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Blood Relatives: Dynamic Regulation of Bioactive Lysophosphatidic acid and Sphingosine-1- Phosphate Metabolism in the Circulation

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Abstract

Lysophosphatidic acid and sphingosine 1-phosphate are bioactive lipid mediators with potent effects on cardiovascular development and vascular function. New studies define dynamic mechanisms that maintain physiologically relevant levels of both lipids in the blood. We review the mechanisms controlling the production, metabolism, and distribution of these lipids between vascular cells, circulating blood components and the plasma.

Introduction

Two simple lysophospholipids, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are now well established as receptor-active mediators with effects on a variety of organ systems (Noguchi et al. 2009; Rosen et al. 2009). LPA and S1P are present in the blood plasma of a variety of mammalian species at physiologically relevant concentrations. In experimental models, LPA has been implicated as a regulator of blood pressure, hemostasis, inflammation and injury responses (Smyth et al. 2008), whereas S1P has potent immunomodulatory actions and may regulate endothelial barrier function, injury responses, and atherosclerosis (Rosen et al. 2008). These effects involve actions on multiple vascular cell types including vascular endothelial cells, vascular smooth muscle cells, platelets, neutrophils and lymphocytes. In fact with the exception of erythrocytes, every vascular cell type examined exhibits responses to one or both of these lipids (Smyth et al. 2008). Much of what we know about the function of these mediators comes from use of mouse models with genetic inactivation of specific LPA and S1P receptor subtypes. More recent studies targeting the enzymes involved in the synthesis and inactivation of these bioactive lysophospholipids have revealed new aspects of their regulation and suggest that this will be an equally fruitful approach for dissecting the biology of LPA and S1P signaling and the roles played by these lipids in cardiovascular physiology and disease.

Production of LPA and S1P in the Circulation

While the molecular diversity of LPA and S1P receptors continues to expand, the number of genes encoding enzymes involved in their synthesis and inactivation is considerably more restricted (Figure 1). The primary mechanism for synthesis of LPA, at least in the blood,

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involves hydrolysis of circulating lysophospholipids, of which the most abundant by far is lysophosphatidyl choline (LPC), by the lysophospholipase D activity of the secreted enzyme autotaxin (van Meeteren and Moolenaar 2007). Although single (or in some cases combined) inactivation of several of the identified LPA receptors in mice results in viable offspring, homozygous inactivation of the autotaxin gene (*Enpp2*) causes embryonic lethality in mice resulting from defects in vascularization (Tanaka et al. 2006; van Meeteren et al. 2006). Genetic manipulation of *Enpp2* in mice established a central role for autotaxin in regulation of LPA levels in the blood: *Enpp2* heterozygotes have reduced circulating LPA levels, whereas transgenic mice overexpressing the enzyme have elevated circulating LPA levels (Pamuklar et al. 2009).

Although autotaxin is readily detected as a soluble protein in blood plasma, recent data indicate that it is recruited to the surface of lymphocytes and platelets by interaction with activated integrin receptors, which may result in specific effects on platelet activation and lymphocyte trafficking through the localized production of LPA (Kanda et al. 2008; Pamuklar et al. 2009). The circulating LPA pool comprises a variety of distinct molecular species that differ in the length, saturation and linkage of their radyl chain substituents (Baker et al. 2001). Distinct LPA receptors exhibit differential agonist responsiveness to some of these different LPA species (Noguchi et al. 2009). Integrin-dependent recruitment of autotaxin to the surface of circulating or vascular cells could provide a mechanism for generation of this diversity by compartmentalization of the enzyme with distinct molecular species of its substrates. In theory, integrin binding might also alter autotaxin enzyme activity.

Evidence for an additional pathway for production of extracellular LPA through the actions of A-type phospholipases has been presented, but molecular details of the process remain to be established (Aoki et al. 2002). Definition of the pathways involved in production of “signaling” LPA is complicated by the obligatory intracellular role of this lipid in phospholipid and triglyceride synthesis. LPA may have additional intracellular signaling functions, for example as an endogenous activator of PPAR γ (McIntyre et al. 2003).

S1P is produced by phosphorylation of sphingosine catalyzed by two sphingosine kinases (SK), SK1 and SK2. Inactivation of either gene does not compromise viability in mice and results in modest (most pronounced in the case of SK2 $-/-$ animals) declines in circulating S1P levels (Maceyka et al. 2005). Remarkably, inducible inactivation of SK2 in SK1 $-/-$ pups produces adult animals with no detectable circulating S1P (Pappu et al. 2007). Adoptive transfer of erythrocytes from wild type mice into the SK1/SK2 $-/-$ animals restores circulating S1P to wild type levels (Pappu et al. 2007). Consistent with this finding, human erythrocytes can take up and release S1P (Hanel et al. 2007), suggesting a role for these cells in “buffering” circulating S1P levels. Despite this central role in circulating S1P homeostasis, erythrocytes are almost certainly not the sole source of circulating S1P. Activated platelets release S1P and may serve as a localized source for the lipid in some situations (Yatomi et al. 1997). A more recent study identifies a key role for vascular endothelium in production of bioactive S1P in the circulation (Venkataraman et al. 2008).

SK1 and SK2 are both ATP-dependent intracellular enzymes, so production of extracellular S1P necessarily requires mechanisms for export of these polar lipids across the plasma membrane. ATP binding cassette (ABC) family transporters are integral membrane proteins responsible for transmembrane movement of a wide variety of molecules. Evidence that certain members of the ABCC class of these proteins are responsible for S1P export from platelets, mast cells and vascular endothelial cells has been presented, but the physiological role for these transporters in regulation of extracellular S1P levels is presently undefined (Hla and Im 2009; Mitra et al. 2006). S1P signaling coordinates cell migration during development of the zebrafish heart (Sehnert and Stainier 2002). Mutations in a zebrafish S1P receptor disrupt

migration of cardiac progenitor cells leading to defects in formation of the primitive heart tube. Two groups have recently reported that mutations in another locus, two of hearts (toh), phenocopies mutations in the S1P receptor (Kawahara et al. 2009; Osborne et al. 2008). The zebrafish toh protein is homologous to a *Drosophila* protein called Spinster (SPN). Interestingly, toh, SPN and their mammalian homologs are 12-transmembrane domain containing integral membrane protein members of the Major Facilitator Superfamily of non-ATP-dependent transporters, which include bacterial homologs that are known to be important for transmembrane movement of lysophospholipids (Law et al. 2008). The developmental defects observed in toh mutant embryos could be rescued by injection of S1P. Overexpression of toh and the mammalian homolog SPNS2 promotes S1P export from cultured mammalian cells (Kawahara et al. 2009). Taken together, these results suggest that these spinster family integral membrane proteins are functionally relevant S1P transporters. It will be very interesting to evaluate their roles in regulating the distribution of S1P between vascular cells and circulating blood cells. In particular, if these proteins function as S1P “uniporters” driven solely by their substrate gradient, they might be responsible for both S1P release and uptake by blood and vascular cells. The relationship between this “facilitated export” mechanism and the apparently exocytotic release of S1P from activated platelets also remains to be established. Perhaps these transporters are involved in “loading” exocytotic vesicles with S1P in addition to direct release of S1P at the plasma membrane?

Measurement and Distribution of Circulating LPA and S1P

Plasma levels of LPA and S1P are in the micromolar range (Baker et al. 2001; Ohkawa et al. 2008). As noted above, the circulating LPA pool is structurally diverse, whereas S1P is found in the blood along with its less abundant dihydro derivative. Levels of both lipids are considerably higher in whole blood although because of their obligatory intracellular roles as intermediates in phospholipid and neutral lipid metabolism, the precise contributions of these cell-associated pools of LPA and S1P to extracellular signaling remain difficult to establish. LPA and S1P in the plasma are largely bound to proteins which prominently include serum albumin and gelsolin. These lipids are also associated with lipoprotein particles, in particular S1P is markedly associated with high density lipoproteins in the plasma (Kimura et al. 2001; Theilmeyer et al. 2006). In support of the proposed “buffering” role for erythrocytes in maintaining plasma S1P homeostasis, plasma S1P levels are significantly reduced in anemic patients (Ohkawa et al. 2008), but paradoxically not reduced as a consequence of experimentally induced anemia in mice (Venkataraman et al. 2008). However, aside from suggestions that elevations in plasma LPA levels are associated with certain malignancies (Xu et al. 1998), which have been challenged by others (Baker et al. 2002), evidence for physiological or pathophysiological alterations in circulating levels of these lipids has not been reported. Furthermore, the affinities of LPA and S1P receptors for their ligands, at least measured in recombinant expression systems using purified lipid agonists, are considerably lower than the plasma levels of these mediators, yet blood and vascular cells that are responsive to these mediators do not appear to be persistently activated. These observations have led to the presumption that the bulk of LPA and S1P in the plasma is in a form that is metabolically and functionally “inert” (Smyth et al. 2008). Recently reported data challenge this idea and indicate that the turnover of LPA and S1P in the circulation may be more rapid and dynamic than previously appreciated.

Degradation of circulating LPA and S1P

While LPA can be degraded by phospholipases, and intracellular S1P can be cleaved by S1P lyase, the primary mechanism for termination of LPA and S1P signaling appears to be dephosphorylation to produce the corresponding alcohol derivatives which are not receptor active. The enzymes that catalyze these reactions are integral membrane proteins with a core of six transmembrane-spanning helices and a conserved active site formed by residues in

adjacent extramembrane loops (Brindley and Pilquill 2009; Sigal et al. 2005). The predicted topology of these enzymes (in some cases supported by experimental data) places the active site facing the lumen of an intracellular organelle or the extracellular face of the plasma membrane depending on the subcellular localization of the proteins. One class of these enzymes are S1P-selective phosphatases that play a role in intracellular sphingolipid metabolism. The lipid phosphate phosphatases (LPPs) are broader specificity enzymes. Considerable experimental evidence localizes them to the plasma membrane of many cells where they are positioned to inactivate LPA and S1P at the cell surface and in the extracellular space (Brindley and Pilquill 2009). Evidence that LPPs can attenuate LPA and S1P signaling responses comes largely from overexpression studies in mammalian cells showing that up-regulation of these enzymes is associated with increased intact cell “ecto” lipid phosphatase activity and decreased responsiveness to lysophospholipid agonists that can be circumvented when cells are stimulated with metabolically stabilized lysophospholipid analogs which are resistant to these enzymes (Tanyi et al. 2003). However, the “ecto” lipid phosphatase activity may not account for all of the effects of these enzymes on cell signaling. LPPs generate the less polar lipids monoacylglycerol and sphingosine, respectively, from LPA and S1P. These products can be taken up by cells and may serve as substrates to generate intracellular LPA and S1P or have independent intracellular signaling effects (Brindley and Pilquill 2009). Consistent with this idea, studies in *Drosophila* identify roles for the LPP enzymes encoded by the *wunen* genes in both “cell autonomous” guidance of migrating germ cells during early development and specific control of germ cell survival (Renault et al. 2004). These studies support the idea that cell surface LPP activity is responsible for both limiting the distribution of a germ cell guidance and survival factor in the embryo and also for generating germ cell specific signals. Although the *wunen* gene products are functional LPPs and their catalytic activity is required for control of germ cell migration, the identity of their (presumed) lipid phosphate substrate remains to be established.

Mouse Models of LPP Function

Mice subjected to genetic manipulations of the three widely expressed LPP genes have been reported (Brindley and Pilquill 2009). Inactivation of the *Ppap2c* gene encoding LPP2 produces animals with no obvious alterations in phenotype, although further examination of these animals may be warranted. By contrast, inactivation of the *Ppap2b* gene that encodes for LPP3 results in early embryonic lethality resulting from defects in patterning and vasculogenesis (Escalante-Alcalde et al. 2003). A recent report augments these studies by describing the characterization of mice with a disruption of the *Ppap2a* gene encoding LPP1 (Tomsig et al. 2009). These animals were derived from ES cells harboring a “gene trap” insertion at the *Ppap2a* locus containing a splice site acceptor in which pre-mRNA splicing generates a disrupted LPP1 mRNA. Although phenotypically unremarkable, these mice exhibit substantial decreases in LPP1 mRNA and LPP activity (measured as LPA phosphatase activity) in multiple tissues. Interestingly, use of the gene trap splice site acceptor appears to be much less efficient in neuronal tissues because LPP1 mRNA and LPP activity are normal in brains from these animals. In light of the severe developmental consequences of inactivation of LPP3, it is possible that alterations in splicing efficiency preserve LPP1 function during development of these animals. Global or tissue-specific knockouts of LPP1 have not yet been reported.

Role of LPPs in Inactivation of circulating LPA and S1P

Radiolabeled LPA or S1P or analogs of these molecules with unnatural radyl chains can be used as probes to monitor metabolism of these lipids in the blood either *ex vivo* or in live animals. Although LPA is essentially stable in plasma, it is rapidly degraded in whole mouse blood *ex vivo* with a half time of ~ 5 min. The half time for clearance of LPA from the blood of live mice is ~3 min, suggesting that both circulating blood cells and a non blood cell vascular component (possibly vascular endothelium) contribute to the rapid removal of circulating LPA.

The rate of removal of intravenously administered LPA from the blood of live mice was markedly slower (half time ~12 min) in animals homozygous for the *Ppap2a* gene trap allele (Tomsig et al. 2009). Clearance of intravenously administered S1P from the plasma of live mice was recently reported to also be relatively rapid, with a half time of ~15 minutes (Venkataraman et al. 2008) but S1P clearance has not yet been examined in the *Ppap2a* gene trap homozygotes.

Dynamic Regulation of LPA and S1P in the Circulation

These new findings have important implications for our understanding of the dynamics of bioactive lysophospholipid metabolism in the circulation and are also of significance for the design and development of therapeutic strategies to target LPA and S1P signaling. The relatively rapid clearance of exogenously supplied LPA and S1P from the circulation of live mice suggests that the turnover of circulating LPA may be much more rapid than previously appreciated. If results obtained using radiolabeled or unnatural radical chain analogs of LPA and S1P as “tracers” are faithfully reporting changes in the bulk of these circulating lysophospholipids, these observations imply that maintenance of the observed steady-state micromolar levels of these lipids in the plasma requires that the rates of degradation are balanced by rates of synthesis and/or replenishment. Consistent with this suggestion, manipulation of activities of enzymes responsible for production and degradation of these mediators clearly alters the “set-point” circulating level of LPA and S1P. For example, plasma LPA levels were elevated in mice homozygous for the gene trap allele of *LPP1* to ~1.5 fold of those of littermate controls (Tomsig et al. 2009), whereas as discussed above, genetic manipulation of activities of the lysophospholipase and kinase enzymes responsible for production of circulating LPA and S1P also result in alterations in the circulating level of these mediators (Pamuklar et al. 2009; Pappu et al. 2007; van Meeteren et al. 2006).

The long-standing observation that LPA accumulates when plasma and serum are incubated *ex vivo* underscores the fact that blood contains both a plasma lysophospholipase D activity (now ascribed to autotaxin) and a sufficient supply of circulating lysophospholipid substrates to sustain a substantial rate of production of LPA (Tokumura et al. 2002). Rough calculations indicate that the rate of production of LPA through this pathway in whole blood would be more than sufficient to counterbalance the rate of degradation of LPA revealed by the *in vivo* and *ex vivo* metabolism experiments discussed above. LPA, acting as a product inhibitor, can regulate autotaxin activity *in vitro* so decreases in circulating LPA may lead to further increases in autotaxin activity by relief of this inhibition (van Meeteren et al. 2005). Several groups have recently reported the identification of small molecule inhibitors of autotaxin (Federico et al. 2008). If these inhibitors can be delivered to the circulation of live mice at effective concentrations, they may provide a direct way to block LPA synthesis and evaluate the possibility that continuous production of LPA is required to maintain circulating LPA levels.

Concluding Comments

LPA and S1P receptors are attractive targets for pharmacological intervention in a variety of settings including the cardiovascular system (Noguchi et al. 2009). Recent progress in the development of S1P mimetic prodrugs typified fingolimod (FTY720) as novel treatments for autoimmune and inflammatory conditions underscores the potential of these mediators as therapeutic targets. The discovery that LPA and S1P are rapidly cleared from the circulation has obvious implications for understanding the pharmacokinetics of synthetic mimetics of these molecules designed to be receptor agonists or antagonists. The opposing pathways of synthesis and inactivation of these mediators that govern their steady-state levels in the blood effectively constitute “futile cycles”, which are a common motif in metabolic regulation allowing significant changes in flux or intermediate levels through a pathway through small changes in activity of the competing enzymes. Accordingly, this suggests that the kinase, phospholipase

and lipid phosphatase enzymes responsible for the synthesis and inactivation of LPA and S1P may also be effective targets for strategies designed to manipulate LPA and S1P signaling by altering endogenous levels of these mediators.

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Figure 1. Structures and metabolic relationships of LPA, S1P and their precursors and degradation products

Figure 2. Compartmentation of LPA and S1P in the blood