Acta Biochimica Polonica

Vol. 55 No. 2/2008, 227–240

on-line at: www.actabp.pl

Review

Lysophosphatidic acids, cyclic phosphatidic acids and autotaxin as promissing targets in therapies of cancer and other diseases

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Received: 17 March, 2008; revised: 20 May, 2008; accepted: 30 May, 2008
available on-line: 14 June, 2008

Lysophospholipids have long been recognized as membrane phospholipid metabolites, but only recently lysophosphatidic acids (LPA) have been demonstrated to act on specific G protein-coupled receptors. The widespread expression of LPA receptors and coupling to several classes of G proteins allow LPA-dependent regulation of numerous processes, such as vascular development, neurogenesis, wound healing, immunity, and cancerogenesis. Lysophosphatidic acids have been found to induce many of the hallmarks of cancer including cellular processes such as proliferation, survival, migration, invasion, and neovascularization. Furthermore, autotaxin (ATX), the main enzyme converting lysophosphatidylcholine into LPA was identified as a tumor cell autocrine motility factor. On the other hand, cyclic phosphatidic acids (naturally occurring analogs of LPA generated by ATX) have anti-proliferative activity and inhibit tumor cell invasion and metastasis. Research achievements of the past decade suggest implementation of preclinical and clinical evaluation of LPA and its analogs, LPA receptors, as well as autotaxin as potential therapeutic targets.

Keywords: autotaxin/NPP2, lysophosphatidic acid, cyclic phosphatidic acid, G protein-coupled receptors

INTRODUCTION

Lysophosphatidic acids (LPA; 1-acyl-2-hydroxy-*sn*-glycero-3-phosphates) (Fig. 1) are simple phospholipids consisting of various compounds bearing both saturated (16:0, 18:0) and unsaturated (18:1, 18:2, 20:4) fatty acid chains. They have been recognized for decades as components of biological membranes. However, they are also ubiquitous bioactive molecules influencing a broad variety of cellular processes, particularly proliferation, differentiation, and migration. Although all cells contain small amounts of LPA associated with membrane biosynthesis, some cellular sources (such as activated platelets) can produce significant amounts of extracellular LPA, which account for the LPA found in serum (Eichholtz *et al.*, 1993). Although a signaling role of lysophosphatidic acids has been recognized, the identification and cloning of cell surface G protein-coupled receptors (GPCRs) having high affinity for LPA (LPA_1–LPA_5) in the last several years has dramatically improved our understanding of the diverse roles of LPA in biological processes (Meyer zu Heringdorf & Jacobs, 2007). Recently, peroxisome

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Abbreviations: AC, adenyl cyclase; ATX, autotaxin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; cPA, cyclic phosphatidic acids; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; LDL, low-density lipoproteins; LPA, lysophosphatidic acids; $LPA_{1\text{-}5}$, LPA receptor type 1-5; LPAAT, LPA acyl transferases; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPLs, lysophosholipids; LPPs, lipid phosphate phosphatases; LPS, lysophosphatidylserine; lysoPLD, lysophospholipase D; MAG, monoacylglycerol; moxLDL, mild oxidation of low density lipoproteins; NPP, nucleotide pyrophosphatase/phosphodiesterase; PA, phosphatidic acids; PKCγ, protein kinase C γ; PPARγ, peroxisome proliferator-activated receptor γ; S1P, sphingosine-1-phosphate; SPC, sphingosyl-phosphorylcholine; TZDs, thiazolidinediones; VEGF, vascular endothelial growth factor; VSMCs, vascular smooth muscle cells.

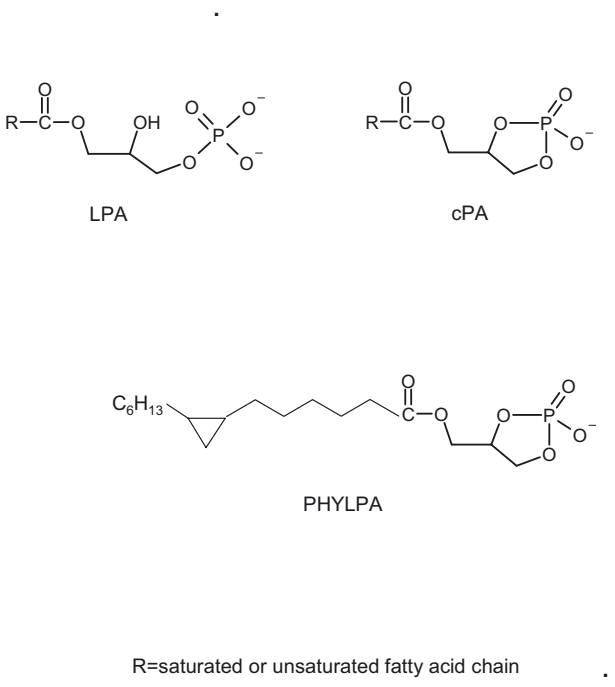

Figure 1. Structures of lysophosphatidic acid (LPA), cyclic phosphatidic acid (cPA), and PHYLPA.

proliferator-activated receptor γ (PPARγ) has been also identified as an intracellular receptor for LPA (McIntyre *et al.*, 2003). The widespread expression of LPA receptors and coupling to several classes of G proteins allow regulation of numerous processes, such as vascular development, neurogenesis, immunity, cancerogenesis (Gardell *et al.*, 2006) and wound healing (Watterson *et al.*, 2007).

It is worth to mention that among the most intensively studied lysophospholipids there is not only LPA but also sphingosine-1-phosphate (S1P). This molecule has diverse signaling functions mediated by five cell membrane receptors ($S1P_1$ to $S1P_5$). One of S1P analogs (FTY720 or 2-amino-2[2-(4-octylphenyl)ethyl]-1,3-propanediol also known as *fingolimod*) acts as an agonist of S1P and is currently tested in Phase III clinical trials for multiple sclerosis, and, independently, in Phase III as a novel immunosuppressant for kidney transplantation (Gardell *et al.*, 2006).

The studies on clinical evaluation of S1P as a therapeutic target have indicated that interventions in LPA signaling might also provide new therapeutic approaches for the treatment of human diseases. The pleiotropic roles of lysophospatidic acids in cancer behavior suggest that novel LPA-related drugs would have wide applicability in the treatment of many types of tumors. A significant progress in this field as well as unexpected results were recently achieved and therefore this review will describe mainly LPA metabolism and LPA-mediated signaling (van Meeteren & Moolenaar, 2007).

LPA RECEPTORS

Cell surface receptors in LPA signaling

An extracellular action of LPA was first mentioned in the 1970s when Tokumura *et al.* (1978) described the effect of LPA on blood pressure. However, clear evidence of LPA as an extracellular signaling molecule only came in the late 1980s (van Corven *et al.*, 1989). The first gene for an LPA receptor (LPA_1) was identified in 1996 during a search for genes with predominant expression in the ventricular zone of the cerebral cortex. Hecht *et al.* (1996) identified ventricular zone gene 1 (*VZG-1*) that was shown to encode a high-affinity GPCR for LPA (further renamed *EDG2* based on its similarity to "*endothelium differentiation genes*" — *EDGs*). Subsequently, sequence similarities allowed rapid identification of further cognate LPA receptors (Contos *et al.*, 2000). The second LPA receptor gene, *EDG4*, was identified by An *et al.* (1998) on the basis of functional studies. The encoded protein was determined to be another LPA receptor (LPA_2). In 1999 Bandoh and coworkers isolated a human cDNA encoding the third LPA receptor (LPA_3) and designated it *EDG7*. Because of the inconsistency of the LPA receptors' nomenclature, in 2002 *EDG2/VZG-1*, *EDG4* and *EDG7* genes were renamed as LPA_1, LPA_2 and LPA_3 following the guidelines of IUPHAR (International Union of Pharmacology). Then, in 2003 Noguchi and coworkers identified p2y(9)/GPR23 as the fourth LPA receptor, LPA_4. With only 20–24% amino-acid homology with Edg-2/LPA_1, Edg-4/LPA_2, and Edg-7/LPA_3, LPA_4 is evolutionarily distant from other LPA receptors, which share 50–57% identity of their amino-acid sequences. Recently, Lee *et al.* (2006a) demonstrated that the orphan GPR92 is activated by LPA, and named it LPA_5. A comparison of amino-acid sequences showed that this receptor had approx. 35% amino-acid identity with the LPA_4 receptor (Lee *et al.*, 2006a) and only between 21.3 and 22.6% homology with the LPA_{1-3} family of receptors (Kotarsky *et al.*, 2006).

LPA receptors couple to members of three major G protein families, G_i, G_q, and G_{12} (Table 1). LPA_1 and LPA_2 are known to interact with members of all three aforementioned G protein families, while LPA_3 interacts with G_i and G_q proteins only. LPA_4 and LPA_5 elevate cAMP levels, but their coupling to G_s proteins has yet to be proven. The LPA_5 receptor additionally interacts with G_q and G_{12} proteins (Meyer zu Heringdorf & Jacobs, 2007). The

Table 1. LPA receptors-mediated signaling pathways

Receptor	Expression in human organs	G family	Effector	References
LPA_1	Widely expressed; low expression in skeletal muscle, kidney, lung and thymus; not detected in liver and peripheral blood leukocytes	G_i, G_q, G_{12}	AC↓ ERK↑ Akt↑ Rho↑ PLC↑ $[Ca^{2+}]_i$↑	An *et al.* (1998), Anliker *et al.* (2004), Meyer zu Heringdorf & Jacobs (2007)
LPA_2	High level in testis and leukocytes; moderate in pancreas, thymus, spleen and prostate; little or no expression in brain, heart, lung, liver, kidney, muscle, ovary, placenta, intestine and colon	G_i, G_q, G_{12}	Rho↑ PLC↑ $[Ca^{2+}]_i$↑ ERK↑ Akt↑ AC↓	An *et al.* (1998) Anliker *et al.* (2004), Meyer zu Heringdorf & Jacobs (2007)
LPA_3	High level in heart, prostate, brain, pancreas and testis; moderate level in lung and ovary	G_i, G_q	PLC↑ $[Ca^{2+}]_i$↑ ERK↑ AC↓	Bandoh *et al.* (1999) Anliker *et al.* (2004), Meyer zu Heringdorf & Jacobs (2007)
LPA_4	Weakly expressed (high level only in ovary)		AC↑ $[Ca^{2+}]_i$↑	Noguchi *et al.* (2003)
LPA_5	Weakly expressed in most organs; strong expression in small intestine, spleen, dorsal root ganglion cells and embryonic stem cells	G_q, G_{12}	AC↑ $[Ca^{2+}]_i$↑	Lee *et al.* (2006a)

major effector systems of LPA receptors include phoshatidylinositol-3-kinase (PI3K), G_i — Ras — extracellular signal regulated kinase (ERK), G_q — phospholipase C (PLC), and a small GTPase (Rho). Activation of PLC, ERK or Rho *via* LPA receptors results in cell proliferation as well as survival and changes in cell morphology.

One cannot exclude that some other LPA receptors (membrane or intracellular) may be identified in the future. One of the candidates for intracellular LPA receptors appears to be peroxisome proliferator-activated receptor γ (PPARγ) (McIntyre *et al.*, 2003).

Interaction of LPA with peroxisome proliferator-activated receptor γ (PPARγ)

The peroxisome proliferator-activated receptors (PPARs) are nuclear fatty acid receptors that have been implicated to play an important role in obesity-related metabolic diseases such as hyperlipidemia, insulin resistance, and coronary artery disease (Lee *et al.*, 2003a). PPARγ controls transcription of genes that in general are involved in energy metabolism (glucose and fatty acids), is essential for adipocyte differentiation and also modulates anti-inflammatory as well as antineoplastic reactions. PPARγ is activated after binding natural ligands such as polyunsaturated fatty acids or prostaglandin metabolites. It can also be activated by synthetic ligands such as thiazolidinediones (TZDs, e.g. rosiglitazone, pioglitazone or troglitazone) (Lehmann *et al.*, 1995). Most of them bind predominantly to PPARγ in adipose cells. The binding of TZDs to their receptors increases insulin sensitivity, and therefore TZDs are frequently administered to patients with insulin resistance associated with type II diabetes.

McIntyre *et al.* (2003) showed that LPA, but not their precursors (phosphatidic acids) displaced rosiglitazone from the ligand-binding pocket of a purified PPARγ protein. Using RAW 264.7 monocytic cells transfected with a luciferase gene controlled by peroxisome proliferator response element, they showed that LPA stimulated transcription of the reporter gene. However, unlike rosiglitazone (a PPARγ agonist), LPA were unable to induce PPARγ activity in adipocytes (Simon *et al.*, 2005). Although LPA were demonstrated to bind to PPARγ in an *in vitro* assay (McIntyre *et al.*, 2003), their ability to activate PPARγ appears to be dependent on the cell type. In adipocytes, activity of high ecto-lipid phosphate phosphohydrolase was observed, which dephosphorylates and inactivates LPA (Simon *et al.*, 2002). This finding also suggests that ecto-lipid phosphate phosphohydrolase activity could be weaker in monocytes than in adipocytes, allowing a higher amount of LPA to enter into the cells and activate PPARγ.

LPA METABOLISM

Enzymes involved in LPA production

LPA have long been known as products of the lipid synthetic pathways generated from glycerol-3-phosphate and acyl-CoA by glycerolphosphate

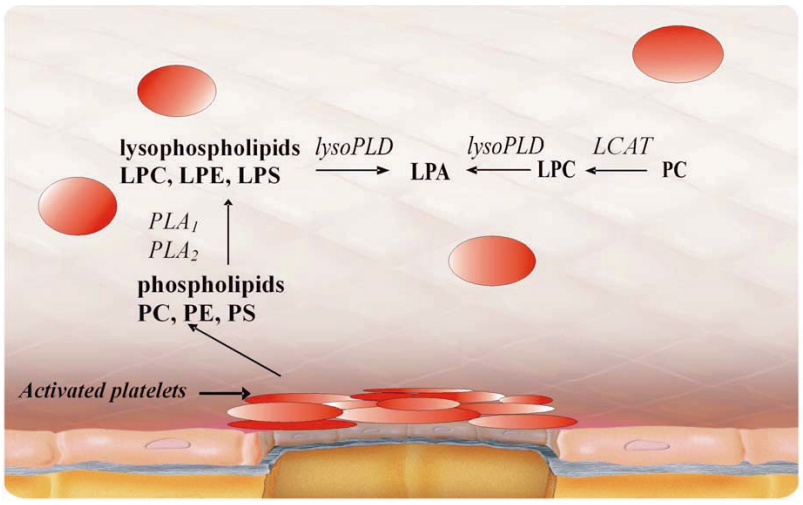

Figure 2. Synthesis of LPA in serum. In a platelet-dependent pathway, activated platelets release large amounts of phospholipids which are then converted by phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2) to lysophospholipids (LPLs) such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS). Subsequently LPA is generated from LPLs by a plasma enzyme lysophospholipase D (lysoPLD). About half of serum LPA is produced by a platelet-independent pathway from lysophosphatidylcholine. Plasma LPC is synthesized mainly by lecithin-cholesterol acyltransferase (LCAT) which catalyzes transesterification of phosphatidylcholine and free cholesterol.

acyl transferase. The conversion occurs in the endoplasmic reticulum and mitochondria (Aoki, 2004). However, new mechanisms of LPA production have also been identified both in cells and in biological fluids, where multiple pathways occur under various conditions. LPA are produced extracellularly by lipoprotein oxidation (Siess *et al.*, 1999) or by the action of secretory phospholipase A_2 on microvesicles released from activated cells (Fourcade *et al.*, 1995). LPA are also produced in plasma by thrombin-activated platelets through the stimulated release of phospholipase A_1, phospholipase A_2 (Sano *et al.*, 2002), and lysophospholipase D (autotaxin) (Tokumura *et al.*, 2002; Umezu-Goto *et al.*, 2002), which act on plasma lipids or secreted lysophosphatidylcholine (Fig. 2).

Mild oxidation of low density proteins (moxLDL) catalyzed by Cu^{2+} changes the biological properties of LDL making them prothrombotic and proatherogenic. Siess *et al.* (1999) discovered that lysophosphatidic acids are formed during mild oxidation of LDL and are responsible for platelet activation induced by moxLDL and minimally modified LDL. They also found that LPA accumulate in the intima of human atherosclerotic lesions. LPA and moxLDL activate Rho and Rho-kinase through $G_{12/13}$ and this pathway mediates the reorganization of the actin cytoskeleton underlying platelet shape change (Retzer & Essler, 2000). In addition, LPA and moxLDL stimulate a different pathway during shape change. Thus the activation of the Src family of tyrosine kinases and the tyrosine kinase Syk occurs, which mediates the exposure of fibrinogen-binding sites on integrin $\alpha_{IIb}\beta_3$ during a shape change, which is a prerequisite for platelet aggregation (Bauer *et al.*, 2001). LPA molecules present in the core region of atherosclerotic plaques trigger rapid platelet activation through the stimulation of LPA_1 and LPA_3 receptors (Rother *et al.*, 2003). Finding of efficient antagonists of platelet LPA receptors might provide a new strategy to prevent thrombus formation in patients with cardiovascular diseases.

In serum and plasma LPA are mainly produced from lysophospholipids. By contrast, in platelets and some cancer cells they are created from phosphatidic acids (PA). Serum is the best characterized source of LPA. In serum incubated for 1 h, total LPA levels are significantly higher (0.85–1.5 μM) than those measured in corresponding plasma samples (0.6–0.7 μM). The overall increase of total LPA content in serum *versus* EDTA-anticoagulated plasma is due to the contribution of platelet-derived LPA, which was determined to be 0.6 μM following a 1-hour incubation at 25°C (Baker *et al.*, 2001). It has been proposed that LPA in serum are produced as a result of blood coagulation and, therefore, platelets are involved, in part, in the production of these phospholipids in serum. Activated platelets produce and release a large amount of lysophosholipids (LPLs) such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS). Aoki *et al.* (2002) propose that in a platelet-dependent pathway platelets supply LPLs which are produced by phospholipase A_1 (PLA_1) hydrolysing fatty acids at the *sn*-1 position and phospholipase A_2 (PLA_2) hydrolysing them at the *sn*-2 position. Subsequently, these LPLs are converted to LPA by a plasma enzyme lysophospholipase D (Fig. 2). This pathway requires activation of platelets. Thus, LPA are produced through this pathway under pathological conditions such as injury, inflammation or artherosclerosis.

About half of serum LPA are produced by a platelet-independent pathway. Their levels in freshly prepared plasma or serum are much lower than in samples incubated for 24 h at 25°C (about 1 μM and 5 μM, respectively) (Baker *et al.*, 2001), indicating that lysophosphatidic acids are also produced in a cell-free system. Tokumura *et al.* (1986) first showed

that LPA are created in plasma from lysophosphatidylcholines by lysophosphospholipase D (lysoPLD). This activity has been detected in plasma and serum in a wide variety of mammalian species including rat, mouse, cattle, and humans (Aoki, 2004). Initially, only LPC was considered as a substrate for serum lysoPLD. However, the enzyme also acts on other LPLs including LPE, LPS and LPI (lysophosphatidylinositols) (Aoki *et al.*, 2002). LysoPLD is specific for the "lyso" forms of phospholipids; this means that the enzyme does not act on phosphatidylcholines (PC) or other diacyl phospholipids. Plasma lysoPLD was purified in 2002 and it was found to be identical with autotaxin (ATX) — an earlier identified cell motility-stimulating factor (Stracke *et al.*, 1992; Umezu-Goto *et al.*, 2002; Tokumura *et al.*, 2002). Since lysoPLD/ATX is a key enzyme of significant biological importance, its detailed characterization will be further discussed (see next chapter). Lysophosphatidylcholines — one of the main substrates for lysoPLD — exist in plasma at a concentration of 125–150 μM, making them the most abundant lysophospholipids in plasma (Tigyi & Parrill, 2003). Plasma LPC are synthesized mainly by two enzymes: liver PLA_1 and lecithin-cholesterol acyltransferase (LCAT), which catalyze transesterification of phosphatidylcholines and free cholesterol.

Lysophosphatidic acids are also produced and released from many types of cells. However, in contrast to the mechanism of LPA production in serum and plasma, the mechanisms of their synthesis in cells are still ambiguous. Fourcade *et al.* (1995) first demonstrated LPA production from microvesicles shed by activated cells, such as erythrocytes, platelets and white blood cells. LPA are generated as a result of cellular activation induced by various stimuli (e.g. phorbol esters, bombesin, ATP, and LPA itself) (Aoki, 2004). The major pathway consists of two steps: production of phosphatidic acids (PA) and subsequent conversion of PA to LPA. The first step of this pathway, conversion of diacyl phospholipids (for example: PC) to phosphatidic acids, is mainly catalyzed by intracellular phospholipase D (PLD) (Cummings *et al.*, 2002). The next step, the conversion of PA to LPA, is catalyzed by PLA_1 and PLA_2 phospholipases.

The co-existence of the various pathways responsible for LPA synthesis suggests that this process has to be highly regulated under *in vivo* conditions, but details of this regulation remain to be explained.

Autotaxin/NPP2 — a secreted lysophospholipase D

A major step towards understanding of the biological activities of LPA was made by the unexpected discovery that the lysophospholipase D producing LPA in serum was identical with autotaxin (ATX, also known as NPP2), a widely expressed nucleotide pyrophosphatase/phosphodiesterase (NPP) (Umezu-Goto *et al.*, 2002; Tokumura *et al.*, 2002). Autotaxin has been known since the early 1990s as an ecto-nucleotide pyrophosphatase/phosphodiesterase that stimulates the motility of A2058 melanoma cells in a pertussis toxin-sensitive manner (Stracke *et al.*, 1992). Molecular cloning of ATX has revealed that it is a member of the NPP family which also includes NPP1/PC-1, gp130RB$^{13\text{-}6}$/NPP3, NPP4, NPP5, NPP6 and NPP7 (Stefan *et al.*, 2005). NPP1-3 hydrolyzes nucleotides and some of their derivatives with the formation of nucleoside-5′-monophosphates. The phosphodiesterase activity of ATX/NPP2 could not explain the involvement of the enzyme in the stimulation of melanoma cells and tumor progression. It was difficult to envision how altered nucleotide processing in the extracellular environment could account for these biological effects of ATX. Finally, the identification of ATX/NPP2 as a lysophospholipase D allowed the previously unconnected areas of cell biology to be linked: the biological activities of the NPP family members and LPA synthesis and signaling. ATX/NPP2 is unique in that it is the only lysoPLD within the NPP family. However, it was found that the K_m values for LPC and nucleotide substrate are 100–150 μM and 0.9–1.0 mM, respectively. This indicates that although ATX/lysoPLD is capable of hydrolyzing nucleotides *in vitro*, it primarily functions as a lipid phosphodiesterase (van Meeteren *et al.*, 2005).

ATX/lysoPLD has no homology to previously characterized phospholipases. Instead, similarly to its closest relatives (NPP1 and NPP3), ATX has a domain structure consisting of two N-terminal somatomedin B-like (SMB) domains, a central catalytic phosphodiesterase (PDE) domain and a C-terminal nuclease-like domain (Stefan *et al.*, 2005). Amino acids from all these domains form a lysophospholipid-binding pocket. The central catalytic domain of ATX (approx. 400 amino acids) is, however, structurally unrelated to those found in typical phospholipases; it has been predicted to fold similarly to the catalytic domains of alkaline phosphatases. The C-terminal domain of NPP1-3 is structurally related to non-specific DNA or RNA endonucleases, however, it lacks the key residues required for enzymatic activity. An intact nuclease-like domain is needed for proper folding, subcellular localization and secretion. Full-length ATX is synthesized as a pre-pro-enzyme and its secretion requires specific proteolytic cleavage. Following the removal of a 27-amino-acid fragment by the signal peptidase, NPP2 is subsequently cleaved by proprotein convertases (PCs). Then it traffics along the classical export pathway and is se-

creted as an active glycoprotein (Jansen *et al.*, 2005). ATX is N-glycosylated on three sites (N52, N410 and N524). Mutagenesis studies showed that the glycosylation of N524, a conserved residue in the catalytic domain, was absolutely required for the activity of ATX (Jansen *et al.*, 2007).

The human ATX-encoding gene, *ENPP2*, is organized in 27 exons. Alternative splicing results in generation of three different isoforms (van Meeteren & Moolenaar, 2007). The shortest and the most abundant form (ATX^{ter}), having 863 amino acids, was initially cloned from a teratocarcinoma cell line and is identical with plasma lysoPLD. The melanoma-derived isoform (ATX^{mel}; 915 amino acids) contains a highly basic insertion (52 residues) in the catalytic domain. A less frequent, 'brain-specific' isoform (originally termed PD-1α) is expressed mainly in oligodendrocytes and contains a 24-residue insertion close to the nuclease-like domain.

NPP2 is expressed in many cell lines and tissues with the highest mRNA levels detected in brain, ovary, lung, intestine and kidney. As a secreted protein, NPP2 accumulates in body fluids such as plasma and cerebrospinal fluid. In addition, its elevated expression has been observed during tumor progression, and this upregulation correlates with the invasiveness of the cancer. ATX was originally identified as a tumor cell motility factor of A2058 melanoma cells (Stracke *et al.*, 1992). This protein is overexpressed in several human cancers, including glioblastoma, lung and breast cancer, renal cell carcinoma, neuroblastoma, thyroid carcinoma and Hodgkin lymphoma (Kishi *et al.*, 2006). The highest ATX expression is detected in glioblastoma multiforme (GBM), a very malignant cancer with high infiltration rate. Autotaxin stimulates cell survival, proliferation, contraction and migration, and these effects can all be due to its ability to generate signaling molecules such as lysophosphatidic acids. The formation of LPA can also account for the ability of ATX to promote tumor progression, angiogenesis and metastasis. In addition to converting LPC into LPA, ATX can also hydrolyze sphingosyl-phosphorylcholine (SPC) to yield sphingosine 1-phosphate (S1P). However, the physiological significance of the SPC-to-S1P conversion is doubtful, since plasma levels of SPC are >1 000-fold lower than those of LPC, and in addition ATX hydrolyzes SPC much less efficiently than LPC (Clair *et al.*, 2003).

Although many problems were solved when the biology associated with ATX was linked to the formation of LPA, one significant question remained, namely, how are plasma LPA levels maintained in such a low nanomolar range? ATX is a ubiquitous plasma protein, and the substrate, LPC, is abundantly present in plasma at a 125–150 μM concentration (Tigyi & Parrill, 2003). Therefore, why do plasma LPA levels reach only 100 nM (Aoki *et al.*, 2002)? van Meeteren *et al.* showed that ATX is a subject of feedback inhibition by enzymatic hydrolysis products, LPA and sphingosine 1-phosphate (2005). This phenomenon offers the most likely explanation of the low plasma LPA levels and provides insight into modulation of ATX activity.

Cyclic phosphatidic acids — naturally occurring analogs of LPA generated by ATX

There are several lines of evidence that ATX is capable of producing not only LPA, but also cyclic phosphatidic acids (cPA) (Fig. 1) which contain a dioxaphospholane ring spanning the *sn*-2 and *sn*-3 positions of glycerol. The first cPA family member was originally isolated from myxoamoebae of a true slime mold, *Physarum polycephalum*, and designated as PHYLPA (Murakami-Murofushi *et al.*, 1992). This compound had a cyclopropane-containing fatty acyl chain and a cyclic phosphate joining the *sn*-2 and *sn*-3 positions of glycerol (Fig. 1). PHYLPA reversibly inhibited activity of eukaryotic DNA polymerases of α-family (Murakami-Murofushi *et al.*, 1992) and cell proliferation (Murakami-Murofushi *et al.*, 1993).

Cyclic phosphatidic acids were also detected as physiological constituents of human serum (Kobayashi *et al.*, 1999). Recently it was found that ATX is largely responsible for their generation in mammalian serum (Tsuda *et al.*, 2006). ATX has the ability to synthesize cPA by catalysis of intramolecular transphosphatidylation in LPC molecules (Fig. 3). Although the structure of cPA is similar to that of lysophosphatidic acids, their biological activities are apparently distinct from those of LPA. Cyclic phosphatidic acids have anti-proliferative activity (Murakami-Murofushi *et al.*, 1992) and inhibit tumor cell invasion and metastasis both *in vitro* and *in vivo* (Ishihara *et al.*, 2004) (Table 2). Previous reports on the anti-invasive properties of cPA suggested their role in increasing cellular cAMP levels (Murakami-Murofushi *et al.*, 1993) and subsequent RhoA inactivation (Mukai *et al.*, 2000). These observations implicated cell surface GPCR activation as the response. However, natural cPA isoforms as well as various

Table 2. Comparison of biological activities of lysophosphatidic acids (LPA) and cyclic phosphatidic acids (cPA)

Biological response	LPA	cPA
Cell proliferation	generally activation	inhibition
Tumor cell invasion and metastasis	generally activation	inhibition
$[Ca^{2+}]_i$	increased	increased
cAMP level	decreased	increased

cPA analogs were found to be poor activators of the LPA_1, LPA_2, LPA_3, and LPA_4 receptors (Baker *et al.*, 2006). On the other hand, it has been shown that cPA and their analogs are potent inhibitors of ATX activity and LPA production and thus these compounds can block cancer cell invasion *in vitro* and tumor cell metastasis *in vivo* (Baker *et al.*, 2006; Uchiyama *et al.*, 2007). Those results raised the possibility for the use of cPA as a potential target in cancer therapy. However, it should be remembered that hydrolytic cleavage of the cyclic phosphate ring will lead to the formation of LPA which are well known to augment tumor invasion and metastasis. Furthermore, cleavage of the fatty acid by phospholipases may limit the effective half-life of cPA. Hence, different analogs of cPA were recently synthesized rather than unmodified cPA. Among these novel compounds, so-called carba derivatives of cPA (ccPA), in which the phosphate oxygen was replaced with a methylene group at either the *sn*-2 or the *sn*-3 position, showed a much more potent inhibitory effect on the migration of cancer cells *in vitro* and metastasis *in vivo* than the natural cPA (Uchiyama *et al.*, 2007).

Degradation of LPA

In view of the pleiotropic effects generated by LPA, not only their synthesis but also degradation is a precisely regulated multistep process. The degradation of extracellular LPA can be mainly attributed to the ectophosphatase activity of plasma membrane lipid phosphate phosphatases (LPPs). LPPs are integral membrane proteins that dephosphorylate a variety of phospholipid substrates. Three mammalian LPP isoforms, termed LPP1, LPP2 and LPP3, have been cloned. In addition, a catalytically active splice variant of LPP1, termed LPP1a, and truncated catalytically inactive forms, termed LPP1b and LPP1c, have been identified, although their physiological relevance is not yet known (Pyne *et al.*, 2004). Various reports of their substrate specificity suggest that, in general, LPP1 and LPP3 can readily hydrolyze PA and LPA thereby producing diacylglycerols and monoacylglycerols, whereas LPP2 is most active against LPA and S1P, producing monoacylglycerols and sphingosine.

The second LPA conversion pathway involves the action of lysophosphatidic acid acyltransferases (LPAAT). These enzymes catalyze the transfer of an acyl group from acyl-CoA to LPA to form PA. Five members of the LPAAT family have been identified and sequenced: LPAATα, LPAATβ, LPAATγ, LPAATδ and LPAATε (Tigyi & Parrill, 2003). Two of them, LPAATα and LPAATβ, appear responsible for most of the LPAAT activity in cells due to their higher catalytic potency in relation to other family members. LPAATα shows a marked preference for LPA over other acyl acceptors, including LPC, LPE and LPI.

BIOLOGICAL ACTIVITIES OF LPA

Lysophosphatidic acids are required to maintain homeostasis of many physiological processes,

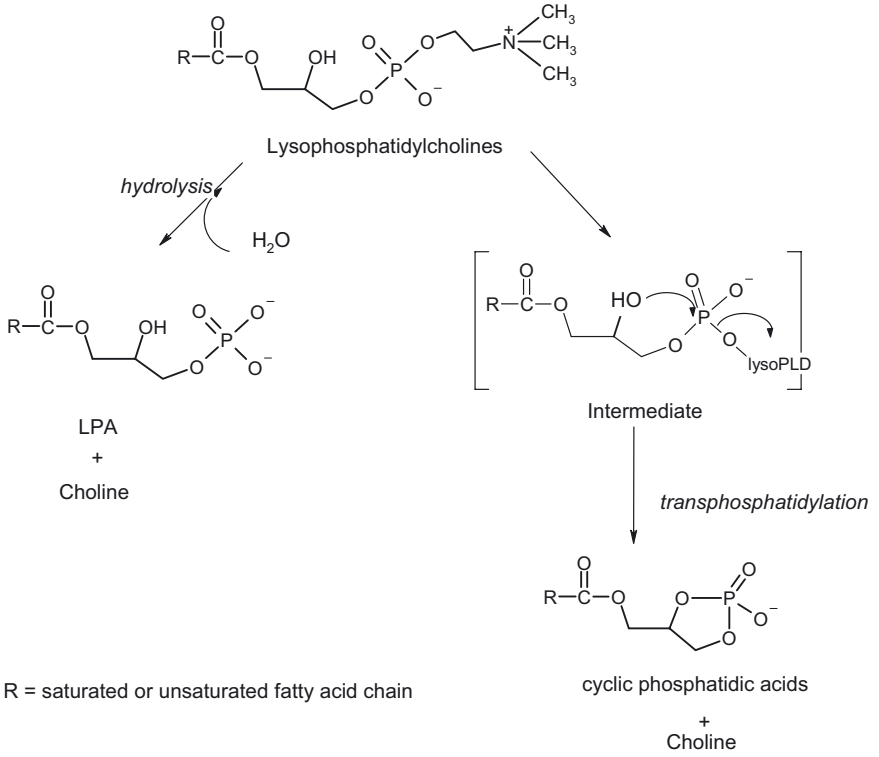

Figure 3. Synthesis of LPA and cPA by lysophospholipase D.
LysoPLD catalyzes hydrolysis of LPC that produces LPA and choline. The enzyme also attacks the substrate to form a transient lysophosphatidyl-lysoPLD intermediate. Then, intramolecular transphosphatidylation occurs with the hydroxyl group at the *sn*-2 position of LPC to form cPA.

including reproduction, vascular development, and functioning of the nervous system. However, they may also trigger the development and expansion of pathological processes, including cancers. Since their initial characterization as growth factors, the list of cellular responses to LPA has expanded considerably and now includes also many non-proliferative effects, ranging from stimulation of cell migration and survival to neurite retraction and gap junction closure.

The role of LPA in the cardiovascular system

Lysophosphatidic acids are constitutively present in serum at physiological concentrations of about 1–5 μM. Under pathological conditions, LPA is released to serum from platelets in response to injury and thrombosis (Aoki *et al.*, 2002) as well as to myocardial infarction (Chen *et al.*, 2003), which indicates a role of these phospholipids in cardiac pathophysiology. LPA may be involved in process of angiogenesis and atherosclerosis, as well as regulation of blood vessel tone. The oxidation of low-density lipoprotein (LDL) is thought to contribute to atherogenesis, which is an inflammatory disease involving activation of phagocytic cells (Dever *et al.*, 2006). Lysophosphatidic acids formed during mild oxidation of LDL initiate platelet activation and stimulate endothelial cell stress-fiber and gap formation (Siess *et al.*, 1999). Moreover, LPA accumulate in and are the primary platelet-activating lipid of atherosclerotic plaques, as observed in human carotid artery specimens where LPA level was highest in the lipid-rich core, the region that is most thrombogenic and prone to rupture. An early change in atherosclerosis is also characterized by neointima formation resulting from the proliferation and migration of dedifferentiated vascular smooth muscle cells (VSMCs). Unsaturated LPAs strongly induce VSMC dedifferentiation *via* the coordinated activation of the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK), resulting in the proliferation and migration of dedifferentiated VSMCs (Yoshida *et al.*, 2003). Zhang *et al.* (2004) have shown that LPA containing unsaturated acyl elicit progressive and long-lasting neointima formation in the rat carotid artery, which requires activation of PPARγ. All these findings suggest that *in vivo* LPA may act as endogenous atherogenic factors.

LPA in wound healing

Not surprisingly for a platelet-produced growth factor, LPA have all the hallmarks of a wound healing agonist: they stimulate the proliferation and migration of endothelial cells (Lee *et al.*, 2000), smooth muscle cells (Siess, 2002) and migration of fibroblasts (Pilquil *et al.*, 2006). LPA can also stimulate fibroblast-collagen matrix contraction (Lee *et al.*, 2003b). They show vasoconstrictive actions and enhance the production of matrix metalloproteinases (Wu *et al.*, 2005), which all are important events in tissue repair. Moreover, platelets themselves are activated by LPA which adds an element of positive feedback in the wound healing response. When applied topically, LPA promote wound healing in skin (Balazs *et al.*, 2001), while applied rectally they stimulate intestinal epithelial wound healing in rats (Sturm & Dignass, 2002). Lysophosphatidic acids may also stimulate wound healing in the upper digestive organs, as they are present in the saliva at physiologically relevant concentrations (close to 1 μM). LPA markedly enhance the growth of cells of esophagus, pharynx, and tongue origin *in vitro* (Sugiura *et al.*, 2002). Lysophosphatidic acids also stimulate the healing responses of human periodontal ligament fibroblasts and interact positively with PDGF (Cerutis *et al.*, 2007).

Lysophosphatidic acids stimulate endothelial cell migration which is a critical feature of several physiological and pathological processes, including angiogenesis and metastasis. Neovascularization (angiogenesis) as the process of restoring of the vascular network is an essential part of repair processes. LPA enhance the expression of genes that promote angiogenesis, including those for vascular endothelial growth factor (VEGF) (Hu *et al.*, 2001) and interleukin-8 (IL-8) (So *et al.*, 2004). Recently, it has been proposed that LPA stimulate the expression of vascular endothelial growth factor (VEGF) through hypoxia-inducible factor-1 (HIF-1α) activation (Lee *et al.*, 2006b). Upon binding to the hypoxia-responsive element within the target gene, HIF-1 activates transcription of various hypoxia-inducible genes like angiogenic factors, including VEGF. LPA have been found to stabilize endothelial monolayer barriers, a later event in angiogenesis (English *et al.*, 1999). Taking into account these data, LPA are potent mediators of tissue repair and wound healing.

LPA and autotaxin in tumor progression

The formation of vasculature by angiogenesis is essential not only for embryonic development or proper wound healing but also for the unrestrained growth of tumors. LPA and their receptors play an important role in the development of ovarian, prostate, breast, head and neck cancers.

Among different pathological processes, the role of LPA in ovarian cancer has been studied most extensively. In ovary cancer, LPA contribute to the development, progression, and metastasis and their concentration is increased in both plasma and ascites of ovary cancer patients, reaching 80 μM (in

comparison to the physiological 1–5 μM concentration) (Fang *et al.*, 2000). Assignment of serum lysophosphatidic acids can be a potential biomarker for ovarian cancer especially in light of the fact that they are not produced by normal ovarian epithelial cells. Ovary cancer cells also produce LPA, thereby maintaining an LPA-rich microenvironment. Elevated LPA levels have been detected in 98% of ovary cancer patients, including 90% of patients with stage I disease, suggesting that LPA promote early events in ovary carcinoma dissemination. In ovarian cancer cell lines, LPA show various activities including the enhancement of cell adhesion/attachment, production of angiogenetic factors such as VEGF (Hu *et al.*, 2001), IL-6 (Fang *et al.*, 2004) and IL-8 (So *et al.*, 2004), enhancement of urokinase plasminogen activator (uPA) expression (Li *et al.*, 2005), and prevention of cell apoptosis (Kang *et al.*, 2004). Ovarian cancers (as well as colorectal cancer) show markedly increased expression of LPA_2 and LPA_3 receptors (Wang *et al.*, 2007).

Lysophosphatidic acids support the progression of breast and ovarian cancer metastasis to bone, acting as inducers of IL-6 and IL-8. Although breast cancer cell lines express LPA_1, LPA_2 and LPA_3 receptors, most of the LPA activities on human breast cancer MDA-BO2 cell proliferation and production of proosteoclastic cytokines are LPA_1-dependent. Blocking the LPA_1 receptor is a promising therapeutic target in cancer, especially for metastasis to bone. Indeed, the LPA_1 receptor antagonist Ki16425 (3-(4-[4-([1-(2-chlorophenyl)ethoxy]carbonyl amino)-3-methyl-5-isoxazolyl]benzylsulfanyl) propanoic acid) blocked *in vivo* tumor cell proliferation and inhibited the production of proosteoclastic cytokines by tumor cells, whereas normal platelet functions were unaffected (Boucharaba *et al.*, 2006).

The LPA_1 receptor could also be a target in prostate cancers' therapy (Hao *et al.*, 2007). LPA_1, LPA_2 and LPA_3 have been detected in prostate cancer cells, however, only LPA_1 was found to be responsible for prostate cancer cell migration *in vitro*, and the overexpression of LPA_1 resulted in increased tumor growth.

There is a growing body of evidence that not only LPA but also autotaxin is involved in tumor growth. ATX was originally identified as a tumor cell motility factor (Umezu-Goto *et al.*, 2002; Tokumura *et al.*, 2002). The enzyme stimulates both proliferation and motility of cancer cells through LPA production. In addition, overexpression of ATX is frequently associated with malignant tumors such as small cell lung cancer, renal cell cancer, hepatocellular carcinoma, breast cancer, Hodgkin lymphoma, thyroid cancer, and glioblastoma (Mills & Moolenaar, 2003). The highest ATX expression is detected in glioblastoma multiforme (GBM), a very malignant cancer with a high infiltration rate. In breast cancer, ATX expression level strongly correlates with the invasiveness of cancer cells. In addition to stimulating proliferation and motility of tumor cells, autotaxin contributes to the progression of tumors by stabilizing preformed blood vessels in their vicinity (Tanaka *et al.*, 2006). **M**uch of the anti-apoptosis effect and intracellular signaling attributed to ATX can be explained by its ability to produce bioactive phospholipids, such as LPA. Whether the generation of an LPA-rich tumor microenvironment can fully account for the ability of autotoxin to promote metastasis remains to be established, especially in view of the fact that ATX expression is strongly upregulated by the v-*Jun* oncogene (Black *et al.*, 2004) and the cancer-associated $\alpha 6\beta 4$ integrin, apparently *via* the transcription factor NFAT1, which binds to the ATX promotor at two distinct sites (Chen & O'Connor, 2005). Nevertheless, the available evidence suggests that the ATX–LPA axis is a promising target for pharmacological intervention. Furthermore, ATX is a druggable target because it is a soluble exo-enzyme that is readily amenable to high-throughput screening. A lead towards developing ATX inhibitors has been provided by the discovery that this enzyme undergoes end product inhibition, for example by LPA (van Meeteren *et al.*, 2005). Indeed, a limited number of ATX inhibitors that are LPA analogs have been reported to date (among them fatty alcohol phosphate analogs, phosphatidic acid derivatives, and recently β-hydroxy and β-keto phosphonate derivatives of LPA) (Cui *et al.*, 2007). Considering the importance of ATX as a determinant of blood LPA level, there are also attempts to apply ATX activity in clinical laboratory testing (Nakamura *et al.*, 2008).

The role of LPA in the immune system

Lysophosphatidic acids are pro-inflammatory factors that have been implicated in the development of inflammation taking place in pathological processes such as asthma or allergy. In the normal individual, airway injury is properly repaired due to the release and actions of appropriate repair mediators, including LPA. The LPA-mediated effects on airway cells that may contribute to airway repair in a physiological setting include fibronectin release, fibroblast proliferation and contraction, and airway smooth muscle cell proliferation. LPA alone can stimulate proliferation of human airway smooth muscle (HASM) cells or they can synergize with epidermal growth factor (EGF) to further increase proliferation of HASM cells (Toews *et al.*, 2002). The chronic inflammation that for instance characterizes asthma leads to damage of the epithelium and epithelial cell shedding, thus exposing the underlying tissue and leading to the release of a variety of inflammatory and repair mediators. In the

asthmatic airway, exaggerated or prolonged responses to LPA contribute to long-term airway remodeling. Fibronectin secretion contributes to the thickening of the lamina reticularis, and fibroblast proliferation and contraction can contribute to the subepithelial fibrosis. Airway smooth muscle proliferation in synergy with EGF and other growth factors are a major stimulus for the thickening of the smooth muscle layer. Additionally, following proliferation, the cells respond by upregulating cytokine production (VEGF, GM-CSF, and TNF-α) leading to increased inflammation.

LPA participate in the motility, polarization, and metabolic burst of human neutrophils, induce haptotactic migration of human monocytes (Zhang *et al.*, 2006), and also enhance monocyte–endothelial cell adhesion and monocyte chemotaxis toward endothelial cells through the respective upregulation of IL-8 and MCP-1 (monocyte chemoattractant protein-1) expressions in endothelial cells, thus promoting inflammation processes. Expression of LPA-induced inflammatory response genes is mediated by LPA_1 and LPA_3, which suggests the utilization of LPA_1 or LPA_3 as drug targets to treat severe inflammation (Lin *et al.*, 2007)

LPA and neuronal cells

LPA receptors have multiple activities in nervous system cells achieved *via* activation of various signaling pathways. LPA cause the collapse of neuron growth cone and tend to inhibit or reverse the morphological differentiation of many neuronal cell lines. In addition to cell intrinsic responses, systemic effects on larger populations of neuronal cells have also been observed. They induced changes both in neuroanatomy and in animal behavior. For example, LPA exposure can produce dramatic receptor-dependent changes in folding of developing cerebral cortex, along with increase in cell number (Kingsbury *et al.*, 2003). The direct stimulation of peripheral nociceptor endings by LPA through LPA_1 receptors also suggests its role in nociceptive processes. It has been reported recently that the activation of the LPA_1 receptor and its downstream Rho/Rho-kinase pathway is required for the development of neuropathic pain (Inoue *et al.*, 2004). The increased expressions of the γ isoform of protein kinase C (PKCγ) in the spinal dorsal horn and the α_2 δ-1 subunit of voltage-gated calcium channels (Caα_2 δ-1) in the dorsal root ganglion are two important markers of neuropathic pain. The intrathecal injection of LPA caused an increase of PKCγ expression in the spinal dorsal horn. An up-regulation of PKCγ was also observed after partial sciatic nerve injury. Similarly, LPA- and nerve injury-induced up-regulations were observed for the Ca_v α_2 δ-1 subunit in spinal dorsal horn. The use of $lpa_1^{-/-}$ mice has also demonstrated that endogenous LPA plays a role in induc-

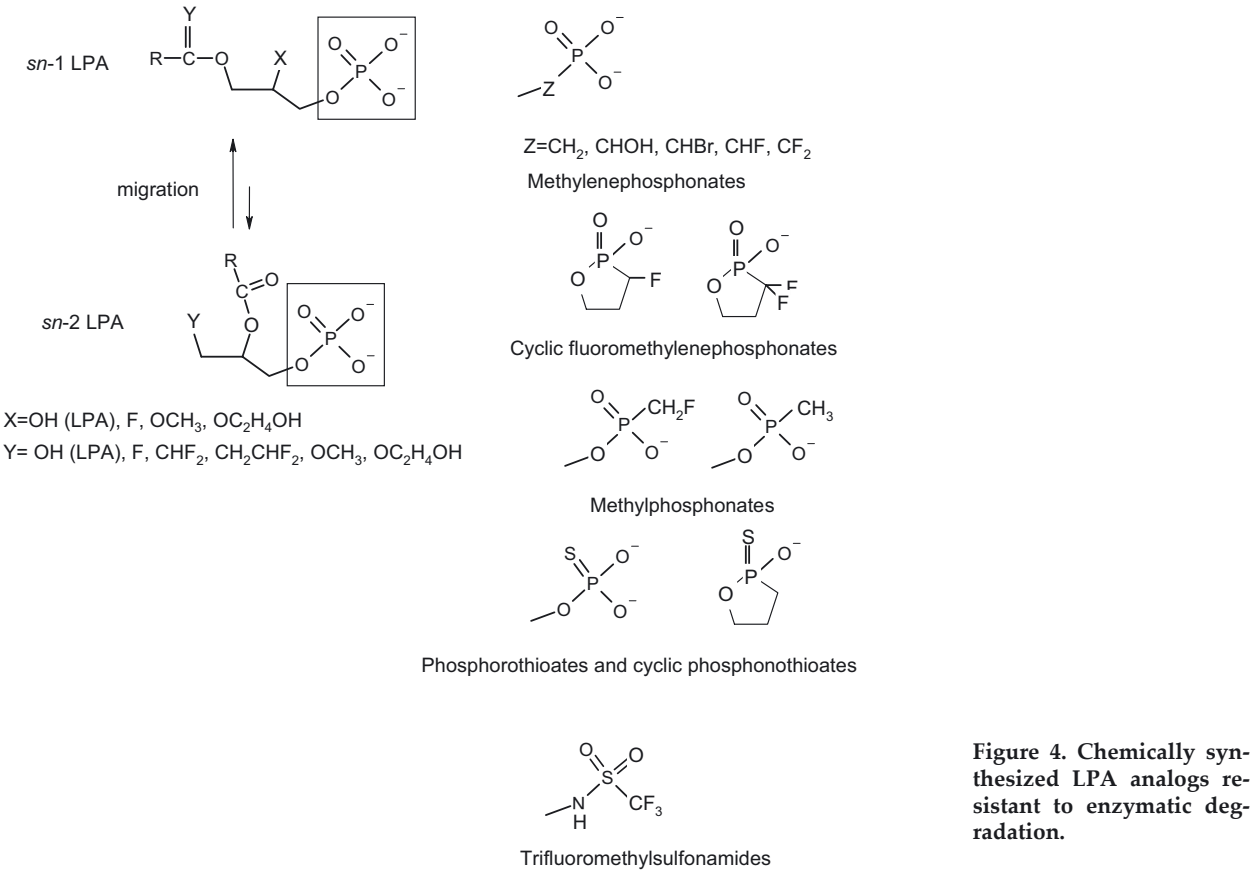

Figure 4. Chemically synthesized LPA analogs resistant to enzymatic degradation.

ing underlying mechanisms such as demyelination, decrease in protein and gene expressions of myelin basic protein (MBP) and peripheral myelin protein 22 kDa (PMP22), which are reduced in the demyelinated status. Up-regulation of PKCγ and of Ca_v α_2 δ-1 in mice giving partial sciatic nerve ligation was also observed (Ueda, 2006). The demyelination and Ca_v α_2 δ-1 up-regulation are thought to underlie the sensitization of Aδ/β nociceptive transmission possibly through ephapsis, ectopic discharge, and collateral sprouting, leading to allodynia and hyperalgesia. PKCγ up-regulation in lamina II of the dorsal spinal cord, on the other hand, may be involved in the process of central sensitization, which is thought to be a common cause of neuropathic allodynia and hyperalgesia. Altogether, these findings suggest that novel analgesics for the treatment of the initial phases of neuropathic pain may consist of antagonists or inhibitors of any of these identified components of the LPA signaling pathway.

Chemically synthesized LPA analogs

The recent progress in elucidation of LPA-dependent signaling and LPA metabolism has been possible due to the use of their analogs in which labile groups have been chemically stabilized. For example, the phosphate group can be replaced with charged or neutral phosphoromimetics including methylene (–CH_2–) phosphonates, α-X methylene phosphonates (where X is –CHF–, –CHBr– or –CHOH–), phosphorothioates or phosphonothioates. These modifications make such analogs resistant against lysophospholipid phosphatases (LPP). The *sn-1* or *sn-2* hydroxy groups can be substituted with fluorine or methoxy groups. Such LPA analogs are unable to undergo acyl migration, effectively "freezing" the acyl chain in the *sn*-1 or *sn*-2 position, respectively. Additional stabilization of LPA analogs can be achieved by replacement of the *sn*-1 O-acyl group with an O-alkyl ether (Fig. 4). Chemically synthesized LPA analogs are not only more resistant to the action of the LPP or LPAAT enzymes. Some of them have been reported to be potent and selective agonists of LPA receptors, especially the LPA_3 receptor or PPARγ. However, taking into account the multitude of LPA receptors, it seems more reasonable to search for specific inhibitors of ATX than of individual LPA receptors. Unfortunately, among the LPA analogs synthesized so far, only a few compounds have shown an inhibitory effect towards this enzyme (Cui *et al.*, 2007). Therefore, a recent report describing phosphorothioate inhibitors of NPP1, an enzyme related to autotoxin, may be useful for further search of ATX inhibitors (Wójcik *et al.*, 2007). Synthesis and optimization of small-molecule inhibitors of ATX as well as characteristics of tissue-specific ATX isoforms will be necessary to elucidate whether autotaxin is a valuable therapeutic target.

CONCLUDING REMARKS

At the beginning of the 2000s Hla *et al.* (2001) concluded that lysophospholipid receptor biology had generated insights into fundamental cellular mechanisms and might provide therapeutic targets for drug development. However, further findings and discoveries have shown that this area of cellular biology is more surprising than one could expect. Research of the last decade has demonstrated that lysophosphatidic acids, their receptors as well as enzymes of LPA metabolism are novel targets for therapeutic intervention in many pathophysiological processes. Targeted deletion of LPA receptors, particularly in conjunction with the use of receptor agonists and/or antagonists, has revealed roles of individual receptors in many disease models, e.g. loss of LPA_1 signaling has been reported to block the initiation of nerve injury-induced pain and alter the balance of adipocytes and their precursors in adipocyte tissue. The effects of a loss of LPA_3 have indicated the importance of LPA signaling in fertility.

Extremely important for LPA signaling is autotaxin expression, activity and regulation. Therefore, ATX is also an attractive pharmacological target since blockage of LPA production *via* this enzyme inhibition could be a useful anticancer chemotherapy. While much has recently been learned about ATX activity and LPA function, many problems remain to be solved. How many tissue-specific isoforms of ATX do exist? What are the differences between them? Which pool of LPC or other substrates is preferentially degraded by autotaxin? How is the enzyme activity regulated? Are there any membrane cPA receptors as is the case for LPA receptors? Which molecular factors switch ATX activity from LPA synthesis to cPA production? Hopefully, answers to these questions will emerge in the near future.

Acknowledgements

This work was supported by a grant (PBZ-MNiSW-07/I/2007) from the Ministry of Science and Higher Education.

I thank Professor Maria Koziolkiewicz for her valuable consultations and help in preparation of this manuscript.

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