iszkémia-reperfúziós károsodás. Ennek ellenére az irodalom szegényes és ellentmondásos e mediátorok és receptorok vazoregulációban betöltött szerepét illetően. Kutatásaink során arra kerestük a választ, befolyásolják-e az EDG családba tartozó LPA és S1P receptorok az értónust.

Eredményeink szerint az LPA_{1-3} receptort aktiváló VPC31143-nak kettős hatása van az értónusra. Ép, előfeszített egér aortában vazorelaxációt okozott, mely hatás endotheliális nitrogén monoxid szintáz-függő volt, de független volt a prosztanoid rendszertől. Endothél-irtott erekben az agonista vazokonstrikciót okozott. Frissen izolált egér aorta ér simaizom sejtek (VSMC) az összes ismert LPA receptort expresszálják. Továbbá kimutattuk, hogy a kontrakció hátterében az LPA_1 receptor aktivációja áll, mely thromboxán A_2 (TXA_2) felszabaduláshoz vezet G_i és cyclooxigenáz-1 aktiváción keresztül. A TXA_2 saját, TP receptorán hatva vazokonstrikciót okoz. A folyamatban részt vesznek a $G\alpha_{q/11}$ és $G\alpha_{12/13}$ fehérjék, pontos szerepük azonban még tisztázásra vár. Eredményeink alapján feltételezhető, hogy az LPA receptorok Janus arcú hatásának szerepe lehet véralvadással összefüggő patológiás folyamatokban, mint az iszkémiás stroke és a szívinfarktus.

Az S1P receptorok aktiválódásának nem volt érdemi hatása az erek alaptónusára és ez nem változott az endothelium eltávolítás után sem. Előfeszített erekben az S1P kontrakciót okozott. Kimutattuk, hogy az S1P-vel való inkubálás fokozza a fenilefrin által kiváltott kontrakciókat egér aortában. Eredményeink szerint frissen izolált egér aorta VSMC-kben az $S1P_{1-4}$ receptorok fejeződnek ki. Megállapítottuk, hogy az S1P vazokonstrikciót potencírozó hatásáért az $S1P_2$ receptor felelős. Figyelembe véve, hogy az S1P lokális koncentrációja megnőhet thrombocyta aktivációkor, feltételezzük, hogy az itt leírt folyamatoknak szerepük lehet olyan koagulációval összefüggő folyamatokban, ahol a vérben előforduló természetes konstriktorok szintje is emelkedett, mint például a szívinfarktus, vagy az iszkémiás stroke.

10. References

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11. Publications

11.1. Publication directly related to this thesis

Ruisanchez, E., **Dancs, P.**, Kerek, M., Nemeth, T., Farago, B., Balogh, A., Patil, R., Jennings, B. L., Liliom, K., Malik, K. U., Smrcka, A. V., Tigyi, G., and Benyo, Z.: Lysophosphatidic acid induces vasodilation mediated by LPA1 receptors, phospholipase C, and endothelial nitric oxide synthase. FASEB J. 2014 Feb; 28(2): 880-90

Dancs, P. T., Ruisanchez, E., Balogh, A., Panta, C. R., Miklos, Z., Nusing, R. M., Aoki, J., Chun, J., Offermanns, S., Tigyi, G., and Benyo, Z.: LPA1 receptor-mediated thromboxane A2 release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction. FASEB J. 2017 Apr; 31(4): 1547-1555

11.2. Publications not related to this thesis

Iring, A., Jin, Y.-J., Albarrán-Juárez, J., Siragusa, M., Wang, S., **Dancs, P. T.**, Nakayama, A., Tonack, S., Chen, M., Künne, C., Sokol, A. M., Günther, S., Martínez, A., Fleming, I., Wettschurek, N., Graumann, J., Weinstein, L. S., and Offermanns, S.: Shear stress-induced endothelial adrenomedullin signaling regulates vascular tone and blood pressure. J Clin Invest. 2019 Jun; 129(7): 2775-2791

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