Chapter 10
Lysophospholipid Activation of G Protein-Coupled Receptors

Tetsuji Mutoh and Jerold Chun

Abstract One of the major lipid biology discoveries in last decade was the broad range of physiological activities of lysophospholipids that have been attributed to the actions of lysophospholipid receptors. The most well characterized lysophospholipids are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). Documented cellular effects of these lipid mediators include growth-factor-like effects on cells, such as proliferation, survival, migration, adhesion, and differentiation. The mechanisms for these actions are attributed to a growing family of 7-transmembrane, G protein-coupled receptors (GPCRs). Their pathophysiological actions include immune modulation, neuropathic pain modulation, platelet aggregation, wound healing, vasopressor activity, and angiogenesis. Here we provide a brief introduction to receptor-mediated lysophospholipid signaling and physiology, and then discuss potential therapeutic roles in human diseases.

Keywords Sphingosine 1-phosphate (S1P · autoimmune diseases · transplantation · cancer · cardiovascular diseases

10.1 Introduction

In addition to being integral for cell membranes and essential sources of energy, lipids also have a major function as signaling mediators. Lysophospholipids (LPs), are simple lipid molecules with a wide range of important signaling effects on many different organ systems. For example, LPs can act as extracellular signaling molecules that affect cardiovascular function, immune responses, pain transmission, embryo implantation, osteogenesis, the circulatory system, and brain development. A lysophospholipid is a 3-carbon backbone phospholipid derived from glycerophospholipids or sphingolipids that contain a single chain

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and phosphate headgroup in the first position. Examples of LPs include LPA (lysophosphatidic acid), S1P (sphingosine 1-phosphate), LPC (lysophosphatidylcholine), SPC (sphingosylphosphorylcholine), LPS (lysophosphatidylserine), and LPE (lysophosphatydilethanolamine).

Despite the high concentration of LPC in blood (several hundred μM) (Croset et al., 2000), the physiological function of LPC remains largely unknown. On the other hand, LPs with relatively low concentrations (low μM range (Aoki, 2004; Okajima, 2002)) such as LPA, S1P, and LPS have documented functions *in vivo*. In particular the LPs, LPA and S1P, as well as their signaling cascades, have been extensively studied. Because lysophospholipids have a chemical makeup that allows them to enter the lipid bilayer, it was previously thought that the effect of LPs and their mechanisms of action were largely non-specific. However, this initial view changed with the identification of specific LP receptors that were essential to the physiological functions of LPs. Cloning and functional characterization of the lysophospholipid receptors represented a significant advance towards understanding this class of lipid signals. Today, ten *bona fide* lysophospholipid receptors have been reported, 5 for LPA ($LPA_{1\text{-}5}$) and 5 for S1P ($S1P_{1\text{-}5}$), with a number of additional putative lysophospholipid G protein-coupled receptors (GPCRs) existing in the literature (Anliker and Chun, 2004; Ishii et al., 2004; Lee et al., 2006; Rivera and Chun, 2007). Recently, a specific receptor for LPS was identified, however the associated signaling cascade(s) for this receptor is not fully understood (Sugo et al., 2006). Many of the LP receptors are necessary for normal embryonic development and have roles in normal adult physiologies as well as disease processes. Furthermore, the GPCRs for specific LPs are intriguing since they are attractive targets for drug discovery.

In this chapter, we will discuss the normal physiological functions of LPA and S1P mediated by their cognate receptors. In addition, we will discuss diseases associated with these bioactive LP molecules. Although LPA belongs to the glycerophospholipid family and S1P belongs to the sphingolipid group, the amino acid sequence of their receptors is generally conserved to a significant extent and they have overlapping but distinct biological functions. Originally known in the 1900s as a lipid metabolite, LPA was reported to have physiologically active properties that functioned to control blood pressure (Sen et al., 1968; Tokumura et al., 1978). S1P was originally identified as a mitogen capable of inducing intracellular calcium mobilization via proposed intracellular mechanisms (Zhang et al., 1991). Continuing research in the field of lipid biology revealed the importance of these two LP signaling molecules *in vivo*. For instance, the phosphorylated metabolite of FTY720, FTY720-P, is an S1P analog that was discovered to be a novel immunomodulator by inducing lymphopenia via S1P receptors (Mandala et al., 2002). While a number of recent reviews have covered many facets of this rapidly growing field, the purpose of this chapter is to provide a basic overview of LP signaling and discuss how LPs are relevant to both normal physiological functions and the pathology of human diseases.

10.2 Biochemistry of LP Signaling

10.2.1 Receptor Mediated Signaling Pathways

Initially, LPs were shown to be precursors and metabolites in the *de novo* biosynthesis of phospholipids. However, other bioactive properties were subsequently discovered. For instance, LPA was shown to function as an anti-hypertensive agent (Sen et al., 1968; Tokumura et al., 1978). LPA was also discovered to act as a cell growth and motility factor present in serum, and the signaling cascades mediated by LPA were shown to involve G proteins (van Corven et al., 1989), suggesting the involvement of GPCRs, although other GPCR-independent mechanisms were also possible in the absence of identified receptors. The first LP receptor was cloned from mouse brain cDNA by degenerate PCR with primers designed against GPCRs (Hecht et al., 1996). This receptor, originally designated VZG-1, and now called LPA_1, was the first LP receptor discovered. Within several years of this initial report, several members of an orphan GPCR receptor family, called "endothelial differentiation genes (Edg)," were identified as GPCRs for both lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) (An et al., 1997; Ishii et al., 2004; Lee et al., 1998; van Brocklyn et al., 2000). All of these LP receptors are GPCRs capable of interacting with a number of heterotrimeric G proteins. The current nomenclature reflects the receptor's cognate ligand and chronological order of the relevant receptor's identification (Chun et al., 2002; Ishii et al., 2004; Table 10.1). LP receptor genes are distributed throughout the genome and are organized in a somewhat similar fashion.

The coding regions for each of the *lpa* genes in the genomes of human and mice, with the exception of LPA_4, are divided between two exons, while the coding region of each *s1p* gene is contained within a single exon, with only non-coding exon(s) upstream (Contos and Chun, 2001; Contos et al., 2000b, 2002). Several structural characteristics are shared between LPA and S1P receptors, including an extracellular N-terminus, seven α-helical THs (transmembrane

Table 10.1 Nomenclature of lysophospholipid receptors

IUPHAR Nomenclature	Chromosomal location (Human)	Natural Agonist Ligand	Previous Names
LPA1	9q32	LPA	Edg–2, LPA1, VZG–1, REC1.3
LPA2	19p12	LPA	Edg–4, LPA2
LPA3	1p22.3–p31.1	LPA	Edg–7, LPA3
LPA4	Xq13–q21.1	LPA	GPR23, P2Y9
LPA5	12p 13.31	LPA	GPR92
S1P1	1p21	S1P > SPC	Edg–1, LPB1
S1P2	19p 13.2	S1P > SPC	Edg–5, LPB2, AGR16, H218
S1P3	9q22.1–q22.2	S1P > SPC	Edg–3, LPB3
S1P4	19p 13.3	S1P > SPC	Edg–6, LPB4, LPC1
S1P5	19p 13.2	S1P > SPC	Edg–8, LPB5, NRG–1

helices), and an intracellular C-terminus (Pierce et al., 2002). Studies of LPA and S1P receptor ligand binding mechanisms suggest that several specific amino acid residues are responsible for ligand interaction, e.g., Arginine 120 in TH3 is thought to be required for ligand binding and Glutamine 121 for ligand specificity recognition (Holdsworth et al., 2004; Parrill, 2005; Parrill et al., 2000), although no formal structural data have been reported for this family of GPCRs.

Lysophospholipid receptors each have a heterogeneous spatiotemporal gene expression pattern and multiple receptors may be expressed by the same cell. These data have been derived by examining mRNA combined with functional assays. Notably, no antibodies or antisera have been clearly proven for use in immunohistochemical studies of native proteins, although many can identify overexpressed proteins in cell lines. For example, LPA_1 was initially called "ventricular zone gene-1 (Vzg-1)" because of its enrichment in the neural progenitor zone of the embryonic cerebral cortex, the so called "ventricular zone." In adult mice, LPA_1 is widely expressed with high mRNA levels in brain, lung, heart, and other organs. LPA_1 and $S1P_1$ expression patterns are generally similar but differ in detail in both embryonic and adult tissues. For example, $S1P_1$ is expressed in the ventricular zone throughout the embryonic telencephalon, however, LPA_1 gene expression is limited to the neocortical ventricular zone as stated above (Anliker and Chun, 2004; Contos et al., 2000b; Hecht et al., 1996; McGiffert et al., 2002). Most cell types express multiple LPA and S1P receptors, and each receptor can activate multiple types of downstream molecules as mentioned below. LP signaling in each cell and tissue can vary depending upon the composition and expression level of the receptor family members and their downstream molecules. In addition, ligand availability, concentration, and half-life are also likely to influence cellular responses mediated by LP receptors. Their desensitization is probably mediated by known mechanisms in other systems of phosphorylation of GPCRs by kinases and or an uncoupling from G proteins by arrestins, followed by receptor internalization and degradation (Lefkowitz and Shenoy, 2005).

LPA and S1P receptors couple to heterotrimeric G proteins, which consist of a G_α and the associated $G_{\beta\gamma}$ subunits. The heterotrimeric G proteins are thought to be bound to the inner surface of the cell membrane. One receptor may couple to several different types of G_α protein subunits to form a complex signaling network (Fig. 10.1). $LPA_{1,2,4,5}$ and $S1P_{2\text{-}5}$ all signal via $G_{\alpha 12/13}$ to activate RhoA, a member of the family of Rho GTPases. $LPA_{1\text{-}5}$ and $S1P_{2,3}$ couple to $G_{\alpha q/11}$ to activate phospholipase C (PLC). $LPA_{1\text{-}4}$ and $S1P_{1\text{-}5}$also couple with $G_{\alpha i}$ to activate PLC, Ras, Phosphoinositide-3 Kinase (PI3K), and to inhibit adenylyl cyclase (AC), but LPA_4 can also couple to $G_{\alpha s}$ to activate AC. When a ligand binds to the receptor, it exchanges GDP for GTP on the G_α subunit, and then G_α-GTP and $G_{\beta\gamma}$ can activate the effector molecule complex for each signaling cascade (Etienne-Manneville and Hall, 2002; Neves et al., 2002).

Furthermore, several reports suggest that LP receptor signaling can involve trans effects via receptor tyrosine kinases, as seen in the synergistic interaction between S1P and platelet derived growth factor (PDGF), as well as signaling via

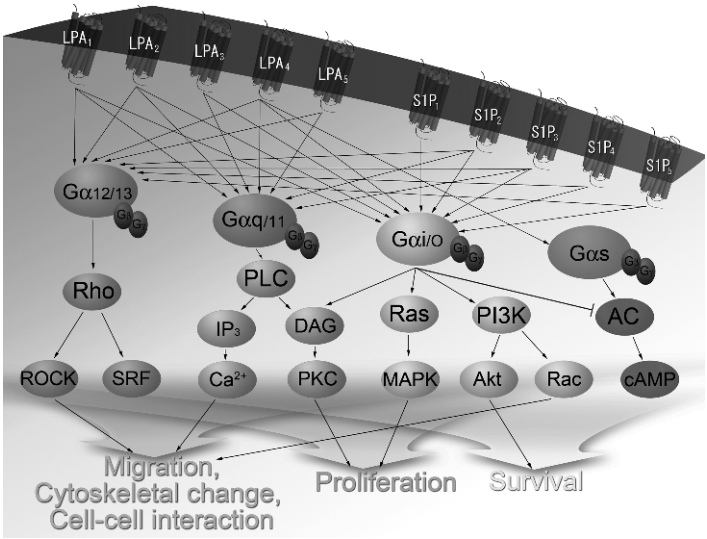


Fig. 10.1 The network of LPA and S1P signaling through G protein-coupled receptors. Each LPA and S1P receptor couples to their specific class of G proteins. Ligand binding activates or inhibits downstream second messenger molecules, and the most prominent cellular effects are illustrated. *Rock*, Rho-associated kinase; *SRF*, serum response factor; *IP3*, inositol 1,4,5-trisphosphate; PLC, phospholipase C; DAG, diacylglycerol; *PKC*, protein kinase C; *MAPK*, mitogen-activated protein kinase; *PI3K*, phosphoinositol 3-kinase; *DAG*, diacylglycerol

other kinases such as p42/p44 MAPK or Akt activation in chemotaxis, and Erk1/2 mediated anti-apoptotic effects (Hobson et al., 2001; Pyne et al., 2007; Wong et al., 2007). In addition, PDGF, VEGF and TNF-α can stimulate sphingosine kinases (e.g., SPHK1) and increase S1P levels in an autocrine fashion. This has important implications for vascular maturation (Spiegel and Milstien, 2003). Interestingly, a recent report suggested a "criss-cross" transactivation between estrogen-S1P-EGFR pathways (Sukocheva et al., 2006), and that other interactions amongst GPCRs and receptor tyrosine kinases/other kinases are likely.

10.2.2 Variable Cellular Responses via LPA and S1P

Before the discovery of specific LP receptors, there were multiple hypotheses proposed to explain the physiological signaling response mechanisms provoked by LPs. For instance, it was thought that LPs could act as calcium chelators, ionophores, membrane disruptors, second messengers, or act via intracellular receptors (reviewed in (Chun, 1999; Fukushima et al., 2001). Heterologous expression of cloned receptors was performed to prove that extracellular receptors mediated the LP signaling pathway. Two cell lines, RH7777 (hepatoma)

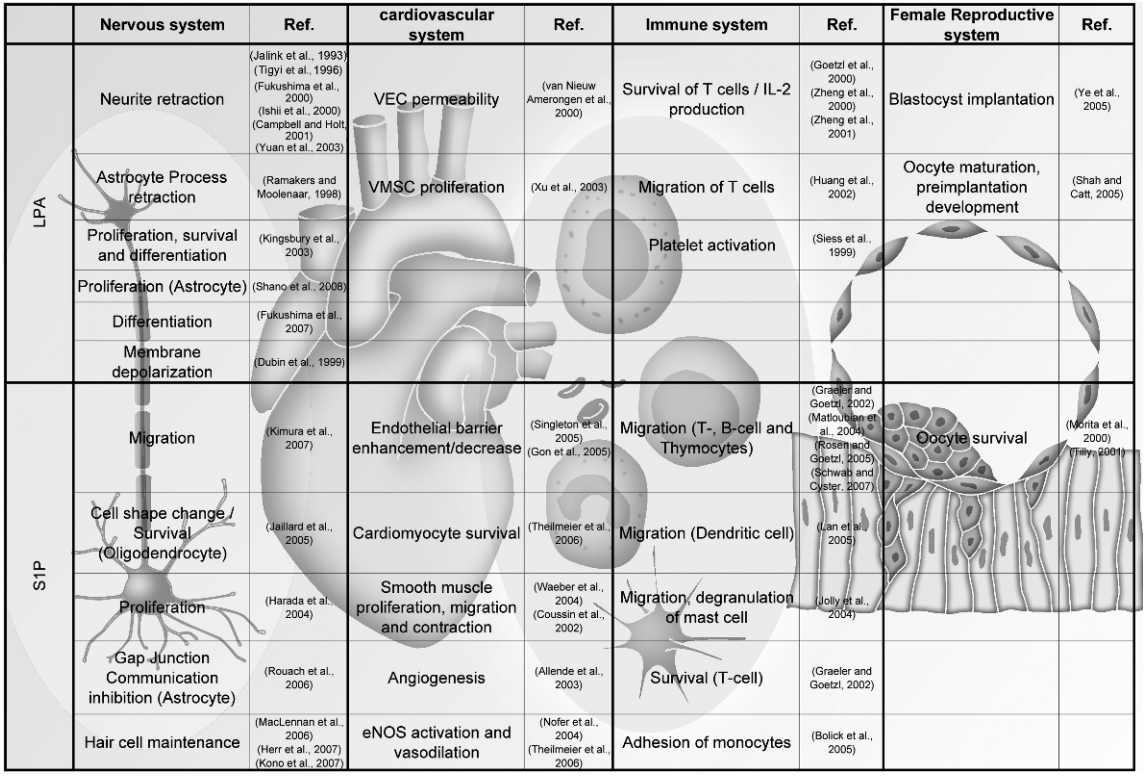
and B103 (neuroblastoma) were identified, which lack endogenous responses to LPA and/or S1P and were useful in these mechanistic studies (Fukushima et al., 1998). Even though several non-GPCR mediated signaling pathways have been reported (Hooks et al., 2001; McIntyre et al., 2003), it is now clear that the dominant mechanism by which extracellular LPs function, at least in vertebrates, is through the actions of specific cell surface receptors (Chun and Rosen, 2006). In terms of pathology, there are numerous functional studies using cancer cells that show cell growth, apoptosis, invasion, cell migration, and extra-cellular matrix reorganization, which are mediated by LPs. Concomitant alterations in cell migration and invasion may further contribute to the growth of metastatic cancer. Studies with primary cells have shown that LPs influence immune responses including cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction (Fig. 10.2).

10.2.2.1 Cell Survival and Growth

LPA and S1P can signal via G_i, $G_{q/11}$, $G_{12/13}$, and Gs. In many well-documented studies using cultured cell lines, LPs function as survival factors (Ishii et al., 2004). LPA and S1P largely couple to the G_i pathway that regulates PI3K and Akt, but other signals generated through different G protein pathways can also be initiated. The signaling cascades that are activated via the G_i and Ras/MAPK pathway or G_q and phospholipases generate second messengers that facilitate cell growth. While it is generally known that the activation of PLC, Ca^{2+}, or PKC signaling pathways are insufficient to promote cell proliferation, additional signaling pathways activated by LP receptors provide complementary proliferative stimuli. Signaling through the $G_{12/13}$ mediated Rho pathway also promotes cell proliferation. G_i-mediated signaling contributes to cell survival through PI3K/Akt (Radeff-Huang et al., 2004; Weiner et al., 2001).

10.2.2.2 Cell Migration

LPs also affect cell migration of diverse normal and transformed cell types (Mills and Moolenaar, 2003). Depending on the combination of receptors expressed and downstream molecules, S1P signaling can also promote and inhibit cell migration (Okamoto et al., 2000; Sugimoto et al., 2003). $S1P_1$ is crucial for angiogenesis and lymphocyte trafficking, which is based on its ability to stimulate cell migration (Chun and Rosen, 2006). On the other hand, $S1P_2$ inhibits Rac and abolishes membrane ruffling and cell migration. This inhibition can be antagonized by concurrent G_i mediated Rac activation (Sugimoto et al., 2003). Many studies have shown that G_i and/or $G_{12/13}$ mediated pathways can control cell motility via changes in cytoskeletal organization (van Leeuwen et al., 2003). The actin cytoskeleton is regulated by the Rho-GTPase family: RhoA, Cdc42, and Rac. G_i activates the PI3K-Rac pathway via RhoA (Neves et al., 2002). It is notable that $S1P_1$ is unique in that it only couples to G_i, which appears to utilize Rac signaling to promote migration via this particular receptor.



	Nervous system	Ref.	cardiovascular system	Ref.	Immune system	Ref.	Female Reproductive system	Ref.
LPA	Neurite retraction	(Jalink et al., 1993) (Tigyi et al., 1996) (Fukushima et al., 2000) (Ishii et al., 2000) (Campbell and Holt, 2001) (Yuan et al., 2003)	VEC permeability	(van Nieuw Amerongen et al., 2000)	Survival of T cells / IL-2 production	(Goetzl et al., 2000) (Zheng et al., 2000) (Zheng et al., 2001)	Blastocyst implantation	(Ye et al., 2005)
	Astrocyte Process retraction	(Ramakers and Moolenaar, 1998)	VMSC proliferation	(Xu et al., 2003)	Migration of T cells	(Huang et al., 2002)	Oocyte maturation, preimplantation development	(Shah and Catt, 2005)
	Proliferation, survival and differentiation	(Kingsbury et al., 2003)			Platelet activation	(Siess et al., 1999)		
	Proliferation (Astrocyte)	(Shano et al., 2008)						
	Differentiation	(Fukushima et al., 2007)						
	Membrane depolarization	(Dubin et al., 1999)						
S1P	Migration	(Kimura et al., 2007)	Endothelial barrier enhancement/decrease	(Singleton et al., 2005) (Gon et al., 2005)	Migration (T-, B-cell and Thymocytes)	(Graeler and Goetzl, 2002) (Matloubian et al., 2004) (Rosen and Goetzl, 2005) (Schwab and Cyster, 2007)	Oocyte survival	(Morita et al., 2000) (Tilly, 2001)
	Cell shape change / Survival (Oligodendrocyte)	(Jaillard et al., 2005)	Cardiomyocyte survival	(Theilmeier et al., 2006)	Migration (Dendritic cell)	(Lan et al., 2005)		
	Proliferation	(Harada et al., 2004)	Smooth muscle proliferation, migration and contraction	(Waeber et al., 2004) (Coussin et al., 2002)	Migration, degranulation of mast cell	(Jolly et al., 2004)		
	Gap Junction Communication inhibition (Astrocyte)	(Rouach et al., 2006)	Angiogenesis	(Allende et al., 2003)	Survival (T-cell)	(Graeler and Goetzl, 2002)		
	Hair cell maintenance	(MacLennan et al., 2006) (Herr et al., 2007) (Kono et al., 2007)	eNOS activation and vasodilation	(Nofer et al., 2004) (Theilmeier et al., 2006)	Adhesion of monocytes	(Bolick et al., 2005)		

Fig. 10.2 Cellular response to LPA and S1P in different organ systems. Examples of receptor-mediated cellular responses to LPA and S1P. Cytoskeletal reorganization, migration, survival, and proliferation operate in many biological systems particularly within the nervous system, the cardiovascular system, the immune system, and the female reproductive system. *IL-2*, interleukin-2; *VEC*, vascular endothelial cells; *VSMCs*, vascular smooth muscle cells; *eNOS*, endothelial nitric oxide synthase

10.2.2.3 Cell Shape Change

As mentioned above, LP signaling regulates F-Actin through G_i and/or $G_{12/13}$. In this context, LPs affect cell shape changes not only by influencing motility, but also neurite retraction, growth cone collapse, repulsive growth cone turning, neuroblast and glial cell rounding, and smooth muscle cell contraction (Ishii et al., 2004; Moolenaar et al., 2004). For assessing the LP effect on cell shape and cytoskeletal changes, cell rounding assays, stress fiber formation assays, membrane ruffling assays with F-Actin staining, and real-time assays are utilized.

10.2.2.4 Cell Adhesion and Aggregation

The activation of $G_{12/13}$ induces Rho and Rho kinase-mediated formation of actin stress fibers, focal adhesions, and cell contraction. Both LPA and S1P also mediate physiological wound healing processes and potentially atherogenic and thrombogenic processes (Siess, 2002). For example, LPA and mildly oxidized LDL (mox-LDL) promote monocyte binding to endothelial cells by increasing the cell surface expression of E-selectin and the vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Rizza et al., 1999). LPA can also induce N-cadherin mediated Schwann cell clustering and Rho-ROCK mediated focal adhesion formation (Weiner et al., 2001). S1P can stimulate adhesion activity, but it can also inhibit cell adhesion via PI3K and nitric oxide synthase (eNOS) activation (Kimura et al., 2006).

10.2.2.5 Inhibition of GAP Junction Communication

LPA inhibits GAP junction communication by connexin 43 phosphorylation in rat liver cells (Hill et al., 1994). MAPK and arachidonic acid cascades may transduce the signal, but the mechanism is still unclear (De Vuyst et al., 2007). Recently, it was reported that high S1P levels negatively affect gap junctions in astrocytes. In this case, the inhibitory effect of LPA is mediated through G_i and Rho GTPases (Rouach et al., 2006).

10.2.2.6 Transcription Regulation

LPA and S1P have been shown to activate NF-kappaB and induce expression of multiple effector genes. In endothelial cells, LPA also increases the levels of various adhesion molecule mRNAs and secreted factors, such as E-selectin, VCAM, and ICAM, as mentioned above (Li et al., 2005; Xia et al., 1998).

10.2.3 Metabolism and Enzymes

To understand the dynamics of LPs *in vivo*, it is necessary to review the enzymes involved in LPA production and degradation. After the identification of the receptors, the identification of enzymes responsible for LP synthesis and degradation has accelerated our understanding of lipid biology (Fig. 10.3).

10.2.3.1 Synthetic and Degradating Enzymes

As signaling mediators *in vivo*, the production and degradation of lysophospholipids should be tightly controlled. The metabolism of LPA has been partially characterized and involves a number of convergent biosynthetic pathways and enzymes of varied specificity (Meyer zu Heringdorf and Jakobs,

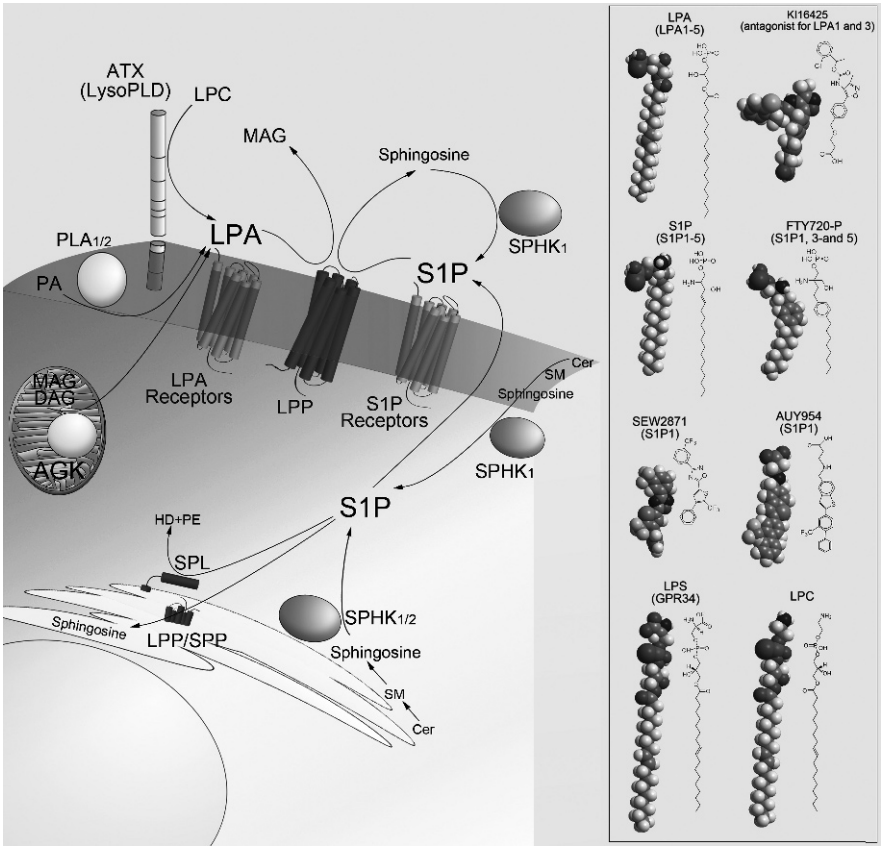


Fig. 10.3 LPA and S1P metabolic pathways. Schematic representation of LPA and S1P metabolism. LPA is produced by ATX ("autotaxin," a lysophospholipase D or lysoPLD), phospholipase A1 and A2 ($PLA_{1/2}$), and acylglycerol kinase (AGK). PA is generated and transported from the inner leaflet of the plasma membrane, then subsequently converted to LPA by PLA_1 or PLA_2. According to a recent report, acylglycerol kinase phosphorylates monoacylglycerol (MAG) and DAG can produce LPA in mitochondria (Bektas et al., 2005). S1P is formed from sphingosine by sphingosine kinase 1 and 2 (SPHK1, SPHK2). Lipid phosphate phosphatases (LPPs) inactivate both LPA and S1P through dephosphorylation. Sphingosine phosphate phosphatase (SPP) specifically dephosphorylates S1P. S1P is also inactivated by S1P lyase (SPL) that produces irreversible cleavage. The space-filling molecular models and structures of LPA, S1P, and some major analogs are shown in the right box. High affinity LP receptors are indicated in parentheses under the name of each ligand

2007). To date, lysophospholipase D (lysoPLD), autotoxin (ATX), phospholipase A1 (PLA1), phospholipase A2 (PLA2), and acylglycerol kinase (AGK) are enzymes reported to be involved in LP synthesis (Chun and Rosen, 2006). There are multiple pathways responsible for LPA production (Meyer zu Heringdorf and Jakobs, 2007).

S1P metabolism involves a number of specific and highly conserved enzymes (Saba and Hla, 2004). Two sphingosine kinase isoforms, sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK 2), produce S1P from sphingosine (Kohama et al., 1998; Liu et al., 2000a). Recently it was shown by specific genetic removal of SPHK 1 and 2 in erythrocytes that these cells are the major source of S1P in blood (Pappu et al., 2007). SPHK activity is not only present in blood, but also in most mouse tissues (with high activity in thymus and lung) (Fukuda et al., 2003).

The duration and strength of LP signaling likely depends, at least locally, on the activity of synthetic and degradative enzymes and their localization relative to the LP receptors. For example, it has been reported that S1P lyase (SPL) has an important role in maintaining a steep gradient of S1P between blood and tissues, which in part controls lymphocyte localization (Schwab and Cyster, 2007; Schwab et al., 2005). The local distribution and potential LP gradients in tissues remain to be elucidated. Pharmacological and molecular manipulation of LP metabolic enzyme activity is also an intriguing approach for cancer therapy or other clinical treatments (see below).

10.2.3.2 Endogenous Concentration of LPA and S1P *In Vivo*

It was previously thought that the major source of lysophospholipids was from blood. Reported concentrations of LPA and S1P vary in the literature, however most publications report around 1000 nM (200–5000 nM) in blood, and 0.2–100 nmol/g in tissues under basal, normal conditions (Aoki, 2004; Berdyshev et al., 2005; Bielawski et al., 2006; Das and Hajra, 1989; Eichholtz et al., 1993; Min et al., 2002; Murata et al., 2000; Okajima, 2002; Olivera et al., 1994; Yatomi et al., 1997). Platelets contain large amounts of LPA and S1P, which can both be released following platelet activation (Benton et al., 1982; Yatomi et al., 1997). For this reason, it was believed that platelets are the major source of S1P. However, it had been shown that erythrocytes can synthesize S1P by enzymatic pathways (Stoffel et al., 1970), and it is now clear that SPHKs present in erythrocytes are responsible for S1P in blood (Pappu et al., 2007). In addition, it has been shown that erythrocytes are able to import and store S1P that can be actively released upon stimulation (Hanel et al., 2007). It was also believed that LPA in blood is mainly derived from activated platelets, however it was recently reported that an LPA producing enzyme, lysophospholipase D(lysoPLD)in plasma, may also contribute to the total amount of LPA found in the blood (Aoki, 2004). The plasma LysoPLD activity was measured directly and half of this activity is attributed to autotaxin (ATX), one of the major LysoPLDs (Tanaka et al., 2006). In addition to the aforementioned pathway,

there might be local LP synthesis in specific tissues. For example, the highest expression of ATX is found in the floor plate of the developing embryo and in the choroids plexus and osteoblasts throughout development (Bachner et al., 1999). Also, neurons are a potential source of LPA in the developing brain as nanomolar concentrations of LPA are found in conditioned medium from embryonic brain primary cultures (Fukushima et al., 2000).

10.2.4 Binding Proteins

Lysophospholipids are usually bound to lipoproteins *in vivo*. Serum LPA binds to albumin, gelsolin, and other proteins (Moolenaar et al., 2004). S1P binds mainly to HDL and albumin (Levkau et al., 2004; Nofer et al., 2004; Okajima, 2002; Sato et al., 2007; Theilmeier et al., 2006). Such lipoproteins stabilize LPs in the hydrophilic environment and possibly protect them from rapid degradation. The stabilization effect of the lipoprotein is currently being studied (Moumtzi et al., 2007).

10.3 Physiology of LPs

As mentioned above, the LPA and S1P receptors are widely expressed throughout the body, however each receptor's expression is temporally and spatially distinct albeit often overlapping. To study the physiological function of each lipid receptor signal, targeted gene mutations in mice have been utilized to remove LP receptor genes or related enzymes. To date, null mutations for the LP receptor genes LPA_1-, LPA_2-, LPA_3, $S1P_1$-, $S1P_2$-, $S1P_3$, and $S1P_5$, and the LP producing enzymes ATX, SPHK1, SPHK2 have been reported.

10.3.1 LPA Receptor Mutant Mice

Deletion of LPA_1 in mice causes a reduction in litter size primarily reflecting a 50% perinatal mortality rate. The observed mortality is due to poor suckling behavior that appears to result from an olfactory defect. However, they have a grossly normal cerebral cortex (Contos et al., 2000a). A smaller body size, shorter snouts, and cranial hematomas are characteristic features of surviving LPA_1 null mutant mice. Mild anatomical defects in the cerebral cortex and defective behavior in pre-pulse inhibition assays have also been reported in distinct null mutants or genetic variants (Estivill-Torrus et al., 2007; Harrison et al., 2003). LPA_1-null mice also show an increased number of apoptotic Schwann cells in the sciatic nerve (Contos et al., 2000a). This is consistent with the fact that LPA_1-null Schwann cells exhibit a reduced response to LPA (Weiner et al., 2001). Indeed, LPA_1-null mice do not show injury-induced dorsal root demyelination and neuropathic pain after peripheral nerve injury (Inoue et al., 2004).

The LPA_2-null mutation produces viable animals that are also grossly normal. The LPA_1 and LPA_2 double knockout animals show only a slight exacerbation of the hematoma defect that is seen in the LPA_1-null. However, primary fibroblasts and the embryonic cortex show vastly reduced responses to LPA (Contos et al., 2002; Kingsbury et al., 2003).

The functional loss of LPA_3 causes severe reproductive defects (Ye et al., 2005). The LPA_3-null litter sizes were less than 50% of wild type and heterozygote controls, and show delayed embryo implantation as well as spacing defects in the uterus. These phenotypes are attributable to the maternal genotype regardless of the male or embryo genotypes. The cyclooxygenase 2 (COX2) expression and prostaglandin levels are also reduced in the LPA_3-null uterus. This study demonstrated that LPA_3 is an indispensable upstream regulator of prostaglandin-mediated on-time implantation and embryo spacing.

10.3.2 S1P Receptor Mutant Mice

$S1P_1$-null mice have severe defects in vascular maturation, and die *in utero* because of hemorrhaging between E12.5 to E14.5 (Liu et al., 2000b). Because of the embryonic lethality, studies with $S1P_1$ conditionally deleted with the Cre-loxP system were used to analyze defects in specific cell and tissue types. Endothelial cell-specific deletion of $S1P_1$ showed that the vascular abnormality observed in $S1P_1$-null mice was due to a maturation defect in vascular endothelial cells (Allende et al., 2003). Also, T cell-specific deletion showed that $S1P_1$ was crucial for mature T cell egress from the thymus to the periphery (Allende et al., 2004a). To study lymphocyte egress using constitutive $S1P_1$ null mutant lymphocytes, hematopoietic precursors from $S1P_1$-null embryos were transferred to irradiated wild type adult mice and allowed to repopulate the lymphoid compartments. These elegant experiments showed that $S1P_1$ was intrinsically required for appropriate lymphocyte egress (Matloubian et al., 2004).

Interestingly, $S1P_2$-null mice show a degenerative and progressive loss of hearing and balance (Herr et al., 2007; Kono et al., 2007; MacLennan et al., 2006). $S1P_2$ is indispensable for maintenance of vestibular and cochlear hair cells *in vivo*. $S1P_2$-null mutants in the C57Bl/6 background have also been reported to show electrophysiological defects and develop seizures (MacLennan et al., 2001). In zebrafish, a single point mutation in the $S1P_2$-related *mil* gene leads to abnormal heart development (Kupperman et al., 2000), however, this defect in not recapitulated in $S1P_2$ knockout mice (Ishii et al., 2002). $S1P_3$-null mutant mice are grossly normal, but lack some of the S1P-mediated responses. For example, they show a loss of the vasodilation response to FTY720 (Tolle et al., 2005) with MEFs from $S1P_3$-nulls showing a marked decrease in PLC activation (Ishii et al., 2001). The knockout studies also revealed some functional redundancy in that mice lacking multiple receptors have new or exacerbated phenotypes. For example, mice lacking both $S1P_2$ and $S1P_3$ receptors have

remarkably reduced litter sizes owing to an increase in perinatal lethality (Ishii et al., 2002). In addition, $S1P_{1,2,3}$ triple knockouts show severe defects in vascular development, to a greater extent than any single or double mutant, and are embryonically lethal at E10.5-11.5 (Kono et al., 2004).

The $S1P_5$-null mouse was recently reported. These mice do not have any apparent behavioral deficits or evident myelin deficiencies and their oligodendrocytes do not show defects in S1P induced process retraction and cell survival (Jaillard et al., 2005). This is surprising since $S1P_5$ expression is highly restricted and is present at significant levels only in oligodendrocytes and some hematopoetic cells. Further analyses of this mutant may reveal unrecognized phenotypes.

10.3.3 Others

The genetic study of lipid metabolic enzymes has yielded complementary data. One of the most well characterized LPA producing enzymes is LysoPLD, originally known for its nucleotide phosphodiesterase activity as a protein called Autotoxin (ATX). ATX mutants have been generated by three different groups (Ferry et al., 2007; Tanaka et al., 2006; van Meeteren et al., 2006). Heterozygous deletion of ATX results in mice that are grossly normal, but have LPA plasma levels half of those in normal mice. Homozygotes are lethal at E9.5 due to severe defects in blood vessel development and neural tube formation (Tanaka et al., 2006; van Meeteren et al., 2006).

Similar to S1P receptor mutants, individual loss of either SPHK1 or SPHK2 does not produce an abnormal phenotype. However, SPHK 1 and 2 double mutant embryos lose detectable SPHK activity. As a consequence, the double mutants are lethal prior to E13.5 with severe vascular and neural tube defects (Allende et al., 2004b; Mizugishi et al., 2005).

Some of the LP kinase and lyase mutant mice have also been reported and their phenotypes are consistent with receptor mutant mice (Escalante-Alcalde et al., 2003; Schmahl et al., 2007). However, a number of enzymes are involved in lipid metabolic pathways so the existence of functional redundancy is therefore conceivable. Ongoing multiple and conditional gene targeting studies are helping to elucidate these pathways.

10.4 Possible Relevance of LPs to Human Diseases

10.4.1 Possible Clinical Applications

10.4.1.1 Immunity/Transplantation

Both LPA and S1P have been shown to act as immunomodulators in the regulation of T-cells, B-cells, and macrophages. These immune cells are likely

regulated by combinations of LP receptors. LPA and S1P acting through $LPA_{1,2}$ and $S1P_{2,3}$ respectively may also serve as survival factors for T-cells by suppressing Bax (Goetzl et al., 1999). LPA induces migration and suppression of IL-2 production in unstimulated T-cells via LPA_2, however, once the T-cell is stimulated, LPA inhibits cell migration but activates IL-2 production via LPA_1 (Zheng et al., 2000, 2001). The expression pattern of LP receptors can also be changed during cell activation (Graler and Goetzl, 2002; Rosen et al., 2003; Zheng et al., 2000). According to recent models, S1P stimulates migration of inactive T-cells via $S1P_1$ and $S1P_4$. Upon activation, T-cells temporarily suppress receptor expression and lose the S1P mediated migration response. For retention in lymphoid organs, terminally differentiated effector T-cells then again upregulate $S1P_1$ to egress from lymph nodes (Graeler and Goetzl, 2002; Matloubian et al., 2004; Schwab and Cyster, 2007). The proper S1P gradient between plasma and lymph node is also important for lymphocyte migration (Schwab et al., 2005).

Studies with FTY720 have been of great importance in demonstrating the role of S1P signaling in immunomodulation. The phosphorylated metabolite of FTY720 (FTY720-P) is being evaluated as a clinically relevant immunosuppressant for organ transplantation. Conventional immunosuppressants like cyclosporine (cyclophilin inhibitor) and FK506 (calcineurin inhibitor) inhibit IL-2 dependent T-cell activation. The unique feature of FTY720-P is that it suppresses the immune system by inhibiting lymphocyte egress from lymphoid organs and acts as an S1P receptor modulator (Brinkmann, 2007). Thus, application of FTY720 with conventional immunosuppressants is expected to reduce the risk of conventional drug side effects like kidney toxicity from cyclosporine (Tedesco-Silva et al., 2005).

10.4.1.2 Asthma

S1P levels are dramatically upregulated in the airways of asthmatic patients following allergen exposure. Cross-linking of IgE receptors on mast cells activates SPHK1 and increases S1P levels. Activation of $S1P_2$, and to a lesser extent $S1P_1$, promotes degranulation and chemotaxis of mast cells (Jolly et al., 2002, 2004). Also, airway smooth muscle cells (SMC) express $S1P_{1\text{-}4}$, and they could modulate the SMC contraction and proliferation via the $G_{12/13}$ and $G_{i/o}$ pathways (Jolly et al., 2002). This potential therapeutic modality has been demonstrated *in vivo* with the observation that FTY720 administration can reduce the Th1 or Th2 cell-mediated lung-inflammatory responses (Sawicka et al., 2003).

10.4.1.3 Autoimmune Diseases

Since FTY720 does not generally impair lymphocyte proliferation and function, it could provide a new strategy for immunosuppression, which would be useful in transplantation, multiple sclerosis (MS), or autoimmune diabetes,

leaving crucial functions of the immune system intact (Gardell et al., 2006; Rivera and Chun, 2007).

10.4.1.4 Cancer

LP signaling has relevance to cancer. One of the better characterized cancer links is ovarian cancer. LPA elevation in the ascites of patients was reported to elicit growth factor-like activity (Mills et al., 1988), although there is controversy over the generality of this initial report. It has also been shown that LP receptors and the enzymes involved in LPA and S1P metabolisms are highly expressed in multiple cancer types, e.g. ovarian cancer and glioblastoma (Murph et al., 2006). S1P has both positive and negative effects on cancer cell growth (Hong et al., 1999). FTY720 has anti-tumor effects *in vitro* and *in vivo*, and this may be due to not only the effect on tumor cells, but also the inhibition of angiogenesis directly or indirectly (Azuma et al., 2002; Ho et al., 2005; LaMontagne et al., 2006).

10.4.1.5 Cardiovascular

Both LPA and S1P have vaso-regulatory functions, such as regulation of heart rate, blood pressure, platelet aggregation, and smooth muscle contraction (Karliner, 2004; Siess et al., 2000). Atherosclerosis is a type of accelerated vasculitis that reduces blood flow leading to heart attacks and strokes (Siess, 2002). It is well known that HDL level correlates with a reduced risk of cardiovascular disease, such as atherosclerosis (Choi et al., 2006), and it has been recently shown that it is the S1P content of HDL that mediates many of its effects. For example, HDL induces vasodilation and myocardial perfusion by activation of $S1P_3$ (Levkau et al., 2004; Nofer et al., 2004). Furthermore, in an *in vivo* mouse study, HDL and S1P reduce the infarction size about by 20 and 40% and also inhibit inflammation caused by the recruitment of polymorphonuclear leukocytes and cardiomyocyte apoptosis via the $S1P_3$ receptor eNOS/NO pathway (Theilmeier et al., 2006).

10.4.1.6 Hearing Loss

As mentioned above, $S1P_2$-null mice lose hearing and have balance defects (Herr et al., 2007; Kono et al., 2007; MacLennan et al., 2006). It may be possible to prevent the degeneration of hair cells with a selective S1P signaling modulator. These studies are ongoing and may offer novel treatment modalities for the prevention of age-related and ototoxic hearing loss.

10.4.1.7 Wound Healing (CNS)

LPA and S1P in blood may enter the brain during central nervous system (CNS) injury. An experimentally caused brain hemorrhage provides an influx of 1–10 μM of LPA in the cerebrospinal fluid (Tigyi et al., 1995). In cerebral

infarction, platelet aggregation can release micromolar concentrations of LPA and could also lead to increased LPA levels in CSF (Eichholtz et al., 1993). Indeed, intracranial injection of LPA or S1P causes astrogliosis *in vivo* (Sorensen et al., 2003). Reactive astrogliosis is a prominent component of CNS injury, and this would benefit from further study of LP signaling modulator applications.

10.4.1.8 Pain

In animal models, nerve injury to the dorsal root results in the development of behavioral allodynia and hyperalgesia paralleled by demyelination. Intrathecal injection of LPA, but not S1P, initiates behavioral, morphological, and biochemical symptoms of neuropathic pain via an LPA_1-mediated Rho/Rho-kinase pathway (Inoue et al., 2004). LPA signaling modulation may be relevant for some forms of neuropathic pain, an area of significant, unmet medical need (Dworkin et al., 2007).

10.4.1.9 Female Reproduction

Recent studies show that LPA_3 has a crucial role in blastocyst implantation through COX-2, which generates prostaglandins (PGs) E_2 and I_2 (Hama et al., 2006, 2007; Shah and Catt, 2005; Ye et al., 2005). S1P can also act to prevent intrinsic, chemical, and irradiation-induced oocyte apoptosis. S1P pretreatment improves the rate of successful pregnancy in irradiated mice (Morita et al., 2000; Tilly, 2001). Thus, controlling LP signaling could be a valuable therapeutic option in human infertility.

10.4.2 Pharmacology (Agonists and Antagonists)

About 40% of drugs on the market in the United States target GPCRs. Furthermore, over 2% of genes in the human genome are estimated to encode GPCRs (over 1000) (Tyndall and Sandilya, 2005). Screening efforts are underway to identify chemicals that agonize and antagonize LP signaling (Chun and Rosen, 2006; Delgado et al., 2007; Herr and Chun, 2007). A computational approach is also being performed to design drugs and assess receptor specificity based on the structure of ligand binding pockets and amino acid residues required for ligand binding (as mentioned in Section 10.2.1.) (Holdsworth et al., 2004; Parrill et al., 2000).

10.4.2.1 LPA Pharmacological Tools

Several LPA receptor agonists or antagonists have been reported, although most show modest selectivity and lack *in vivo* validation, which should be considered in any experimental usage, particularly for *in vivo* studies.

Agonists: N-acyl ethanolamide phosphate (NAEPA) is an LPA analog which has an ethanol amine backbone (Lynch et al., 1997). A screening of a 2-Oleoyl LPA derivative which had a pyran ring to stabilize the head group was performed and one LPA_1-selective agonist, two LPA_3-selective LPA agonists, and one LPA_3-selective antagonist were identified with this scheme (Tamaruya et al., 2004).

Antagonists: VPC-12449 is an LPA_1- and LPA_3-selective compound that can protect against LPA_3-mediated renal ischemia-reperfusion injury in a mouse model (Okusa et al., 2003). A natural lipid metabolite, diacylglycerol pyrophosphate (DGPP), was shown to act as an LPA_1 and LPA_3 specific antagonist (Fischer et al., 2001). Ki16425 is an LPA_1 and LPA_3 selective antagonist with little resemblance to LPA (Ohta et al., 2003). This compound can inhibit breast cancer cell proliferation and bone metastasis in mice (Boucharaba et al., 2006).

10.4.2.2 S1P Pharmacological Tools

There are also several S1P receptor agonists and antagonists which have different receptor selectivities.

Agonists: AAL-(R) is non-selective S1P receptor agonist which has structural and functional similarities to FTY720 (Brinkmann et al., 2002; Rosen et al., 2003). Another agonist, KRP-203 prevents allograft rejection, but does not affect $S1P_3$ signaling (Fujishiro et al., 2006). KRP-203 ($S1P_1 > S1P_3$) is currently in Phase I clinical trials for the treatment of multiple sclerosis (Novartis). SEW2871 and AUY954 are $S1P_1$ specific agonists and have been shown to function to prevent appropriate lymphocyte egress and inhibit allograft rejection, respectively (Pan et al., 2006; Sanna et al., 2004). Receptor-selectivity is expected to show greater efficacy with minimal undesirable side effects.

Antagonist: JTE-013 is an $S1P_2$ specific antagonist (Yokoo et al., 2004). A recently reported $S1P_1$ specific antagonist called W146 can induce loss of capillary integrity (Sanna et al., 2006). In addition, there are a number of agonists/antagonists that have been described with varying affinities for the different receptor subtypes (Clemens et al., 2003, 2004; Davis et al., 2005; Im et al., 2001).

FTY720 (FTY720-P): FTY720 is perhaps the best characterized S1P receptor agonist and deserves special consideration. FTY720 is currently in Phase III clinical trials for the treatment of multiple sclerosis (Novartis). FTY720 was initially isolated from the fungi *Ascomycetes* in 1995 and identified as an immunosuppressive agent (Adachi et al., 1995). FTY720 administration significantly increases the survival rate of canine kidney allograft recipients (Suzuki et al., 1996). Recent reports have shown that FTY720 is phosphorylated by SPHK2 but not SPHK1 (Allende et al., 2004b; Kharel et al., 2005), and it inhibits T and B-cell egress from lymph nodes by modulating S1P signaling (Schwab and Cyster, 2007; Zemann et al., 2006). An increase in S1P levels, as seen by S1P lyase inhibition, also inhibits lymphocyte egress from lymph nodes (Schwab et al., 2005). Other $S1P_1$ antagonists, AAL-(R) and SEW2871, can inhibit thymocyte egress *in vivo* (Rosen et al., 2003), and an $S1P_1$ partially

selective agonist, KRP-203, sequesters circulating lymphocytes into peripheral lymphoid organs (Shimizu et al., 2005). These data suggest that FTY720 and related agonists mimic high dose S1P exposure. FTY720 can also induce poly-ubiquitination and proteasomal degradation of $S1P_1$ (Oo et al., 2007), that could remove receptors from further agonism. In addition, an $S1P_1$ deficiency arrests thymocyte development at the CD69 positive stage, and prevents lymphocyte egress, as mentioned above (Allende et al., 2004a; Matloubian et al., 2004). These data suggest that FTY720 can result in degradation of the S1P receptor, acting as a functional antagonist of S1P signaling. However, some controversies concerning the immunosuppression mechanism by which FTY720 operates still remain (Chun, 2007), and this is still an active research area.

FTY720 represents the first generation of LP receptor modulators that may have therapeutic value. Other data concerning the efficacy of FTY720 administration for the treatment of type I diabetes, uveoretinitis, thyroiditis, myocarditis, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis in animal models have been reported (Fujino et al., 2003; Hozumi et al., 1999; Kurose et al., 2000; Matsuura et al., 2000; Okazaki et al., 2002; Suzuki et al., 1998; Webb et al., 2004). The potential to treat medically important diseases through LP receptor modulation represents an attractive and technically tractable approach that is being actively assessed.

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