Lipids in Platelets

Lipids are low molecular weight, typically hydrophobic and amphipathic molecules found in all cell types. They are either generated endogenously or incorporated into cells from dietary sources. Their formation, trafficking, and metabolism are tightly controlled by cellular proteins, which include members of large families of phospholipases, lipid synthetases, ligases, oxidases/reductases, and transporters. Lipids fulfill 3 primary roles: structural, energy storage, and signaling.

Platelet Lipidomics: Modern Day Perspective on Lipid Discovery and Characterization in Platelets

What Can Proteomics Tell Us About Platelets?
Assessment of Platelet Function and the Arguable Need for More Antiplatelet Drugs
Genomics of Megakaryocytes and Platelets

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minor structural differences, including positional isomers, fatty acid (FA) chain length, and hydrocarbon saturation, which render a complex mixture of molecular species. In common with all mammalian cells, the major structural lipids in platelets are phospholipid, which arrange themselves in membranes with hydrophobic FAs orientated to the core and polar headgroups facing the aqueous phase (Figure 1). Phospholipid membranes include both the plasma membrane and also the numerous intracellular organelle membranes in platelets. During activation, they provide substrates that are converted enzymatically to bioactive species, including 1,2-diacylglycerol, FAs, eicosanoids/prostaglandins, phosphatidylinositides (PI), lysophospholipids, and lysophosphatidic acid (LPA). They are also indirectly oxidized to form phospholipid-esterified eicosanoids and prostaglandins by lipoxygenases and cyclooxygenases. Major remodeling of lipids occurs during platelet activation and is associated with significant structural alterations to platelet membranes, including shape change, spreading, microvesicle formation, and degranulation, as well as generation of bioactive prothrombotic species. Platelet membranes contain sphingomyelins and free cholesterol that are enriched in specialized signaling areas termed lipid rafts. Platelets also contain appreciable amounts of neutral lipids, including 1,2-diacylglycerol, triglycerides, and cholesteryl esters (CE). Some additional lipids present in smaller amounts, but with important signaling roles include members of the sphingolipids and glycolipids/ceramide families.

In this review, we will summarize what is currently known about each lipid class in platelets, how they are metabolized, and their major functions. Their roles in platelet-dependent pathologies will also be described.

Historical Perspective, Early Studies on Platelet Lipids

Before the advent of lipidomics, cellular lipids were studied using traditional techniques that included thin layer chromatography, gas chromatography/mass spectrometry (MS), and high-pressure liquid chromatography coupled with radiochemical, ultraviolet, or fluorescence detection. Research using these approaches still informs most of what we know about platelet lipids today.

One of the earliest comprehensive analyses of platelet phospholipids was performed in 1962 by Marcus et al.1–4 After this, studies were performed in the 1970s to 1980s comparing platelet lipid composition in a diverse and somewhat unusual array of population groups and species.5–15 Also, with interest in the role of lipids causing vascular disease increasing around that time, the effects of dietary supplementation with lipids on

Figure 1. Phospholipids in the membrane. A. Typical structure of a phospholipid: 1-stearoyl-2-arachidonylphosphatidylcholine. B. Structure of the plasma membrane lipid compartment and its relationship to formation of lipid mediators. Note, for simplicity, proteins have not been shown in this figure. DAG indicates diacylglyceride; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PL, phospholipid; PLD, phospholipase D; PS, phosphatidylserine; Ins(2,4,5)P3, inositol triphosphate; SM, sphingomyelin; and TX, thromboxane.
Platelet eicosanoids and related species were first identified in the early 1970s by Hamberg and Samuelsson19 and Hamberg et al20 in the Karolinska Institute, Stockholm, in parallel with Sir John Vane, in London. In 1974, the major platelet products 12S-HETE and 12-hydroxyeicosatetraenoic acid were demonstrated; then in 1975, thromboxane A2 (TXA2) was discovered as a platelet-derived lipid that causes irreversible aggregation. This seminal work contributed to the awarding of a Nobel Prize to Samuelsson and Vane for discoveries on prostaglandins, TXs, and related biologically active substances. In the early 1980s, thrombin was demonstrated to cause significant alterations to membrane lipids, with PI showing losses of ≤45%. Arachidonic acid (AA) specifically decreased in PI and phosphatidylcholine pools, suggesting these to be likely sources of substrate for eicosanoid generation.21,22 Release of AA from PI was proposed to involve the sequential action of phospholipase C generating 1,2-diacylglycerol followed by diacylglycerol lipase acting on 1,2-diacylglycerol, representing an alternative to phospholipase A2 (PLA2).22 These studies established the overall lipid composition of resting and activated platelets, in particular about the more abundant species such as phospholipids and FAs. In contrast, 1980s to 2000s saw intense interest in characterizing the role of specific platelet signaling lipids in regulating platelet function and contributing to both physiological hemostasis and human disease. Examples that will be discussed herein and that deserve special mention include (1) the central role of TXA2 in regulating hemostasis and contributing to pathological clot formation, (2) the role of 1,2-diaclyglycerol, PI, and inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) in promoting calcium mobilization after receptor-dependent activation of platelets, and (3) the role of anionic phospholipids in promoting coagulation.

**Lipidomics: Current State-of-the-Art**

Since the mid-2000s, the advent of `omics, largely driven by the development of sensitive benchtop mass liquid chromatography–MS (LC/MS) instruments, for example, electrospray ionization coupled to tandem (triple quadrupole or MS/MS) or time-of-flight instruments, has revolutionized our ability to study small amounts of complex mixtures of diverse lipids in biological samples, hence the term lipidomics. These days, the term tends to be synonymous with the MS of lipids. The major disadvantages of older approaches instead of LC/MS were (1) low sensitivity and selectivity (thin layer chromatography and high-pressure liquid chromatography), (2) the need for time-consuming derivatization methods (eg, for gas chromatography/MS), and (3) the requirement for radioisotopes with their inherent health issues, notably in regard to 32P-orthophosphate. When compared with this, LC/MS combines high sensitivity with the ability to detect, characterize, and quantify individual molecular species directly without derivatization or purification. Older methods were generally unable to analyze specific lipids directly (eg,}

![Figure 2. Summary of traditional and new lipidomic technologies used to discover and characterize cellular lipids. GC/MS indicates gas chromatography/mass spectrometry; HPLC-UV, high-pressure liquid chromatography/ultraviolet; and LC/MS/MS, liquid chromatography/tandem mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; and TLC, thin layer chromatography.](http://circres.ahajournals.org/)

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1-stearoyl-2-arachidonyl-phosphatidylcholine would not have been measurable as a single species in a complex mixture. Traditional and newer lipidomic methodologies are summarized in Figure 2.

Lipidomics is broadly divided into 2 separate approaches: (1) liquid chromatography-tandem MS (LC/MS/MS), which is targeted, highly sensitive, and quantitative and (2) shotgun lipidomics, which is high throughput but can only detect the most abundant species. In LC/MS/MS, several molecular species are analyzed in the same sample after separation, by their characteristic parent m/z (mass:charge ratio, where mass is divided by the total net charge of the molecule) and daughter ions that form after collision-induced-fragmentation (where molecules are collisionally activated using an inert gas then break apart into small daughter ions that can be separately analyzed). These methods typically use a tandem MS instrument (eg, tandem in space as a triple quadrupole or tandem in time, as an ion trap). These comprise 3 separate chambers: the first and third of which are mass analyzers that house 4 parallel gold-plated rods (quadrupoles). The second is a collision cell where nitrogen or argon fragments the molecule for MS/MS analysis (Figure 3A). Thus, LC/MS/MS methods are best suited to studies where researchers are interested in specific lipids and require accurate quantitation. However, separations can be long (eg, ≥1 hour for phospholipids). However, shotgun lipidomics involves direct infusion of complex mixtures, without separation, into a mass spectrometer, followed by MS scanning of a defined mass window, often using high-resolution instruments (where the MS can distinguish molecules that are extremely close in mass, eg, differing in mass by ≤1–5 ppm). The advantage is that many samples can be analyzed in a relatively short time; however, it is not generally quantitative, and low abundance (often highly biologically relevant) species are missed. A newer quantitative approach combining LC separation with high-resolution MS/MS on rapid scanning Fourier transform or time-of-flight instruments, termed multidimensional MS, is now increasing in popularity in particular for profiling studies. In Fourier transform MS, the m/z is determined based on the cyclotron frequency of the ions in a fixed magnetic field. One example of this is the Orbitrap, a benchtop instrument currently popular for lipidomics that contains an outer barrel-like electrode and a coaxial inner spindle-like electrode that together generate an electrostatic field (Figure 3B). In contrast, time-of-flight instruments calculate mass based on the length of time it takes for molecules accelerated by an electric field to reach a detector at a known distance, with heavier particles having lower speeds (Figure 3C).

Up to now, platelet lipids have not been extensively studied using lipidomics. LC/MS/MS has been applied to quantitation of eicosanoids, such as TX B2 (TXB2) and HETE, and to identification of sphingolipids molecular species (Figure 4A). Shotgun methods have been applied to the characterization of platelet phospholipids, lysophospholipids, CEs, and ceramides in a small number of studies. Using traditional methods, individual lipid pools first had to be isolated using thin layer
chromatography, then hydrolyzed to release FAs for determination as free acid species, a considerably slower approach that requires more material.1–4 Recently, the composition of stored platelets and extracellular vesicles was characterized, showing that vesicles became enriched with LPA, cholesterol, and other lipids.28,29

In the past 2 to 3 years, MS technology has undergone significant increases in both sensitivity and scanning speed, with new benchtop instrument configurations becoming available, and only now are researchers beginning to apply these to the study of cells and tissues. At this time, we are only learning what these new technologies are capable of, and what questions they might answer. One approach, ion mobility (Waters Synapt; AB Sciex SelexION), allows separate detection of lipids that have the same accurate mass (eg, elemental composition) and retention time on high-pressure liquid chromatography, giving an extra layer of molecular differentiation that was not possible before. This is based on the differing mobility of the ions in a carrier gas. For example, 2 triacylglycerides each containing 2 stearic acids and 1 palmitic acid, but with the palmitic acid at different positions on the glyceride backbone can be distinguished. A second approach, using Fourier transform instruments, such as the Orbitrap (ThermoFisher), allows rapid high-resolution scanning that differentiates between lipids based on mass differences down to 1 to 5 ppm (eg, can distinguish coeluting lipids with masses of m/z 516.280 and 516.289; Figure 4B). Coupled with high-pressure liquid chromatography, these instruments are particularly suited to structural characterization and global lipidomic screening approaches. Another configuration of relevance to lipidomics is the combination of matrix-assisted laser desorption/ionization (MALDI) mass spectrometric image of the positive ions (m/z 788.6 blue color) derived from 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC) a phospholipid found in the cells of the airways. MALDI mass spectrometric image of the positive ions (m/z 703.6, red) derived from the sphingomyelin molecular species SM(d18:1/16:0) an abundant phospholipid present in the pulmonary blood vessels. MALDI mass spectrometric image of the positive ions (m/z 810.6, green) derived from 1-oleoyl-2-arachidonoyl-phosphatidylcholine (SAPC) localized to both airways and blood vessels. Merged MALDI mass spectrometric images indicating colocalization of phospholipids in cells of the lung parenchyma.

Methodological Issues: Working With Platelet Lipids In Vitro

Washed human platelets are isolated from whole blood using centrifugation. Care needs to be taken during bloodletting, thus it is important to use a needle that allows the blood to flow freely to ensure that platelets are not exposed to shear. This can itself activate the cells to generate lipid mediators even before isolation. Several protocols are listed in detail by Watson and Authi11 and generally involve a low-speed centrifugation of whole blood anticoagulated with acid citrate-dextrose (lowers pH and chelates Ca2+) to generate platelet-rich plasma followed by a faster centrifugation of the recovered plasma to pellet the platelets themselves. This is followed by a single wash step in Tyrode buffer with acid citrate-dextrose (9:1). Anticoagulants,
such as prostacyclin and indomethacin, are included during washing by many investigators but should be avoided in studies of lipids, to avoid interference to lipid-sensitive signaling pathways. In this case, care needs to be taken because the platelets may activate after the last spin when anticoagulant is removed. The plasma/cells need to be maintained at ≈20°C and gently pipetted at all times. On final resuspension in Tyrode buffer, they may appear slightly clumpy but after ≈15 to 20 minutes will have dispersed to a single cell suspension, without requiring manipulation. It is advisable to avoid repeated pipetting, or shaking/inverting of the cells during isolation and handling. Once isolated, platelets should be kept at room temperature and used within 2 to 3 hours.

Platelet lipids can be isolated using several different methods, including liquid/liquid extraction methods such as Bligh and Dyer using chloroform/methanol, or as in our laboratory, hexane/isopropanol based solvent mixtures that broadly extract most molecular species. The advantage of the latter is lipid extraction into the upper rather than lower organic phase. Specific lipids, such as eicosanoids, can also be more selectively isolated using solid phase extraction methods, such as C18 columns. Once isolated, we typically resuspend in a small volume of methanol or chloroform/methanol when highly concentrated and stored at −80°C before analysis, under inert gas.

Unsaturated lipids can undergo facile oxidation ex vivo, so they need careful handling. Lipid extracts should be kept on ice when in use but stored under inert gas at −80°C. Although some investigators routinely include metal chelators and antioxidants when studying oxidized species, this is not generally necessary with platelet lipid extracts, if they are handled appropriately, and analyzed within a few weeks of generation. As confirmation, we only detect expected enantiomeric species of eicosanoids specific to platelet enzymes (e.g., prostaglandin [PGE]2, 12-HETE, but little 8-iso-PGE2 or related isomers) in our assays. Although antibody-based ELISA kits for eicosanoids are commercially available and may seem attractive where MS lipidomics is not locally available, these should be avoided for measurement of lipids in complex samples because of lack of antibody specificity. However, they may be acceptable for washed platelet eicosanoid measurements because only a selected low number of eicosanoids and related species are formed, but careful validation and comparison should be performed with MS before use.

Specific Lipid Families in Platelets

Aminophospholipids

Like all mammalian cells, the prominent phospholipids in platelets are phosphatidylcholine and phosphatidylethanolamine accounting for ≈40% and 28% of total phospholipids, respectively. Phosphatidylinositol and phospatidylerserine are also relatively abundant (≈18% and 10%) with smaller proportions of PI (3%–5%). The FA composition of platelet phospholipids is described in Historical Perspective, Early Studies on Platelet Lipids of this article and varies for each phospholipid class. Studies in the mid-1970s, using chemical labeling or exogenous phospholipases, established that platelet phospholipids are distributed asymmetrically between plasma membrane bilayers, with the aminophospholipids phosphatidylethanolamine and phosphatidylserine facing the cytosol, and phosphatidylcholine and sphingomyelin facing the outside. On stimulation, a substantial proportion of aminophospholipid is externalized generating a procoagulant or thrombogenic surface. In contrast, lipids required for intracellular signaling are generated from lipids that face the inside. These include Ins1,4,5P3 and 1,2-diacylglycerol generated from PI 4,5-bisphosphate (PI(4,5)P2), hydrolysis of AA to act as a substrate for TX generation and formation of PI-3,4,5-trisphosphate (PI(3,4,5)P3 [PIP3]). Thus, phospholipids act as a major reservoir for substrates for enzymes that generate key platelet signaling mediators. These will be described in more detail below.

In 1977, Zwaal et al. noticed that unactivated platelets were inert to plasma coagulation because of the absence of phosphatidylserine on their surface. Later, Bevers et al. found that activation of platelets with thrombin/collagen caused exposure of aminophospholipid and correlated with procoagulant activity. Around this time, and for many years later, studies on mechanisms of phospholipid translocation, primarily in erythroctyes, revealed several regulatory mechanisms that maintain phospholipid asymmetry. These included a flipase (proposed as P4 ATPase), and a flopase that is promoted by ATP-binding cassette protein transporter C1/multidrug resistance protein multidrug resistance protein 1. The identities and mechanisms of action of these proteins are still not fully known. Opposing enzymes, termed scramblases, are Ca2+-dependent and required for effective exposure of aminophospholipid on the platelet surface to promote coagulation. The role of the platelet membrane in acting as a critical mediator of coagulation, through promoting factor activity, was first proposed in the 1980s. It is now known that at physiological pH, surface exposed phosphatidylserine provides the negatively charged platform that enables calcium ions to form bridges with γ-carboxyglutamic acid–containing Glu domains on coagulation factors. In the case of the prothrombinase complex, FVα is thought to undergo a conformational change that forms a high-affinity binding site for FXa. This brings thrombin generation to the site of platelet activation, enabling coagulation and aggregation to be focused together at the site of hemostatic need. The hunt for the platelet scramblase has been of clinical importance because its absence accounts for the rare bleeding disorder, Scott Syndrome, first described in 1979 by Weiss et al. In 2010, the involvement of a protein, TMEM16F, in supporting scramblase activity in platelets was identified. Since then, the 5 patients with Scott Syndrome to date identified have been reported to have different mutations in the gene encoding TMEM16F, including in intron 6 (G-to-A), disrupting the donor splice site consensus sequence, and in exon 11 as a single-nucleotide insertion that predicts a frameshift and premature termination of translation at codon 411. At this time, whether TMEM16F is the scramblase itself or an accessory protein is the subject of debate. There seem to be ≥2 independent mechanisms for achieving aminophospholipid externalization. Recent studies have established that TMEM16F is required for agonist-triggered scramblase but not that mediated during platelet aging/apoptosis. Also, only agonist-triggered trafficking of aminophospholipids requires influx of extracellular Ca2+. Up to
now, aminophospholipid externalization has generally measured using the flow cytometry probe, annexin V-fluorescein isothiocyanate. However, this gives no information on either the molecular species of aminophospholipid or amounts. Using a newly developed lipidomic assay, where amine headgroups of external facing aminophospholipid are derivatized using a cell impermeable-reagent, we recently showed that platelets (agonist activated, apoptotic, or energy depleted) externalize $\approx$3% to 5% of the total cellular pool of 5 phosphatidylethanolamine and 2 phosphatidylserine distinct molecular species (Figure 5). Those externalized were the most abundant platelet aminophospholipid molecular species, with the same for either apoptosis, agonist activation, or energy depletion, indicating that the process is not selective for aminophospholipid with particular FA chains. We also showed that these aminophospholipid were not efficiently externalized in Scott Syndrome platelets, and that for phosphatidylethanolamine, the species externalized by platelets were the most effective in in vitro thrombogenic assays when compared with those with longer or shorter FA chains. Thus, FA side chains are molecular determinants that can contribute to the coagulation-regulating activity of phospholipid. The detailed biophysical mechanisms of this are not known but may relate to accessibility of aminophospholipid phosphate groups with $\text{Ca}^{2+}$ and Gla domains. Next, it will be important to compare how other membrane lipids, for example, CEs, triglycerides, and sphingolipids with platelet FAs, influence coagulation factor activities. For this, knowing which FAs predominate in platelet plasma membrane lipids of these classes would also be important. Lipidomics will be of central importance in helping answer these questions through defining the composition of the platelet plasma membrane during both health and disease and different activation states. This could be achieved through either targeted or global lipidomic strategies.

Phosphatidylinositol

PIs comprise several interrelated species that contain an inositol headgroup. Two are shown in Figure 6, along with a diagram describing their inter-relationship. These lipids play important roles in cell signaling in health and disease, through reversible phosphorylation of the inositol headgroup that is tightly controlled by enzymes. The phosphoinositide PI(4,5)P$_2$ is metabolized in response to receptor activation through 2 distinct pathways, namely (1) irreversible cleavage of the phosphodiester bond and (2) reversible phosphorylation of the inositol headgroup. The latter generates the major product of the PI 3-kinase pathway, PIP$_3$, and the former, the 2 second messengers, 1,2-diacylglycerol and Ins(1,4,5)P$_3$, which activate protein kinase C and mobilize intracellular $\text{Ca}^{2+}$, respectively.

1. The phosphodiester cleavage of PI(4,5)P$_2$ is mediated by the phospholipase C (PLC) family of phosphodiesterases, which is composed of 10 isoforms, several of which are regulated by the major G-protein–coupled and tyrosine kinase–linked receptors in platelets. 1,2-Diacylglycerol

![Figure 5. Molecular species of phosphatidylethanolamine (PE) and phosphatidylserine (PS) that are externalized by activated, aging, or apoptotic human platelets.](http://circres.ahajournals.org/Downloaded from)
activates several isoforms of protein kinase C which, in combination with the release of intracellular Ca\textsuperscript{2+} by Ins(1,4,5)P\textsubscript{3}, leads to powerful platelet activation responses, such as shape change, aggregation, and secretion. Platelets express variants of PLC-\(\beta\) (\(\beta_1, \beta_2, \beta_3, \beta_4\), and PLC-\(\gamma\))\textsuperscript{54–57} PLC-\(\beta\) is stimulated by G-protein–coupled receptors (including TX and protease activated receptor 1/4), whereas PLC-\(\gamma\) is stimulated by single transmembrane glycoprotein receptors (eg, GPIIb–IIIa, GPIb\(\alpha\), GPVI).\textsuperscript{58} Ins(1,4,5)P\textsubscript{3} activates receptors that function as Ca\textsuperscript{2+} channels in the dense tubular system.

2. PI(4,5)P\textsubscript{2} is also converted to the lipid second messenger, PIP\textsubscript{3}, by the class I family of PI3-kinases. These catalyze the phosphorylation of PI, PI(4)P, or PI(4,5)P\textsubscript{2} at position 3 of the inositol ring.\textsuperscript{59} PI3Ks are divided into class I, II, and III, with class I further subdivided into \(\alpha, \beta, \delta,\) and \(\gamma\) isoforms. Platelets contain all class I isoforms of PI3K although the level of the \(\delta\) isoform is lower than others.\textsuperscript{60} Stimulation of platelet G-protein–coupled receptors results in phosphorylation of PI(4,5)P\textsubscript{2} by the PI3K\(\gamma\) isoform to generate PIP\textsubscript{3}. Deficiency of \(\alpha, \beta,\) and \(\gamma\) isoforms of PI3K in mice results in a mild platelet aggregation defect and impaired thrombosis in vivo.\textsuperscript{61} In vitro, PI3K\(\gamma\)-deficient platelets disaggregate faster after ADP activation and show mildly impaired ability to mobilize intracellular Ca\textsuperscript{2+}.\textsuperscript{62} Several studies show that this isoform controls a major part of platelet ADP responses.\textsuperscript{61,63,64} The protein targets for PIP3 binding and activation downstream of PI3K\(\gamma\) that promote ADP signaling include Akt isoforms. The PI3K\(\beta\) isoform has been proposed to play several roles in regulating platelet activation, including via promoting integrin-dependent Ca\textsuperscript{2+} flux and G\textsubscript{i}-dependent activation of Rap1b. In recent years, a small molecule inhibitor of PI3K\(\beta\), TGX-221, has been developed as a potential antithrombotic therapy.\textsuperscript{60,65,66}

PI3K\(\gamma\) binds to a domain of \(\approx\)120 amino acids, known as a pleckstrin homology (PH) domain, which takes its name from the major protein kinase C substrate in platelets, pleckstrin (which is one of few proteins to have 2 PH domains). PH domains are found in a variety of signaling and cytoskeletal proteins, including, in platelets, (PLC)\(\gamma\)2, and the tyrosine kinase Btk. PH domains are usually found with other domains, such as SH2 and SH3 domains, that bind to phosphorylated and proline-rich regions, respectively. PH domains can also bind to other PIs. In addition, PIP3 is rapidly metabolized in platelets to PI(3,4)P\textsubscript{2} by the Src homology 2 domain-containing inositol 5-phosphatase 1, which itself is regulated by tyrosine phosphorylation. This enzyme removes the phosphate at D-5 of the inositol ring generating PI(3,4)P\textsubscript{2} which can then be degraded.
by additional phosphatases. PI(3,4)P_2 is generated in large amounts, accumulating slowly, but independently of aggregation and integrin activity.\textsuperscript{67,68} It was originally suggested to regulate aggregation, integrin signaling, and thrombus growth, but its targets and their overall significance in platelets remain unknown.\textsuperscript{59-70} Although Src homology 2 domain-containing inositol 5-phosphatase 1 is clearly established as a regulator of PI lipid levels in platelets, the roles of additional phosphatases also expressed in these cells (notably SHIP2 and phosphatase and tensin homolog) are currently unclear.

Thus, there is bewildering number of pathways of metabolism of PIs and regulation of PH domain-containing proteins in platelets such that we still have a relatively poor understanding of the role of the PI 3-kinase pathway at the molecular level. However, the functional significance of PI 3-kinase in platelets has been widely demonstrated using mutant mice and both broad spectrum (eg, wortmannin and LY294002) and PI 3-kinase isoform-specific inhibitors.

Many PIs are generated rapidly and degraded at distinct cellular sites by specific PI-metabolizing enzymes that include lipid kinases, lipid phosphatases, and phospholipases, some of which have been described herein (Figure 4).\textsuperscript{71,72} Several of these enzymes are involved in platelet function during agonist-induced activation. For example, PI-4-phosphate-5-kinase type 1 phosphorylates PI-4-phosphate (PI(4)P) to generate PI(4,5)P_2 on the plasma membrane.\textsuperscript{73} Platelets lacking PIP4K1-γ show impaired generation of PI(4,5)P_2 and a significant defect in anchoring their cell membranes to the underlying cytoskeleton.\textsuperscript{74-75} PI(4,5)P_2 has also been proposed to bind talin and to support its role in integrin activation.\textsuperscript{76-78} PIP3 is generated rapidly during platelet activation and plays a key role in recruitment and activation of PLCγ2 and other PH domain containing enzymes.\textsuperscript{79}

Measurement and quantitation of PI molecular species using MS-based lipidomics approaches has been a major goal for several years, particularly in the area of cancer research. However, these lipids are extremely difficult to analyze using LC/MS primarily because of (1) difficulty in achieving efficient extraction and (2) in-source fragmentation that occurs on ionization of poly-phosphorylated forms, leading to loss of phosphate groups. A recent review highlighted available methods that allow detection and quantitation of some individual PI species, but to date, there is no single methodology that allows robust identification and quantitation of all.\textsuperscript{80} This has significantly hampered the study of these species in living cells because they form transiently and at extremely low concentrations during cell signaling. For example, the specific molecular species of PI regulated in activated platelets including in terms of FA chain length and saturation are still unclear. The development of sensitive and specific methods for quantifying PI molecular species is thus a major ongoing goal in lipidomics that may become possible because newer methodologies become available and is of importance in furthering our understanding of the roles of these important platelet lipids in platelet activation and platelet-driven pathologies.

**FAs, Eicosanoids, and Related Species**

A major early response of platelets to activation is the switching on of several phospholipases, including cytosolic PLA\textsubscript{2,α} isoforms, which cleave FAs from the sn2 bond of phospholipids. Early studies demonstrated that the prominent FAs released were AA, linoleic acid, palmitic acid, and stearic acid.\textsuperscript{81-83} However, the most important in terms of signaling is AA, the precursor for oxidative transformation to several eicosanoids via lipoxygenase and cyclooxygenase enzymes (Figure 7). Platelets express several PLA\textsubscript{2} isoforms, including Ca\textsuperscript{2+}-sensitive and insensitive forms. In general, the Ca\textsuperscript{2+}-sensitive cytosolic PLA\textsubscript{2,α} is considered the source of AA for eicosanoid generation. In support, a recent study demonstrated that genetic deficiency of this isoform leads to significantly impaired platelet eicosanoid generation, along with platelet dysfunction in humans.\textsuperscript{84}

Platelet-generated eicosanoids are best exemplified by the potent proaggregatory TXA\textsubscript{2}, generated by coordinated action of cyclooxygenase-1 and TX synthase. This lipid is extremely unstable and rapidly rearranges to TXB\textsubscript{2}, which is released by platelets at ng amounts per 2×10\textsuperscript{8} cells (Figure 5). TXA\textsubscript{2} binds the G\textsubscript{αi}-coupled receptor TP\textsubscript{αi} with high affinity, triggering activation of PLCβ. A number of receptor agonists to TP have been developed, including U46,619 and SQ 26,655 that mimic the action of TXA\textsubscript{2}. Because of its short plasma half-life, in vivo generation of platelet TX in both humans and mice is best measured through analysis of urinary metabolites, 11-dehydroTXB\textsubscript{2}, or 2,3-dinor TXB\textsubscript{2}.\textsuperscript{85-86} This has been achieved using either gas chromatography or LC/MS and is considered a reliable surrogate for measurement of in vivo platelet reactivity, which is significantly raised in both cardiovascular disease and smokers.\textsuperscript{82-86}

However, 12-HETE, generated by 12-lipoxygenase, is quantitatively more abundant but does not display any potent bioactivity toward platelets in vitro. In contrast, the 12-lipoxygenase–deficient mice display a mild hyper-responsiveness to ADP; however, their collagen responses were normal.\textsuperscript{96} 12-Hydroxyeicosatetraenoic acid is also abundant, accounting for ≤30% of the total AA flux through cyclooxygenase-1. This is formed through conversion of prostaglandin H2 (PGH\textsubscript{2}) either nonenzymatically or via TX synthase although its function is unknown.\textsuperscript{97-99} Platelets also generate smaller amounts of PGs, particularly PGE\textsubscript{2} and prosstaglandin D2 (PGD\textsubscript{2}), through nonenzymatic rearrangement of PGH\textsubscript{2}. They also generate isoprostanes via radical-based biochemistry, notably 8-epi-PGF2α, but levels are ≤1000-fold less than corresponding 12-HETE and TX.\textsuperscript{100,101} PGE\textsubscript{2} is either pro (low dose)– or anti (high dose)– aggregatory, through activation of either EP3 or IP receptors, respectively.\textsuperscript{102} In contrast, 8-epi-PGF\textsubscript{2α}, a specific isoprostane isomer, is primarily antiaggregatory through acting as a TX receptor antagonist although the physiological relevance of this is unclear.\textsuperscript{103-105}

The importance of eicosanoid signaling in terms of platelet physiology and vascular disease is underscored by the widespread use of the cyclooxygenase inhibitor aspirin in prevention of cardiovascular events, as well as an anti-thrombotic agent in stroke, antiphospholipid syndrome, and other prothrombotic conditions.\textsuperscript{106-108} This was established by seminal work from several laboratories notably those of Vane, Samuelsson, Roth and Majerus, Patrono, and FitzGerald\textsuperscript{109-115} and was recently the subject of the 2013 Grand Prix Scientific (Lefoulon-Delalande Foundation, Institute of France) awarded to Garret
FitzGerald and Carlo Patrono. More recently, a number of studies found that low-dose (considered platelet-selective) aspirin can reduce the spread of existing cancers and the risk of developing others, in particular adenocarcinoma of the gut, some lung cancers and breast and prostate cancer. The mechanisms have not been elucidated but have been suggested to involve an antiplatelet effect, particularly in terms of preventing spread of existing cancers through the bloodstream.116–121 The biological basis of this is uncertain, in part because platelets are implicated in multiple stages of cancer progression, but it may involve platelet-derived signaling lipids that are generated either primarily or secondarily via cyclooxygenase-1–dependent signaling. Lipidomics could help address this question in the future. For example, through targeted or untargeted analysis of platelet lipids that are generated on activation in an aspirin-sensitive manner, candidate molecules that may play a role in cancer progression could be identified. Known lipids that could already be considered would include PGE2, which is known to help cancer cells both evade the immune system and resist drug treatment.

Additional eicosanoids, including leukotrienes and lipoxins, can be generated through transcellular pathways involving platelet–leukocyte interactions. In 1989, Maclouf and Murphy122 demonstrated that platelets efficiently converted neutrophil-derived leukotrienes \( \Lambda_4 \) to leukotrienes \( \Lambda_3 \) in mixed cell incubations. After this, neutrophil–platelet incubations were shown to generate lipoxin \( \Lambda_4 \) from endogenous substrate, but this required activation with ionophore.123 In 1990, receptor-mediated interactions were also shown to promote this activity.124,125 However, whether transcellular biosynthesis itself is a significant source of these lipids in vivo is still unclear because determining cellular origin of lipids in complex biological samples is extremely difficult.

Lipidomics has already been extremely powerful in facilitating studies of platelet eicosanoids, primarily through enabling rapid and accurate, high-sensitivity quantitation of these species using LC/MS/MS approaches in both isolated cells and in human and murine disease. Newer lipidomics technologies could further our understanding of the biology of these species, through a number of ways including imaging their spatial and temporal generation in tissues (eg, matrix-assisted laser desorption/ionization-MS), and discovery and structural characterization of new members of this important class of biomolecules, using high-resolution scanning methods.

**Oxidized Phospholipids**

Oxidized phospholipid has long been known as biproducts of nonenzymatic peroxidation, and several are found at relatively high concentrations in atheroma lesions. Recently, we used a targeted lipidomic approach to demonstrate that human platelets generate significant amounts of specific oxidized phospholipid molecular species via enzymatic mechanisms,
during acute activation by thrombin, collagen, or Ca\(^{2+}\) ionophore.\(^\text{26,126}\) This indicates that phospholipid oxidation is not simply an accidental consequence of inflammatory disease but a regulated process of likely importance during physiological hemostasis. To date, 3 main families have been described, 2 from 12-lipoxygenase (10 total lipids) and 4 families of 16 esterified PGs generated via cyclooxygenase-1 (total 26 lipids; Figure 8).

The approach used for their discovery, precursor-LC/MS/MS, is a tandem MS mode that enables molecules to be detected that contain a specific functional group. In this case, we used precursor LC/MS/MS to search specifically for esterified eicosanoids because on collision-induced fragmentation, they generate a robust carboxylate anion signal in negative ion mode. Initial studies demonstrated a family of six 12-HETE–containing phospholipids, comprising 4 phosphatidylethanolamines and 2 phosphatidylcholines. The phosphatidylethanolamines include several plasmalogens and all represent oxidized forms of the most abundant phosphatidylethanolamine and phosphatidylcholine species in platelets. The positional and enantiomeric specificity of the HETE was confirmed using several chromatographic approaches, and the lipids are absent in platelets from 12-lipoxygenase–deficient mice (M. Aldrovandi and V.B. O’Donnell, unpublished data, 2012). Up to 30% of the total 12-HETE generated by platelet 12-lipoxygenase is incorporated into phospholipid, representing a significant proportion of the endogenous pool.\(^\text{26}\) HETE-phospholipid generation is highly coordinated using intracellular signaling pathways that include Src tyrosine kinases, Ca\(^{2+}\) mobilization, and phospholipases and they remain cell associated after their generation.\(^\text{26}\) Their formation requires PLA\(_2\)-dependent hydrolysis of AA from the plasma membrane, followed by its oxidation by 12-lipoxygenase, then re-esterification using Co-A–dependent ligases.\(^\text{26}\) In vitro, liposomes containing physiological amounts of HETE-phospholipids significantly enhance tissue factor-dependent thrombin generation in plasma.\(^\text{26}\) This indicates that they may play a key role in hemostasis and is consistent with the observation that in myeloproliferative disorders, patients with 12-lipoxygenase deficiency hemorrhage is greater than those with normal levels of the enzyme.\(^\text{127}\) Pathways for their formation and cellular localization are summarized in Figure 9.

Platelet 12-lipoxygenase also generates 4 oxidized phospholipids containing 14-hydroxydocosahexanoic acid, in place of 12-HETE.\(^\text{126}\) Recently, we scanned for precursors of m/z 351 and uncovered 4 new families of

![Figure 8. Generation of oxidized phospholipids (PLs) by enzymes. PL substrates (phosphatidylethanolamine [PE] is shown here) are hydrolyzed by phospholipase A2 (PLA2) generating arachidonic acid (AA) that is oxidized by cyclooxygenase (COX)-1 or 12-lipoxygenase (LOX) to form eicosanoids, which are then re-esterified into PE by fatty acyl Co-A ligases (FACL). PGD\(_2\) indicates prostaglandin D2; and PGE, prostaglandin.](http://circres.ahajournals.org/)}
phosphatidylethanolamine-esterified PGs, including 4 molecular species each of PGE$_2$-phosphatidylethanolamine and PGD$_2$-phosphatidylethanolamine. Similar to the other esterified eicosanoids, these are generated acutely via coordinated pathways and remain cell associated. Their inhibition by aspirin or indomethacin in vitro or in vivo by low-dose aspirin indicates a requirement for platelet cyclooxygenase-1. PGs are considerably more hydrophobic than HETEs and might not be expected to remain within the cell membrane itself. Thus, they may protrude out, anchored by the sn1 FA, in a manner similar to that proposed in the Lipid Whisker Hypothesis, available to interact with receptors on adjacent cells and mediate paracrine signaling. Currently, their physiological functions are unknown.

In summary, human platelets generate a diverse array of oxidized phospholipid acutely on activation. We have also found that lipoxygenase isoforms in other innate immune cells, including neutrophils and monocytes/macrophages, generate analogous lipids on activation, indicating that this is a common theme of likely importance in acute response to injury. All these cell types undergo significant changes to the plasma membrane compartment on activation, including shape change, vesiculation, phagocytosis, and microvilli generation. Although we know much on how proteins control these events, far less is known about how lipid oxidation influences the dynamic behavior of the cell membrane. Chemical oxidation of model membranes has long been known to cause changes in headgroup distance, membrane thickness, and water permeability, whereas high concentrations (milligram per milliliter) of purified 15-lipoxygenase can cause pore formation in purified organelle membranes, through lipid peroxidation, and its overexpression in nonerythroid cells is associated with mitochondrial membrane collapse. Thus, the significant oxidation that occurs on platelet activation might have similar consequences. Future work using biophysical methods will address these questions.

Lysophospholipids and LPA
Lysophospholipids and LPA are generated from phospholipids via the actions of phospholipases: PLA$_2$, phospholipase

![Figure 9. Enzymatic pathways that form oxidized phospholipids (PLs) in platelets. A, Thrombin activation of protease activated receptors (PAR) triggers formation of HETE-phosphatidylethanolamine (PE) and HETE-phosphatidylcholines (PCs), via several intracellular signaling cascades. Glutathione peroxidase (GPX) is required to reduce the initial hydroperoxide product to 12-HETE-PLs. B, Formation of prostaglandin (PGE$_2$)- and prostaglandin D2 (PGD$_2$)-PE in platelets after thrombin activation requires several intracellular signaling pathways, and most likely occurs in dense tubular membranes, where cyclooxygenase (COX)-1 has been localized. FACL indicates fatty acyl Co-A ligases; MAPK, mitogen-activated protein kinase; PL$_A_2$, phospholipase A2; and LOX, lipoxygenase.](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.113.301057)
D (PLD), or through phosphorylation of 1,2-diacylglycerol. They can also be generated nonenzymatically although, however, there is no evidence currently that this happens in platelets. The structure of lysophosphatidylcholine is shown (Figure 10). During platelet activation, powerful stimulation of cytosolic PLA₂ occurs, generating AA for eicosanoid generation and resulting in formation of lysophospholipids. Using [¹⁴C]-stearic acid to prelabel phospholipids, generation of stearic acid-lysophospholipids of several classes, including PI, phosphorylcholine, phosphatidylethanolamine, and phosphatidylerine, was reported in response to stimulation by collagen. Lysophosphatidylethanolamine and phosphatidylcholine appeared within 30 s, with lyso-PI and phosphatidylerine appearing ≈2 to 3 minutes. However, the overall increase from the baseline was relatively small (2–3-fold). Although only stearic acid–containing species were studied, platelets might be expected to generate phosphatidic acid and plasmalogens species also for phosphorylcholine and phosphatidylethanolamine, reflecting the predominant molecular species of phospholipid in these cells. The temporal generation of specific lysophospholipid species in platelets is not currently known. These questions have not yet been addressed using lipidomics but could easily be through either targeted quantitative or multidimensional MS approaches.

LPA is generated by thrombin-stimulated platelets and can itself stimulate aggregation through G-protein–coupled receptors. High concentrations exist in serum (5–10 μmol/L) and were suggested to originate directly from platelets although this would seem unlikely given their rate of production of lysophospholipid precursors. More recently, a key role for autotaxin, a plasma lysophospholase D, in cleaving platelet-derived lysophospholipid to generate high serum levels of LPA has been demonstrated using heterozygous knockout mice (the homozygous knockout is embryonically lethal). Lysophospholipid substrates themselves originate at least in part via the action of a platelet-derived PLA₂. At this time, the exact physiological significance of platelet-derived LPA is still not clear although it is thought to contribute to the development of the vasculature potentially and possibly in atherosclerosis and in hypertension.

Neutral Lipids: Glycerides, CEs, and Free Cholesterol
Neutral lipids are uncharged species that include glycerides, CEs, and free cholesterol. In platelets, only small amounts of these are present, with the most abundant being cholesterol at >90%. Free cholesterol is essential for lipid raft function, described in more detail in Sphingolipids and Ceramides of this article. Glycerides include triglycerides and 1,2-diacylglycerols, with triglycerides representing ≈2% of neutral lipids. Although present at only trace amounts in resting cells, 1,2-diacylglycerol is formed during platelet activation by the action of PLC, as described earlier, and activates classical and novel isoforms of protein kinase C. Thus, 1,2-diacylglycerol is an important second messenger involved in receptor-dependent activation of platelets. Currently, the detailed composition of platelet 1,2-diacylglycerol is generated endogenously, in terms of FAAs is not known, or whether distinct 1,2-diacylglycerol species are more effective as second messengers. These questions could potentially be answered in the future using new generation lipidomic approaches (eg, through identifying and quantifying platelet-specific 1,2-diacylglycerol by LC/MS/MS or multidimensional MS methods).

Sphingolipids and Ceramides
Sphingolipids are a class of lipids that contain a sphingoid base backbone, the simplest of which is ceramide and consists of sphingosine and a FA (Figure 10). They are involved in both dynamic membrane functions (eg, lipid raft–dependent signaling) and acting as intracellular signaling messengers although their role in platelets is uncertain. Sphingosine is phosphorylated by sphingosine kinases 1 and 2 to form sphingosine-1-phosphate (S1P; Figure 10), which binds to specific G-protein–coupled receptors to influence vascular development, carcinogenesis, chemotaxis, and proliferation. S1P is generated in platelets via the action of sphingosine kinase, then stored and released on degranulation, contributing to the potent angiogenic activity of serum. Separately, a lipidomic study characterized the sphingolipid composition of murine platelets and demonstrated that resting cells do not contain S1P, in contrast to human. Instead, they contained large amounts of dihydro-S1P and C₂₀ and C₂₀:₁ ceramides. Thrombin activation of mouse platelets in vitro leads to loss of dihydro-S1P and ceramide, with major increases in sphingosine and dihydrosphingosine. In vivo, this was observed as an elevation in plasma dihydro-S1P. In contrast to this, human plasma contains S1P and also dihydrosphingosine but little ceramide. Separately, stored platelets were found to transfer phospholipids and sphingolipids to newly released extracellular vesicles, resulting in increased ceramide and decreased S1P in the cells.

S1P has long been recognized as a regulator of hematopoietic cell trafficking, immune regulation, vascular development, and brain inflammation, released from activated...
platelets. More recently, a role for this lipid in thrombopoiesis was demonstrated using genetically deficient mice. S1P signaling via the multifunctional S1P receptor is required for two specific events involved in platelet formation and release, the directional migration of proplatelet-containing cytoplasmic extensions into the circulatory compartment, and the shedding of proplatelets in a Rac-dependent manner. Because it is known that S1P couples to Rac activation, this suggests that active signaling via S1P is required for key stages of thrombopoiesis. In support, S1P deficient mice display thrombocytopenia.

Currently, relatively little is known about how lipid molecular dynamics during cell activation can regulate platelet function; however, one area of intense investigation in recent years relates to the formation and action of cholesterol and sphingomyelin-rich lipid rafts. These are specialized membrane microdomains that compartmentalize cellular processes relevant for signaling, membrane fluidity, and protein trafficking. Lipid rafts were proposed in the 1970s using biophysical approaches, but more formally recognized through the work of Simons and Toomre in the early 2000s. Raft microdomains are estimated to account for ≤ 10% of the total surface area, with each containing ≈ 3500 sphingolipids molecules and 10 to 20 protein molecules. They are thought to enhance signaling functions by providing platforms that allow clustering of receptors, kinases, and adaptor proteins involved in signaling pathways. The ability of functions of 2 additional proteins involved in adhesion, GP Ib-IX-V and GP VI/Fcγ to activate platelets is dependent on their translocation to lipid rafts.

Furthermore, additional platelet proteins also reported to localize to rafts include the scavenger receptor CD36 and protein trafficking. Lipid rafts were proposed in the 1970s using biophysical approaches, but more formally recognized through the work of Simons and Toomre in the early 2000s. Raft microdomains are estimated to account for ≤ 10% of the total surface area, with each containing ≈ 3500 sphingolipids molecules and 10 to 20 protein molecules. They are thought to enhance signaling functions by providing platforms that allow clustering of receptors, kinases, and adaptor proteins involved in signaling pathways. The ability of functions of 2 additional proteins involved in adhesion, GP Ib-IX-V and GP VI/Fcγ to activate platelets is dependent on their translocation to lipid rafts.

Lipidomic methods have not yet been applied to the characterization of platelet lipid rafts, in terms of profiling individual molecular species. In other cells (KB oral and KBC pancreatic tumor cell lines), these domains are enriched in AA-containing plasmalogen phosphatidylethanolamines although the molecular species of sphingomyelin and phosphatidylserine were not determined. Whether platelet rafts differ from this is currently not known. Furthermore, matrix-assisted laser desorption/ionization imaging methodologies are not yet sufficiently high resolution to enable subcellular localization of lipids to be determined directly. If this were possible, then determination of raft lipids through approaches that do not involve cellular disruption using detergents would significantly increase our knowledge of the composition of these specialized domains and generate new information on how lipids regulate key signaling events in platelets.

General Summary: Future Perspectives and New Horizons

New lipidomic methodologies, in particular those afforded by the latest high-resolution rapid scanning instruments and imaging methodologies, are allowing us to open an exciting window into the world of lipid mediators in platelet and other cells, both in health and in disease. The opportunities presented are considerable, but their potential is yet to be fully realized. Understanding the diversity and number of unique lipid species in cells is of potential importance for elucidating mechanisms of cell biology and disease and also identifying biomarkers for stratified/personalized medicine. This is one area where new generation lipidomic MS has the potential for transforming our understanding of lipids in health and disease. Although prelipidomic techniques reported overall composition of PAs in a particular lipid pool (eg, phospholipids), individual molecular species could not be detected. Analyzing all molecular species is important if particular species display biological actions not shared by others or are key biomarkers of early disease. Although this field of research is still in its infancy, it is exemplified by the observation that a particular molecular species of triglycerides identified using MS can be used for early diabetes mellitus prediction, and that a specific phosphatidylethanolamine integrates hepatic lipogenesis and peripheral FA use.

Combining this level of analytic ability with the study of large cohort sample sets, new generation MS technology has the potential to transform our understanding of disease mechanisms. While cohort collections become larger and more comprehensive, it becomes important that omics technologies, including lipidomics are both analytically robust and used to investigate relevant questions. Considerable development work is still required to analyze large data sets from clinical studies using lipidomic MS screening, but the potential in terms of understanding disease mechanisms is significant. A recent study that highlights this approach used MS to demonstrate a series of phosphatidylethanolamine metabolites in plasma that predict risk of cardiovascular disease. These were found to be generated in the gut through bacterial metabolism of dietary phosphatidylcholine, absorbed into the bloodstream, and exerted proinflammatory effects of relevance to cardiovascular disease.

A major challenge lies in the development of computational and bioinformatic tools for analysis of the large data sets generated by high-resolution instruments. A number of packages for processing MS data have been developed both by instrumentation companies and by research groups, but postprocessing software is still lacking. Several databases provide spectral libraries for lipids, including LipidMaps, HMDB, and Metlin; however, coverage of each alone is incomplete. Recently, Kind et al described the creation of a database including in silico generated tandem MS spectra using cheminformatics, which is now freely available (LipidBlast: http://fiehnlab.ucdavis.edu/projects/LipidBlast/). This begins to address the bioinformatics gap, but much remains to be done.

The application of lipidomics technologies can be either exploratory or hypothesis driven. Many cohort and large data set studies use lipidomics to compare sets of tissue or plasma samples, where large numbers of abundant lipids (eg, phospholipids and CEs) are profiled without a definitive biological or hypothesis-driven question. This can be accomplished using either targeted approaches (eg, monitoring several lipids from well-defined pathways, such as eicosanoids, quantitatively using a triple quadrupole LC/MS/MS approach with deuterated internal standards for all species) but increasingly uses multidimensional MS to profile several families of complex lipids simultaneously but with less accurate quantitation (a single deuterated standard for 1 class of lipid is often used). This is a powerful approach that may lead to discovery of biomarkers that could
guide individualized treatment strategies or facilitate monitoring of drug responses (eg, which patients should be prescribed aspirin, statins, or other drugs of relevance to disorders of lipid metabolism) or open new avenues for understanding disease mechanisms and could be considered analogous to genomic approaches, such as genome-wide association studies. However, targeted lipidomic approaches ask specific hypothesis-driven questions about the behavior of lipid mediators in health and disease. As examples, in platelets, we have used targeted approaches to demonstrate that FA side chains regulate the procoagulant actions of phosphatidylethanolamine and phosphatidylserine, and that platelets generate novel families of procoagulant oxidized phospholipid through enzymatically controlled pathways. 

Both approaches are equally relevant and powerful, but with the new era of personalized/stratified medicine and bioinformatics in medicine, it is likely that a new dawn of discovery for lipid mediators will be driven by exploratory approaches that will advance our understanding of their direct roles in human disease. At this time, we can only speculate on the potential applications for lipidomics approaches in furthering our understanding of disease mechanisms and in the development of new therapeutic approaches, but these could involve: (1) predicting risk of disease (eg, cardiovascular disease or cancer), (2) making decisions on preventative or treatment strategies, and (3) monitoring treatment efficacy and guiding ongoing clinical decisions, using plasma, urine, or tissue global lipidome features or specific lipid levels.

We currently have no robust estimates of the total number of individual lipid molecular species in platelets or in any other mammalian cell type. To address this, we are currently developing high-resolution LC/MS/MS and in-house generated bioinformatic methodologies to define the total number of unique platelet lipids (knowns and unknowns) and how these change on agonist stimulation (eg, thrombin) and cyclooxygenase inhibition (eg, aspirin) in genetically unrelated donors (M. Mondhe and V.B. O’Donnell, unpublished data). This is a challenging endeavor because the number of false signals is high and ensuring that accurate identification of real lipids is laborious.

Lipidomic MS can be used for screening, the identification of new lipids based on the presence of a characteristic functional group, or to study the movement of lipids from inside to the outside of the cell. In our laboratory, we applied a targeted lipidomic method, termed precursor scanning, to uncover several families of new platelet lipids comprising phospholipids with PGs, eicosanoids, or docosanoids attached, that form within 2 to 5 minutes of platelet activation. This was based on the idea that during collision-induced fragmentation, a characteristic eicosanoid or prostaglandin carboxylate anion would be generated. This is described in the Oxidized Phospholipids section of this article. Separately, we developed a method that identifies molecular species of phosphatidylethanolamine and phosphatidylserine that traffic to the cell surface on platelet activation, apoptosis, or aging. This uses derivatization of external facing amino-containing phospholipids using a cell-impermeable reagent and has allowed us to identify not only which lipids are externalized but also which platelet-specific phosphatidylethanolamine/phosphatidylserine are more procoagulant based on side chain FA composition (see Aminophospholipids of this article).51

An important area where lipidomics can be used to provide new information is in the characterization of lipid addition to nonlipid molecules during cell activation, signaling, and proliferation. This generates novel species that are readily amenable to MS structural analysis. One example is the reaction of lipid electrophiles with key transcriptional regulators via Michael addition, which occurs during prostaglandin-dependent activation of the transcription factor Nrf2. In addition, lipids can adduct to proteins to form membrane anchors, for example, via palmitoylation, which is used to localize Src family kinases and the adapter linker of activated T-cells to lipid rafts. Lipidomics combined with proteomic MS could then be used to inform on position of lipidation within the protein critically, as well as the specific lipid itself.

Although many challenges remain in ultimately defining the platelet lipidome, the new advances in technology are likely to make this a reality both in terms of characterization and quantitation of all lipid species. However, this on its own is only the beginning of a new era. The challenge will be to establish the functional significance of this vast amount of information. Perhaps the biggest area of effect in relation to platelet regulation will be around the therapeutic manipulation of products of phospholipases, in particular, phospholipases A2, C, and D and in the regulation of PI 3-kinases. It remains to be seen to what extent the variation in lipid backbone influences signaling, and how this varies between donors, health/disease, and with diet/drug therapy. With an aging population, and the critical role of platelets in thrombosis and bleeding, the question will emerge as to whether the knowledge of the lipid composition and functional activity of second messengers, such as 1,2-diacylglycerol, can be targeted therapeutically or in the diet. However, the challenge of this is perhaps illustrated by the demonstration of a critical role for PLD in pathologic thrombus formation and ischemic stroke as shown using both mutant mice and a small pharmacological inhibitor. Activation of PLD contributes to GPIb-mediated activation of platelet integrins, but the molecular basis of this effect, and the possible involvement of formation of 1,2-diacylglycerol from phosphatidic acid, remains to be established.

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None.

**References**


of glycolipid-enriched membrane domains in glycoprotein VI- and
Leo A, Schraven B, Horejsí V, Shattil SJ, Watson SP. Differential role
2008;1780:606–611. Biochim Biophys Acta
and serum. J
abundantly stored in platelets, is a normal constituent of human plasma
Ozaki Y, Kume S. Sphingosine 1-phosphate, a bioactive sphingolipid
Biochem J
naling by the platelet receptor glycoprotein VI.
Functional association of the platelet collagen receptor glycoprotein
Ezumi J, Kita Y, Kodama K, Uchiyama T, Takayama H. Constitutive and
Garcia JG. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J. 2000;14:2255–2265.
Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, López JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma mem-
Pike LJ, Han X, Chung KN, Gross RW. Lipid rafts are enriched in arachidonic acid and plasmeneylethanolamine and their composition is independent of caveolin-1 expression; a quantitative electrospray ionization/mass spectrometric analysis. Biochemistry, 2002;41:2075–2088.
Platelet Lipidomics: Modern Day Perspective on Lipid Discovery and Characterization in Platelets
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