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Visions & Reflections (Minireview)

New developments in the biological functions of lysophospholipids

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Abstract. Lysophospholipids have long been recognized as membrane phospholipid metabolites, but only recently has their role as intercellular signaling molecules been appreciated. Two of the best-studied lysophospholipids, LPA and S1P, signal through cognate G-protein-coupled receptors to activate many well-known intracellular signaling pathways, leading to a variety of biologically important cell responses. Lysophospholipids and their receptors have been found in a wide range of tissues and cell types, indicating their importance in many physiological processes, including reproduction, vascular development, cancer and nervous system function. This article will focus on the most recent findings regarding the biological functions of lysophospholipids in mammalian systems, specifically as they relate to health and disease.

Keywords. Lysophosphatidic acid, sphingosine 1-phosphate, G-protein-coupled receptor, angiogenesis, embryo implantation, myelination, nervous system development.

Over the last decade, a new type of cell-to-cell signaling has been characterized – lysophospholipid molecules signaling through membrane-bound receptors. Although well established as phospholipid metabolites, lysophospholipids as receptor-mediated intercellular signals represent a comparatively recent and rapidly growing area of research. Several lysophospholipids have been analyzed for their intercellular signaling properties, but the best characterized are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). These two lipids act through G-protein-coupled receptors (GPCRs) named $LPA_{1–4}$ and $S1P_{1–5}$ [1, 2]. They use classic G protein signaling pathways which have been examined in a variety of cell types, including: $G_q \rightarrow$ phospholipase C $\rightarrow$ calcium mobilization; $G_{12/13} \rightarrow$ Rho $\rightarrow$ actin rearrangment; and $G_i \rightarrow$ extracellular signal-related kinase (ERK), phosphoinositide 3kinase (PI3) and inhibition of adenylate cyclase (AC). These signaling pathways have been covered extensively in several excellent reviews [3–5] and will not be discussed further here. A comprehensive description of all of the functions of lysophospholipids is beyond the scope of this review, and instead we will highlight some of the exciting recent findings on the biological functions of receptor-mediated lysophospholipid signaling.

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Reproduction

New results demonstrate that the lysophosphatidic acid receptor LPA_3 is involved in female reproduction. Knockout mice lacking *lpa*$_3$ show reduced litter size due to defects in implantation and embryo spacing [6]. In *lpa*$_3$ –/– pregnant females, implantation was delayed approximately 1 day, and there were fewer implantation sites. Furthermore, the positioning of these sites was altered, resulting in abnormal embryo spacing and embryo crowding, even

to the point of multiple embryos sharing one placenta. Blastocyst transfer experiments demonstrated that these implantation defects were due to the lack of LPA_3 in the mother and not in the embryos.

Normally, implantation is the result of two-way communication between the blastocyst and the uterine wall that induces cellular changes in both the blastocyst and the uterus [7]. Hormonal regulation primes the uterus to be receptive to blastocyst implantation on embryonic day 4 (E4) in mouse, and prostaglandin signaling between the blastocyst and the uterine wall is, at least in part, responsible for establishing the implantation site [8]. Prostaglandins are synthesized from arachidonic acid, a process catalyzed by the rate-limiting cyclo-oxygenase (COX) enzymes. In the uterine wall, the inducible form of COX, COX-2, is upregulated at the site of blastocyst attachment [9]. The induction of high levels of COX-2 results in prostaglandin biosynthesis at the site of future implantation and prepares the uterine wall to accept the embryo. Treatment of mice with indomethacin, a COX inhibitor, results in implantation defects [10, 11] similar to those seen in *lpa*$_3$ –/– mice. Furthermore, *cox-2* knockout mice have significant defects in reproduction, including almost complete failure of implantation [8, 12]. In *lpa*$_3$ –/– mice, COX-2 expression was reduced in the uterus during the implantation period, and the levels of the prostaglandins PGE_2 and PGI_2 were both greatly reduced in the uterus at E3.5. Injection of PGE_2 and carbaprostacyclin (cPGI, a stable analogue of PGI_2) into *lpa*$_3$ –/– pregnant mice rescued the delayed implantation defect; however, the abnormal embryo spacing was still apparent. This suggests the involvement of other signaling pathways in controlling embryo spacing, or may simply represent the inability of a systemic injection of prostaglandins to rescue this defect [12, 13]. A phenotype very similar to the *lpa*$_3$ –/– mice is seen in *pla2g4a* –/– mice lacking cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) [14], a critical enzyme for synthesis of arachidonic acid [15, 16]. Thus it appears that LPA signaling through LPA_3 impacts the arachidonic acid/COX/prostaglandin pathway to regulate implantation.

Although LPA signaling pathways in uterine epithelial cells have not been characterized, LPA has been shown to induce COX-2 transcription in other cell types via $G_{i/o}$ → Rac/Cdc42 signaling [17–19], $G_{12/13}$ → Rho pathways [20] and LPA-mediated transactivation of the epidermal growth factor receptor [17]. When heterologously expressed in B103 neuroblastoma cells, the LPA_3 receptor has been shown to couple to $G_{i/o}$ and G_q but not $G_{12/13}$ [21], suggesting that LPA induction of COX-2 through LPA_3 in the uterine epithelium may involve $G_{i/o}$ signaling through Rac or Cdc42. Since COX-2 is localized at the nuclear envelope [13], the resulting prostaglandins likely signal through nuclear receptors, and evidence suggests that the nuclear receptor PPARδ may be involved [8]. Furthermore, recent evidence suggests that LPA signaling may interact with the Wnt/β-catenin pathway in implantation, since blocking the Wnt/β-catenin pathway in the uterus results in delayed implantation and abnormal spacing, as seen in *lpa*$_3$ –/– females [6, 22]. However, the exact details of the downstream signaling pathways in LPA-mediated implantation remain to be determined.

Vascular development

Initial receptor expression studies [23] and *in vitro* experiments [24–28] suggested that the lysophospholipid S1P and the receptor $S1P_1$ likely play a role in vascular development. This was substantiated when a knockout mouse for $S1P_1$ was generated and shown to die during early embryonic development because of defects in vascular maturation [29]. The $S1P_2$ and $S1P_3$ receptors may also function in vascular development, as the *s1p*$_1$–/– *s1p*$_2$–/– *s1p*$_3$–/– triple knockout mice die even earlier with more severe vascular defects [30], although *s1p*$_2$–/– and *s1p*$_3$–/– single knockout mice show no major phenotypes. The requirement for S1P in vascular development has been confirmed by a knockout of the two isoforms of sphingosine kinase, the enzyme responsible for phosphorylating sphingosine to S1P [31]. Mice homozygous for loss of sphingosine kinase 1 and 2 (*SphK1* –/– and *SphK2* –/–) are embryonic lethal, showing vascular defects similar to the S1P receptor knockout mice. Although loss of either of the sphingosine kinases results in reduced S1P production, there is no vascular phenotype in the single sphingosine kinase knockout mice [31, 32]. Thus, S1P production from either sphingosine kinase enzyme appears sufficient for normal vascular development.

In addition to embryonic vascular development, S1P receptors may be involved in adult angiogenesis (for review, see [33]). S1P is present in follicular fluid, and it can stimulate endothelial cell proliferation and ovarian angiogenesis *in vitro* [34]. Furthermore, S1P and its receptors may also be involved in angiogenesis of tumors; RNA interference (RNAi) of the $S1P_1$ receptor inhibited endothelial cell migration *in vitro*, and suppressed tumor growth in an *in vivo* model [35]. Thus, S1P signaling pathways may represent a potential therapeutic target for cancer through blocking tumor angiogenesis.

Cancer

In addition to its role in angiogenesis, S1P and its receptors influence cancer growth and development; however, there is still much work to be done to fully elucidate the specific roles of S1P signaling. S1P receptors, especially $S1P_2$ or $S1P_3$, are often elevated in a variety of cancer cell lines [36–39], although this expression is variable and differs between types of cancer. In gastric cancer, S1P

can influence cell migration and proliferation [39], and it plays a well-known role in proliferation of human breast carcinoma cells [36]. Thus, S1P receptor signaling plays a significant role in cancer and is a potential target for therapeutic intervention.

Indeed, recent studies suggest that targeting S1P or its receptors may have therapeutic benefits in treating some types of cancer. In a metastatic melanoma model in mouse, S1P treatment inhibited metastasis, even if administered before melanoma cell injection, and overexpression of the $S1P_2$ receptor potentiated this inhibition of metastasis by S1P [38]. In another study, a monoclonal antibody to S1P reduced tumor progression in mouse xenograft and allograft models; this was attributed to both anti-angiogenic and anti-tumorigenic effects [40]. Therefore, targeting S1P or S1P receptors holds promise as a novel cancer treatment, but it likely will depend greatly on the type of cancer and the specific oncogenic cellular changes.

Nervous system

Much of the pioneering work on lysophospholipids as signaling molecules came from the nervous system. Early *in vitro* experiments on peripheral nervous system-derived cell lines (PC12, NIE-115, NG108-15) showed LPA treatment could induce neurite retraction [41–47]. Later, the first LPA receptor (LPA_1, initially called vzg-1) was identified from cortical cell lines and shown to be a GPCR that could mediate G_i responses [48]. LPA_1 was also shown to be highly expressed in the ventricular zone of the cerebral cortex, suggesting its involvement in cortical development [48–54]. Recently, experiments using *ex vivo* cortical cultures have shown that LPA treatment induces cortical folds resembling gyri as well as increased cortical thickness [55]. These changes are due to decreased apoptosis and increased terminal mitosis in the neural progenitor cell population, and they require the receptors LPA_1 and LPA_2.

LPA_1 and LPA_2 are the major LPA receptors expressed in the nervous system, and knockout mice have been generated as single mutants as well as lpa_1–/– lpa_2–/– double mutants [56, 57]. Mice lacking lpa_1 are viable at birth but show craniofacial abnormalities (such as shortened snout) and have a significant perinatal lethality due to impaired suckling behavior [56]. Those mice that survive appear neurologically normal, with no major behavioral or histological abnormalities [56–58]. However, Harrison et al. [58] recently found a decrease in serotonin (5-HT) turnover and 5-hydroxyindole acetic acid (5-HIAA, a serotonin metabolite) levels in several brain regions of lpa_1–/– mice as well as lower levels of aspartate and serine in frontal cortex (but not other brain regions examined). Behavioral tests revealed a small but significant deficit in prepulse inhibition (PPI) that may be due to this altered brain chemistry. However, no electrophysiological differences were observed in hippocampal synaptic function between lpa_1–/– and wild-type mice. Knockout mice for lpa_2 show no obvious phenotype, and lpa_1–/– lpa_2–/– double mutant mice do not show a more severe phenotype than lpa_1–/– mice (except for an increased incidence of hematoma) [57].

Another exciting finding is that LPA and LPA receptors appear to be involved in the condition of neuropathic pain in the adult [59]. In one rodent model of neuropathic pain, injury to the sciatic nerve causes pain to previously non-noxious stimuli, referred to as allodynia, as well as sensitization to mildly painful stimuli, referred to as hyperalgesia. Intrathecal injection of LPA mimicked nerve injury and produced thermal hyperalgesia and mechanical allodynia in a paw test, and hyperalgesia in a thermal tail-flick test [59]. Both LPA injection- and nerve injury-induced neuropathic pain were abolished by pretreatment with compounds that eliminated Rho activity. Furthermore, both LPA and nerve injury caused a transient demyelination of the dorsal root. Mice lacking LPA_1 did not show the nerve injury-induced hyperalgesia and allodynia or demyelination, indicating this receptor may be involved in the pain response to nerve injury. The specific cell types and LPA signaling pathways involved in neuropathic pain remain to be elucidated.

Myelination

Lysophospholipids have been proposed to play a significant role in myelination based on receptor expression studies. The first LPA receptor, LPA_1, was found to be highly expressed in white matter tracts in adult rodents, specifically in oligodendrocytes [60–63]. *In vitro* studies have shown lpa_1 to be highly expressed in mature oligodendrocytes but not in oligodendrocyte precursor cells [61–64]. Further work has demonstrated that LPA receptors on oligodendrocytes are functional and stimulate calcium signaling and ERK phosphorylation [65, 66].

Despite these expression and signaling studies, the role of LPA receptors in oligodendrocyte function and in myelination is still unclear. No effect of LPA on the survival, maturation, cytoskeleton organization, or myelination of oligodendrocytes has been observed in culture [64]. In addition, LPA produced process retraction in oligodendrocyte precursor cells in culture but not in mature oligodendrocytes [67], although LPA_1 is highly expressed in differentiated oligodendrocytes and possibly not in immature oligodendrocytes (see [64]). However, most of this work was done in the immortalized cell line CG-4, which may differ from oligodendrocyte precursor cells *in vivo* (see [66, 68, 69]). Analysis of knockout mice lacking lpa_1 found no obvious defects in myelination or neuro-

logical deficits suggestive of impaired myelination [56]. These results suggest that LPA_1 may not be necessary for normal oligodendrocyte differentiation, development or myelination *in vivo,* but could have other functions that are as yet unidentified.

LPA_1 has also been proposed to be involved in myelination in the peripheral nervous system (PNS) because of its high expression in Schwann cells [61]. LPA can activate multiple G_i-mediated signaling cascades, PI3, AKT (protein kinase B) and MAPK (mitogen-activated protein kinase) pathways to enhance Schwann cell survival *in vitro* [70, 71]. LPA also induces morphological changes and rearrangement of the actin cytoskeleton in Schwann cells, and this response is reduced in Schwann cells from mice lacking *lpa$_1$* [72]. *In vivo* analysis of the sciatic nerve in mice lacking *lpa$_1$* showed an increase in apoptotic Schwann cells identified by in situ end labeling plus ($ISEL^+$) [56]. A recent report indicates that LPA_1 may be involved in peripheral demyelination in the adult [59]. In a neuropathic pain model involving sciatic nerve injury (described earlier), transient demyelination of the dorsal root is observed. This neuropathic pain response is abolished in *lpa$_1$* knockout mice, and can be mimicked by intrathecal injection of LPA. Whether LPA_1 is required in Schwann cells for this injury-induced demyelination, or in other cells involved in the injury response, remains to be determined.

A role for S1P receptors in oligodendrocyte function and myelination has also been postulated. Again, this hypothesis was based on expression studies that found the $S1P_5$ receptor is expressed almost exclusively in white matter tracts and in oligodendrocytes and their precursors in adult rodents [73–75]. *In vitro*, S1P stimulates a calcium response in oligodendrocytes, although it appears to affect only a small subset of oligodendrocytes [66, 68, 69], and it also induces process retraction in pre-oligodendrocytes, but not mature oligodendrocytes [67, 75]. Furthermore, it can be a survival factor for mature rat oligodendrocytes *in vitro*. These S1P effects are dependent on $S1P_5$ [75], although no defects in myelination or myelinated pathways have been observed in these *s1p$_5$* knockout mice [75].

The overall role of lysophospholipid receptors in oligodendrocyte development and myelination is still unclear. Although there is strong expression of two receptors, LPA_1 and $S1P_5$, in oligodendrocytes, there are no obvious defects in oligodendrocyte development or myelination with the loss of either receptor. In the knockout mice, there could be compensation by other receptors, and it will be interesting to examine mice lacking multiple receptors. LPA_1 is also expressed in Schwann cells, but again there are no defects in peripheral myelination, although there is an effect on pathological demyelination. It is possible that the loss of LPA_1 or $S1P_5$ produces subtle defects that have not yet been detected or that these receptors are required not for development but for responses in adults to injury or other insults.

Other aspects of development

In addition to the specific areas highlighted above, there are many other intriguing aspects of lysophospholipid function in biology that include non-mammalian vertebrate models. For instance, LPA receptors have been demonstrated to have a role in the correct patterning of *Xenopus* embryos at the blastula stage through cortical actin assembly [76]. Furthermore, the *Xenopus* homologue of LPA_2 is required for proper embryonic development, including neurulation. Indeed, the LPA receptors may be part of a set of GPCRs that regulate cortical actin assembly in the developing *Xenopus* embryo [77].

In the zebrafish, the mutation *miles apart* (*mil*) results in the formation of two laterally positioned hearts (cardia bifida) due to failure of migration of myocardial precursors to the midline. When *mil* was cloned, it was found to be an S1P receptor [78], implicating S1P in early heart formation. Recent experiments in an *in vitro* murine model of heart formation suggest that S1P may have a similar function in mammalian heart development [79], demonstrating the relevance of these non-mammalian studies.

Conclusion

Recent work indicates many important biological functions for lysophospholipids in cell signaling through GPCRs, and this review highlights only a few of these diverse functions. Our current understanding of lysophospholipid signaling is just the tip of the iceberg. The field is rapidly expanding, but many questions remain to be answered. In most cases, the specific cell types involved and the effects of lysophospholipids on these cells need to be determined. There have been many provocative *in vitro* experiments, but they remain to be correlated with *in vivo* function. Although lysophospholipid receptors are widely expressed, only a few biological functions have been characterized and other important functions likely remain to be discovered. In addition, it is probable that other as yet unidentified receptors also exist for a number of lysophospholipids. Finally, although we are beginning to understand lysophospholipid functions through receptor studies, the regulation of their production as cell signaling molecules is relatively new territory [49, 72, 80–82]. Thus, these are early and exciting days in discovering roles of lysophospholipids in development and disease.

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