thematic review

Thematic review series: Systems Biology Approaches to Metabolic and Cardiovascular Disorders

Lipidomics: a global approach to lipid analysis in biological systems

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Abstract **Lipids are water-insoluble molecules that have a wide variety of functions within cells, including: *1*) maintenance of electrochemical gradients; *2*) subcellular partitioning; *3*) first- and second-messenger cell signaling; *4*) energy storage; and *5*) protein trafficking and membrane anchoring. The physiological importance of lipids is illustrated by the numerous diseases to which lipid abnormalities contribute, including atherosclerosis, diabetes, obesity, and Alzheimer's disease. Lipidomics, a branch of metabolomics, is a systems-based study of all lipids, the molecules with which they interact, and their function within the cell. Recent advances in soft-ionization mass spectrometry, combined with established separation techniques, have allowed the rapid and sensitive detection of a variety of lipid species with minimal sample preparation. A "lipid profile" from a crude lipid extract is a mass spectrum of the composition and abundance of the lipids it contains, which can be used to monitor changes over time and in response to particular stimuli. Lipidomics, integrated with genomics, proteomics, and metabolomics, will contribute toward understanding how lipids function in a biological system and will provide a powerful tool for elucidating the mechanism of lipid-based disease, for biomarker screening, and for monitoring pharmacologic therapy.—**Watson, A. D. **Lipidomics: a global approach to lipid analysis in biological systems.** ***J. Lipid Res.*** **2006.** 47: **2101–2111.**

Supplementary key words inflammation • mass spectrometry • eicosanoids • lipid peroxidation • atherosclerosis

Systems biology is the computational integration of genetic, transcriptomic, proteomic, and metabolomic information with the intent of understanding all of the molecular elements within a cell or organism (1). Network models can be constructed from this information to develop hypotheses and predictions about how the system will respond under certain circumstances, such as a disease state (2, 3). Comprehensively cataloging the interactions between genes, proteins, and other biological molecules is complicated, to say the least. Well-studied signal transduction pathways such as the epidermal growth factor receptor and toll-like receptor pathways provide some glimpse of this complexity (4, 5).

The sequencing of the human genome, the development of gene arrays, and the availability of soft-ionization mass spectrometry techniques have led the way for high-throughput genomics and proteomics (6, 7). Although the human genome comprises just over 25,000 genes, the human proteome, considering a multitude of posttranslational modifications, is composed of millions of different proteins. The end product of genetic and protein expression is the metabolome, the total complement of metabolites within a cell or organism. Although genomics, transcriptomics, and proteomics are fundamental to the functional integrity of the organism, its metabolites reflect the most downstream effects of gene and protein regulation and thus may provide vital information regarding the biological state of the system (8). There are many examples of genetic manipulation based on sound rationale that produce less than desirable results because of unclear knowledge of how the manipulations affect the metabolome (8, 9). Metabolomics represents a paradigm shift from looking at individual metabolites to examining complete metabolic networks in an entire cell or organism.

Lipidomics is more than just the complete characterization of all lipids in a particular cell type. It is the comprehensive understanding of the influence of all lipids on a biological system with respect to cell signaling, mem-

Manuscript received 3 August 2006.

Published, JLR Papers in Press, August 10, 2006.
DOI 10.1194/jlr.R600022-JLR200

Abbreviations: APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; CI, chemical ionization; COX, cyclooxygenase; EI, electron ionization; ESI, electrospray ionization; ESI-MS, electrospray ionization-mass spectrometry; LOX, lipoxygenase; LTA_4, leukotriene A_4; LXR, liver X receptor; MALDI, matrix-assisted laser desorption ionization; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; PC, phosphatidylcholine; PI, phosphatidylinositol; SPE, solid-phase extraction.

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brane architecture, transcriptional and translational modulation, cell-cell and cell-protein interactions, and response to environmental changes over time. There is immense combinatorial structural diversity among lipids, the importance of which is not entirely clear. The possibility that each individual lipid has been conserved throughout evolution for a particular purpose is intriguing.

Once thought to be useful only as membrane-forming and energy storage molecules, lipids have been found to have a variety of important physiological roles, including cell signaling, protein modification, and membrane anchoring. The understanding of the pathologic processes to which lipids contribute has solidified the importance of lipids, lipid metabolism, and lipid oxidation.

This review will cover *1*) the physiochemical nature of lipids and their extraction from biological tissues and fluids; *2*) separation techniques for lipid classes and species; *3*) methods of lipid detection, with emphasis on mass spectrometry; and *4*) some practical uses of lipidomic techniques for understanding systems biology.

NATURE OF LIPIDS

The major difference between lipids and other major components of living tissue (carbohydrates, proteins, nucleic acids) is their solubility in organic solvents. Lipids are defined either by these solubility characteristics or by the presence of long hydrocarbon chains; however, not all lipids satisfy both definitions. For example, phosphatidylinositol (PI) has two hydrocarbon chains in the *sn*-1 and *sn*-2 positions of the glycerol backbone, thus satisfying the definition of possessing fatty acids and being structurally similar to other lipids; however, because of the polar carbohydrate moiety in the *sn*-3 position, PI is only moderately soluble in organic solvents such as hexane or chloroform. This is also the case with gangliosides, which contain extensive oligosaccharides (10). On the other hand, steroids and polyisoprenoids, which do not possess the prototypical hydrocarbon chain, are highly soluble in organic solvents. Therefore, there is some controversy about the exact physiochemical properties that define a lipid.

Recently, a new nomenclature system has been proposed for lipids, which classifies them into eight major categories: *1*) fatty acyls, *2*) glycerolipids, *3*) glycerophospholipids, *4*) sphingolipids, *5*) sterol lipids, *6*) prenol lipids, *7*) saccharolipids, and *8*) polyketides (11). Intrinsic to this system is the ability to subdivide the categories into classes and subclasses in order to accommodate newly discovered lipid species. Each lipid is given a "LIPID ID," a unique 12-character identifier, which includes the source database (two letters), the category code (two letters), the class code (two numbers), the subclass code (two numbers), and the unique identifier code (four numbers). This system has the capacity to specify 1.68 million individual lipids. One obvious advantage of this alphanumeric system of lipid nomenclature is the amenability to database storage and retrieval as well as bioinformatics manageability.

Another unique characteristic of many lipids (particularly glycerophospholipids and sphingolipids) is spontaneous arrangment into micelles or bilayer vesicles in an aqueous environment based on the influence of thermodynamics, interaction forces, and molecular geometry (12). The polar head groups tend to remain associated with water, whereas the hydrocarbon tails form hydrophobic interactions. The relative bulk of the polar head group compared with the acyl chains determines, in large part, the location of the lipid within a bilayer vesicle and the shape of the vesicle. Diacylphosphatidylethanolamine, for example, has a small head group relative to its acyl chains and fits better on the inner leaflet of a spherical lipid bilayer (13). On the other hand, diacylphosphatidylcholine and sphingomyelin have a larger polar head group and fit better in the outer leaflet. Lysophospholipids tend to favor the outer leaflet in mixed lipid bilayers, but form micelles as a pure lipid (13, 14). The original description of the "fluid-mosaic" model suggested that membrane proteins were floating in a homogenous bed of excess lipid arranged in a bilayer (15). However, the lipid-lipid and lipid-protein interactions appear to be much more dynamic than first appreciated. Lipid microdomains rich in cholesterol, sphingomyelin, and glycolipids, called "lipid rafts," play a role in cell signaling by their relative abundance of glycosylphosphatidylinositol-anchored proteins (16) as well as receptor and nonreceptor kinases (17, 18). Cholesterol depletion disintegrates lipid rafts and disrupts many of the cell signaling pathways dependent upon this intricate architecture (19).

LIPIDS AS SIGNAL TRANSDUCTION MOLECULES

We now realize that lipids, once regarded as simply a structural component of cells to maintain an electrochemical gradient, play a fundamental role as mediators of signal transduction. The number of individual biologically active products derived from arachidonic acid alone is staggering, inasmuch as each of these evokes a particular response depending upon the location, abundance, and cell type (20). Nearly all arachidonic acid remains esterified to glycerophospholipids within the cell membrane, and its release is carefully regulated by phospholipases. Interesting, the availability of arachidonic acid for enzymatic metabolism may be heavily dependent on the rate of re-esterification by lysophosphatidylcholine:acyl CoA acyltransferase, at least in polymorphonuclear cells (21).

Arachidonic acid can be metabolized by several enzymes to form eicosanoid lipid mediators such as prostaglandins, leukotrienes, lipoxins, epoxyeicosatrienoic acids, and thromboxanes. Prostaglandin G_2/H_2 synthases [cyclooxygenases, (COXs)] located in the endoplasmic reticulum or nuclear envelope convert arachidonic acid into prostaglandin G_2 and H_2. Nonsteroidal anti-inflammatory drugs inhibit these enzymes and decrease the sensation of pain caused by some prostaglandins (22). Tissue-specific isomerases and synthases generate distinct prostaglandins, which activate G-protein-coupled receptors (20). Prostacyclin activates the IP receptor in the endothelium, kidney, platelets, and brain; thromboxane A_2 stimulates the TP_α and TP_β receptors in platelets, smooth muscle cells, mac-

rophages, and kidney; prostaglandin D_2 stimulates the DP_1 and DP_2 receptors in mast cells, brain, and airways; prostaglandin E_2 stimulates the EP_{1-4} receptors in the brain, kidney, smooth muscle cells, and platelets; and prostaglandin F_2 stimulates the FP_α and FP_β receptors in the uterus, smooth muscle cells, and eye (23). Arachidonic acid can also be converted to biologically active lipids through the lipoxygenase (LOX) pathway by 5-LOX, 12-LOX, and 15-LOX. The initial product of 5-LOX is 5(*S*)-hydroperoxy-eicosatetraenoic acid, which is then converted to the allylic epoxide 5(*S*)-*trans*-7,9-*trans*-11,14-*cis*-eicosatetrenoic acid [leukotriene A_4, (LTA_4)] by the same enzyme (24). LTA_4 can undergo hydrolysis to LTB_4, a potent leukocyte chemoattractant, or combine with glutathione to form LTC_4, LTD_4, and LTE_4 (25). Lipoxins are compounds derived from arachidonic acid that contain a conjugated tetraene structure and three hydroxyl groups (26). They are produced by the concerted action of 5-LOX and 12/15-LOX. LTA_4 can be converted to lipoxins by 12/15-LOX or, alternatively, 15-hydroperoxy-eicosatetraenoic acid, can be converted to lipoxins through 5-LOX (27).

Oxidation products of cholesterol, like those of arachidonic acid, can be produced by enzymatic and nonenzymatic mechanisms. Some members of the cytochrome P_{450} system, as well as sterol-27-hydroxylase and cholesterol-24-hydroxylase, catalyze the addition of a hydroxyl group onto cholesterol to form hydroxycholesterols (28). Hydroxylation of cholesterol is an essential step in the formation of hormones in steroidogenic tissues. The nonenzymatic oxidation of cholesterol has received close attention for its contribution to the oxidation hypothesis of atherosclerosis (29). Oxysterols, many of which are cytotoxic to vascular wall cells (30), are found in abundance after oxidation of LDLs with copper. Certain epoxysterols, 7-ketocholesterol, and 25-hydroxycholesterol are elevated in the plasma and atherosclerotic lesions of cholesterol-fed rabbits (31). Liver X receptors α (LXRα) and β (LXRβ) are both activated by 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, 27-hydroxycholesterol, and 24(*S*),25-epoxycholesterol at physiological concentrations (32), which inhibits the development of atherosclerosis in animal models (33).

Benveniste, Henson, and Cochrane (34) described a "soluble factor" released from leukocytes that caused aggregation of platelets. This platelet-activating factor (PAF) was found to be 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (35), a phospholipid with potent, diverse physiological functions, particularly related to inflammation (36, 37).

Sphingolipids are amphipathic lipids usually found in the cell membrane that contain a sphingosine core structure (**Fig. 1**). The addition of a fatty acid by an amide bond

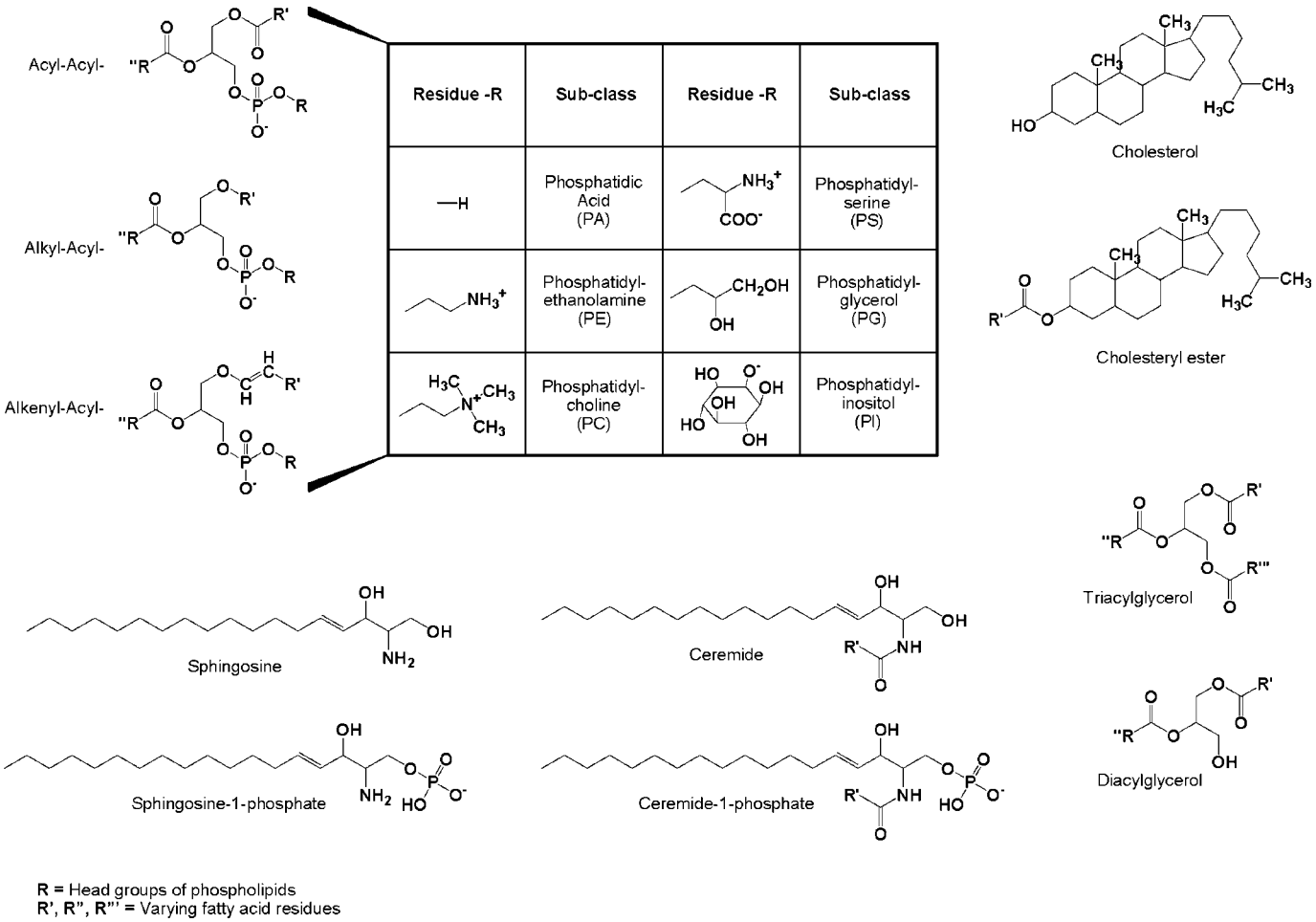

Fig. 1. Classes of lipids. The molecular structure of important naturally occurring lipids.

forms ceramide. Phosphorylation of sphingosine or ceramide results in sphingosine-1-phosphate (S1P) or ceramide-1-phosphate (C1P), respectively (38). Sphingolipids are emerging as important mediators of cellular signaling, cell growth, and cell death, in addition to their structural contribution to membrane architecture (38–40). Whereas ceramide appears to inhibit tumor growth, S1P promotes tumorogenesis through its stimulation of angiogenesis (41, 42). Sphingolipids are abundant components of the nuclear envelope in mammalian cells, where they modulate cell signaling to profoundly affect the function of the cell (43). S1P, C1P, and lysophosphatidic acid (another potent signaling molecule involved in tumorogenesis) are highly regulated by lipid phosphate phosphatases, which dephosphorylate these lipids (44).

Eicosanoids, PAF, sphingolipids, and oxysterols are just some of the lipids involved in cell signaling within cells. Others include lysophospholipids, oxidized phospholipids, and PIs (and their hydrolytic products diacylglycerol and phosphoinositides).

LIPID EXTRACTION FROM BIOLOGICAL SAMPLES

Most methods of lipid isolation from biological samples exploit the high solubility of hydrocarbon chains in organic solvents. Typically, a phase separation is created between immiscible solvents, with the lipids partitioning into the hydrophobic phase. One of the more common techniques for lipid extraction involves the addition of chloroform and methanol at a ratio of 2:1 (v/v) to an aqueous biological solution or tissue (45, 46). The final ratio of chloroform, methanol, and water should approach 8:4:3, by volume, to achieve the most efficient partitioning of lipids into the organic phase. After collection of the chloroform-rich lower phase, it is recommended that additional chloroform be added to the upper phase to remove residual lipids remaining in the aqueous phase during the initial extraction. The lower phases are then pooled to obtain the total lipid extract. Modifications on the chloroform-methanol extraction procedure have been made to maximize the ability to extract lipids of particular interest with high efficiency. Saunders and Horrocks (47) used isopropanol-hexane (2:3; v/v) to extract lipids from bovine brain with a 12–37% greater recovery of prostaglandins, compared with traditional chloroform-methanol extraction. An additional benefit of this method was the use of inexpensive and less toxic solvents, but a drawback was poor recovery of gangliosides.

Lipid extraction with organic solvents is usually the first step toward lipidomic analysis, but no standard method for lipid extraction from biological samples has been established. Therefore, results between research groups using different methods of lipid isolation may vary, and this should be taken into consideration.

Method of separation. The original separation of chlorophyll was described in the early 1900s using a liquid adsorption column containing calcium carbonate (48). All methods of chromatography utilize differential solubility and adsorption of compounds at the interface of a stationary and mobile phase. TLC, developed from paper chromatography in the 1930s (49), has been used routinely for the analysis of lipids since the early 1960s (50–52). The technique uses a thin layer of stationary phase like silica or cellulose on a flat support, usually a glass or aluminum plate. The combination of a wide variety of silica-based solid-phase and organic mobile-phase recipes in multiple dimensions enable the separation of virtually all lipid classes. Methods of visualization include iodine vapor, specialty stains (such as molybdate for glycerophosphocholines), and charring with sulfuric acid. Although TLC is not as sensitive as other methods of lipid detection, it offers a rapid and comprehensive screening tool prior to more sensitive and sophisticated techniques.

Solid-phase extraction (SPE) chromatography is useful and practical for rapid, preparative separation of crude lipid mixtures into different lipid classes. Prepacked columns are available commercially containing several stationary phases (diol, silica, octadecylsilyl, aminopropyl, phenylsulfonic acid) for a variety of applications. A rapid method for the isolation of phospholipids, fatty acids, cholesteryl esters, monoglycerides, diglycerides, triglycerides, and cholesterol from a crude lipid mixture using aminopropyl SPE columns was described by Kaluzny et al. (53). Recovery of lipids using this technique was greater than 95%. Octadecylsilyl (ODS or C_{18}) SPE columns are particularly useful for the isolation of leukotrienes, prostaglandins, and other related eicosanoids from biological samples (54, 55).

HPLC is commonly used in lipidomic analysis to separate lipids prior to mass analysis (see below), although there are other methods of detecting lipids, such as spectrophotometric analysis in the UV range, evaporative light scattering, and flame ionization. There are probably thousands of published HPLC methods for lipid separation, so only general principles will be discussed.

Fatty acids are nearly always separated on a reverse-phase ODS (C_{18}) column using a methanol- or acetonitrile-based gradient solvent system in water. Separation occurs on the basis of polarity, effective chain length, and degree of unsaturation, where short, polar, unsaturated fatty acids elute sooner than long, nonpolar, saturated fatty acids. Columns containing a chiral stationary phase can effectively separate enantiomers of lipids such as hydroxyeicosatetraenoic acids (56, 57).

Separation of phospholipids can be achieved by either normal-phase HPLC or reverse-phase HPLC. Normal-phase HPLC effectively separates glycerophospholipids on the basis of class (58, 59), whereas reverse-phase HPLC effectively separates on the basis of fatty acid residues (60). Oxidized glycerophospholipid species of a common class can be separated by reverse-phase chromatography because of the polarity caused by the position and number of oxygen atoms in the molecule (61).

IONIZATION AND MASS ANALYSIS

Fundamentally, a mass spectrometer is an instrument capable of measuring the mass of molecules that have an

electrical charge. This technique has had such an impact on analytical biochemistry that several Nobel Prizes have been awarded for its development. Most recently, the 1989 Nobel Prize in Physics was awarded to Norman F. Ramsey, Hans G. Dehmelt, and Wolfgang Paul for the development of the ion trap technique and to John B. Fenn and Koichi Tanaka for the development of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Before the availability of these techniques, more destructive forms of ionization were used.

Electron ionization (EI) is commonly used as a means of mass analyzing molecules separated by gas chromatography while in the gas phase. EI generates ions by bombarding the eluting gaseous sample with a beam of high-energy electrons. The energy that is transferred from the electrons results in ionization of the samples as well as fragmentation into smaller ions. The size and abundance of these fragments is dependant upon the bond energies within the molecule, which are unique, thereby generating a specific fragmentation profile or fingerprint.

Chemical ionization (CI) relies on the ionization of a reagent gas (such as ammonia or methane) to indirectly ionize a sample molecule that is too energetically labile for traditional EI analysis. Originally used with gas chromatography, CI is now commonly done at atmospheric pressure in the liquid phase.

MALDI mass spectrometry is a laser-based soft-ionization method often used for analysis of large proteins, but has been used successfully for lipids. The lipid is mixed with a matrix, such as 2,5-dihydroxybenzoic acid, and applied to a sample holder as a small spot. A laser is fired at the spot, and the matrix absorbs the energy, which is then transferred to the analyte, resulting in ionization of the molecule.

The concept of ESI was introduced in the early 1900s by Zeleny and developed for larger molecules by Dole and colleagues in 1968 (62). ESI allows the analysis of large, nonvolatile molecules directly from the liquid phase (such as liquid chromatography or capillary electrophoresis). It is a soft-ionization method that rarely disrupts the chemical nature of the analyte prior to mass analysis. The sample is introduced through a capillary tube into the ion source of the mass spectrometer in a water-rich solvent at atmospheric pressure. A high voltage (2–5 kV) is applied to the capillary relative to the entrance of the mass spectrometer, which creates an electric field gradient along which the charged droplets travel. As they travel, the solvent rapidly evaporates in a curtain gas of nitrogen and the density of the charges increases to the point of Coulombic repulsion (62). At this point, large droplets divide into smaller droplets and eventually into individual charged molecules, which enter the mass spectrometer (**Fig. 2**).

In a multisector mass spectrometer, such as a triple quadrupole machine, it is possible to mass analyze ions in the first sector, select an ion of interest to pass into the collision cell, and mass measure fragmentation products in the second mass analyzer (**Fig. 3A**). This is termed collision-induced dissociation, tandem mass spectrometry (MS/MS), or product ion scanning. This type of analysis

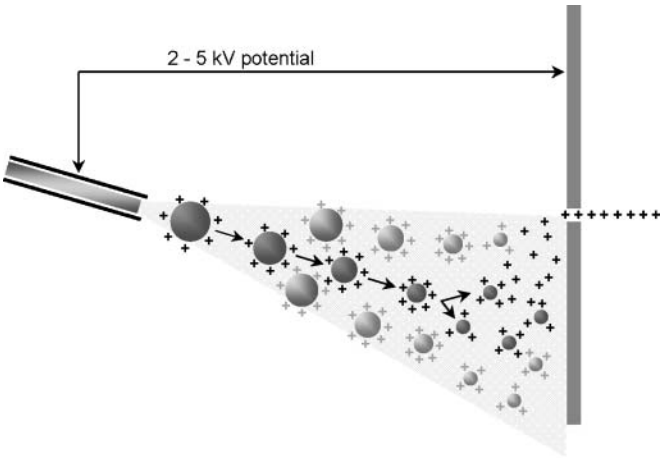

Fig. 2. Electrospray ionization mass spectrometry. Solvent is pumped through a capillary within a hollow needle that has a 2–5 kV electrical potential relative to the orifice of the mass spectrometer. A continuous flow of nitrogen gas through the needle sprays the eluent as it emerges from the capillary. At atmospheric pressure, the nebulized solvent evaporates rapidly, which increases the density of the charged particles on the droplets. As the charge density increases, so does the Coulombic repulsion, which results in the dissociation of molecules from the droplets and eventually into single ions that enter the mass spectrometer for analysis.

can be useful for obtaining information about the molecular structure of unknown molecules as well as comparing product ion fingerprints to known MS/MS spectra. One caveat is that mass spectrometers are incapable of separating isomers with the same molecular weight. Therefore, if a sample contains more than one isomer of the ion of interest, all isomers will be allowed to pass into the collision cell, thus producing a product ion spectrum to which all isomers contribute. Liquid chromatography must be used to separate isomers prior to mass analysis to avoid this problem, and the use of chiral columns may be necessary.

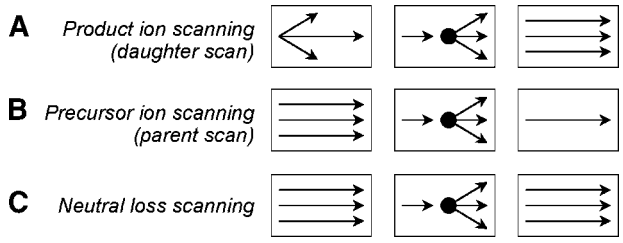

Fig. 3. Methods of using tandem mass spectrometery to analyze biomolecules. A: Product ion scanning is used to analyze the fragments of an ion of interest. The mass spectrometer is set to scan in the first quadupole (Q1), and only the selected ion of interest is allowed to pass into the collision cell (Q2). The parent ion is fragmented into daughter ions, which are mass measured in the third quadupole (Q3). B: For precursor ion scanning, Q3 is set to detect a fragment of a particular size while Q1 scans to determine the m/z of precursor ions that produce that fragment. C: Neutral loss scanning refers to the detection of ions in Q1, which loose a specified mass in the collision chamber as measured in Q3. Scanning in Q3 follows scanning in Q1 except for the offset designated by the neutral loss.

Triple quadrupole mass spectrometers can also do the opposite of product ion scanning, which is parent ion scanning. Instead of the second mass analyzer scanning to monitor product ions from the parent, the first mass analyzer scans and the second mass analyzer is static on a particular product ion of interest (Fig. 3B). The result is a spectrum of precursor ions, all of which fragment to produce the same daughter ion. If one was interested in detecting all phospholipids containing phosphocholine [such as phosphatidylcholine (PC) and sphingomyelin], one could scan in the first mass analyzer between *m/z* 450 and 950 and dwell in the second mass analyzer on *m/z* 184.1 (phosphocholine). Any parent ion that produces a daughter ion with *m/z* 184.1 will be detected in a quantitative manner. A similar type of analysis is "neutral loss" scanning, whereby the second mass analyzer detects ions that are a predetermined amount smaller than the parent ion scanned in the first mass analyzer (Fig. 3C).

Multiple reaction monitoring (MRM) is often used for quantitative analysis of lipids with known fragmentation profiles with up-front liquid chromatography. The first mass analyzer stays static at the *m/z* for the parent ion and the second mass analyzer stays static on the *m/z* of a known daughter ion produced by that parent. The monitoring of this "reaction" can be performed on multiple parent and daughter ion combinations in a single HPLC run. Stable isotopes that have a different parent mass but the same fragmentation profile are used as internal standards for quantitative analysis.

Two other methods of atmospheric pressure ionization are atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). The source for APCI is similar to ESI except that ions are formed by the interaction of the heated analyte solvent with a corona discharge needle set at a high electrical potential (63). Primary ions are formed immediately surrounding the needle, and these interact with the solvent to form secondary ions that ultimately ionize the sample. APCI is particularly useful for the analysis of nonpolar lipids such as triacylglycerols, sterols, and fatty acid esters (64).

APPI utilizes a krypton lamp, which emits photons at 10.0–10.6 electron volts; these have an energy level high enough to ionize many organic compounds, but not enough energy to ionize air or common HPLC solvents. A comparison of lipid analysis using three methods of atmospheric pressure ionization, ESI, APCI, and APPI, showed that APPI was two to four times more sensitive than APCI, which was more sensitive than ESI (65). APPI also had the ability to ionize fatty acid esters, monoacylglycerols, diacylglycerols, and triacylglycerols with higher efficiency than the other two methods of atmospheric pressure ionization (65).

A hybrid technique has been described that has the advantage of both MALDI and electrospray ionization-mass spectrometry (ESI-MS) called desorption electrospray ionization mass spectrometry, which propels charged droplets of solvent into a solid-phase sample. This releases proteins and other large molecules from the sample under atmospheric pressure, and these enter the ion source of the mass spectrometer (66). This technique, as well as variations including direct analysis in real-time and desorption atmospheric pressure chemical ionization, introduces the possibility of creating ions from intact or even living tissues.

MASS SPECTROMETRY OF LIPIDS

Gas chromatography-mass spectrometry has been used for decades as a method for analyzing lipids. A prerequisite for gas chromatography is the ability of the lipid to enter the gas phase under conditions that do not alter its molecular structure. Because most lipids in nature are nonvolatile, chemical derivatization is required. The carboxylic acid moiety of fatty acids must be converted to a methyl-, ethyl-, propyl-, or picolinylester, and polar groups such as carbonyls and hydroxyls must be derivatized to methoxylamines and silyl ethers, respectively. Other nonvolatile lipids, such as glycerophospholipids, require the removal of the polar head group in the *sn*-3 position, or base saponification of the fatty acid groups in the *sn*-1 and *sn*-2 positions with subsequent formation of fatty acid esters. This can also be done in a one-step transesterification process (67). One limitation of analyzing fatty acids esters obtained from phospholipids or other polyacylglycerolipids is the inability to determine which fatty acid group came from which *sn* position. This problem can be overcome by the use of a site-specific phospholipase, such as phospholipase A_2, which can enrich a sample with fatty acids from the *sn*-2 position of a biological sample containing phospholipids.

The availability of newer ionization technologies (discussed earlier) has revolutionized the mass spectrometry of lipids so that volatility is no longer an issue. Fatty acids can be injected directly into the ESI-MS and analyzed in the negative-ion mode as a carboxylate anion, $[M - H]^-$, or in the positive mode as the lithiated adduct, $[M - H + 2 Li]^+$. MS/MS of saturated fatty acids produces few daughter ions, whereas unsaturated and oxidized fatty acids produce abundant daughter ions, the size and intensity of which can be used as a signature for identification (68).

Nearly all phospholipids can be analyzed by ESI-MS (69). PC forms an abundant positive ion $[M + H]^+$ in mildly acidic solvents. MS/MS of PCs in the positive mode produces an ion at *m/z* 184 for phosphocholine, but little information is obtained regarding the fatty acids moieties. Using an elevated orifice voltage, a $[M - CH_3]^-$ ion can be produced in the ion source of the mass spectrometer that can be used as a parent for product ion scanning. Using this method, daughter ions corresponding to the carboxylate anions of the fatty acids can be visualized (70, 71). PI can form an ion in positive and negative mode, but negative mode is used more frequently because of the additional information obtainable with collision-induced dissociation. This information includes the carboxylate anions of the fatty acids and unique fragments produced by the polar head group, which are diagnostic for PIs (72). Glycerophosphatidylethanolamine (PE) can also be ionized in both positive and negative mode. In the positive mode, MS/MS produces an abundant ion corresponding to the neutral loss of phosphoethanolamine [M + H −

141]$^+$. MS/MS analysis of the negatively charged [M − H]$^-$ ion can provide information about the fatty acid species of PE (73).

SHOTGUN LIPIDOMICS

The availability of ESI-MS has enabled the development of methods designed to analyze many lipids from a biological source rapidly and with high sensitivity. One of the first studies to explore this concept analyzed phospholipids extracted from erythrocyte plasma membranes (74). From the equivalent of just 25 nl of blood, it was discovered that in addition to a variety of glycerophosphochlolines, these membranes contained an abundance of plasmenylethanolamine possessing arachidonic acid and other highly unsaturated acyl groups in the *sn*-2 position. Han et al. (75) performed an exhaustive search for molecular species of cardiolipin in total lipid extracts from mouse heart, liver, and skeletal muscle. ESI-MS was performed with either a triple quadrupole machine (in low and high mass resolution settings) or quadrupole time-of-flight (ToF). Exploiting the natural abundance of linoleate and the neutral loss of ketene to form doubly charged triacyl monolysocardiolipins, they found that cardiolipin species were quantifiable within a wide concentration range, down to 10 fmol/μl, and the presence of coexisting lipids in the tissue extracts did not hamper analysis. Oxidized cardiolipin was also detected, which may have important implications for programmed cell death (76). These studies have provided a tool to probe the functional changes in cardiolipin, a major phospholipid of the mitochondrial membrane, under various conditions and over time.

Nearly 75 species of PI and PI phosphates were identified, including fatty acyl chain composition, in the "targeted" electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis of macrophages and macrophage-like cell lines (77). Selective enrichment of the polar phosphoinositides was achieved by adding ice-cold chloroform-methanol (1:1; v/v) to the cell pellet, followed by centrifugation. Soluble, neutral lipids were removed and the polar phosphoinositides in the pellet were extracted with acidified chloroform-methanol (2:1; v/v) and water. Using this technique, changes in phosphatidylinositol-4-phosphate (PIP), PIP_2, and PIP_3 in the cells could be documented in response to several stimulatory factors down to the level of <9 fmol/μl.

Guan et al. (78) used "non-targeted" profiling of lipids extracted from the hippocampus of rats treated with kainite to induce neuronal cell injury and seizures. Lipids were extracted with chloroform-methanol, and negative-ion ESI-MS was performed on the total lipid extract using a quadrupole ToF mass spectrometer. Differences detected between kainite-treated and control hippocampal lipids led to more focused "targeted" lipid analysis focusing on *N*-acylated glycerophosphatidylethanolamines and ceramides. This included determination of molecular structure by MS/MS/MS using an ion trap mass spectrometer and quantification using MRM.

Lee et al. (78a) cleverly adapted electron capture negative chemical ionization-mass spectrometry of fatty acid pentafluorobenzyl (PFB) esters for liquid chromatography-mass spectrometry. The corona discharge of an APCI source was used to generate low-energy gas phase electrons, which caused dissociative electron capture of the PFB moiety and abundant [M-PFB]$^-$ ions. They found this method to be 10–20-fold more sensitive (down to the 100 fmol level) for the detection of eicosanoids, compared with traditional methods of APCI without electron capture. Using this method, combined with normal-phase chiral chromatography, they were able to observe changes in the eicosanoid lipid profile in cells treated with aspirin. Prostaglandin E_2 and 15(*S*)-hydroxyeicosatetraenoic acid (HETE) (both produced by COX) were decreased in a dose-dependent manner, whereas nonenzymatically produced 15(*R*)-HETE increased with higher doses of aspirin.

Lipid arrays have been developed that can identify and quantitate nearly 450 individual species of phospholipids by ESI-MS flow injection analysis from crude lipid extract of biological fluid or tissue (79, 80). Using sophisticated computational analysis, these lipid arrays can be used to detect subtle changes in the lipid composition of cells secondary to biological perturbations. This method has been tested in the setting of mast cell degranulation, in which it was shown that changes in glycerophospholipids were dependent on the type of stimulus as well as the cell type used (79).

LIPID OXIDATION AND LIPIDS AS MARKERS OF DISEASE

PUFAs possess a bis-allylic carbon that is particularly susceptible to oxidation because of a relatively weak bond energy with its protons (81). These protons can be abstracted, either by enzymes (such as LOX and COX) in the initial step of eicosanoid production or by free radicals, leaving a carbon with an unpaired electron (82). This carbon-centered free radical undergoes molecular rearrangement to form a conjugated diene structure and then reacts with oxygen (O_2) to form a peroxyl radical. In the setting of a membrane bilayer or lipoprotein particle with abundant lipids in the immediately vicinity, this peroxyl radical can abstract protons from the bis-allylic carbons belonging to adjacent PUFAs. In the absence of antioxidants and other stable proton donors, oxidative propagation can theoretically continue indefinitely. It is this process that is thought to be common to many chronic inflammatory diseases (83, 84). There are products that form in vivo during free radical-induced lipid peroxidation that are unique and provide information regarding the location, substrate, oxidative source, and extent of peroxidation (85–87). The possibility of detecting, quantifying, and monitoring these products is an attractive tool for assessing the oxidative/inflammatory state of a biological system.

Isoprostanes are prostaglandin-like molecules produced by nonenzymatic, free radical-induced oxidation of ara-

chidonic acid (88–90). They resemble eicosanoids, although isoprostanes are produced by free radical oxidation of arachidonic acid. The peroxyl radical of arachidonic acid undergoes endocyclization and further oxidation to form a prostaglandin G_2-like molecule without, however, the obligate stereochemistry associated with enzymatic oxidation. The side chains of isoprostanes are oriented *cis* compared with enzymatically produced eicosanoids which are *trans* (91). Spontaneous hydrolysis of the endoperoxide and reduction of the hydroperoxyl group yields the F_2 isoprostane designated as either a 5-, 8-, 12-, or 15-series regioisomer, based on the carbon to which the hydroxyl group is attached (92).

F_2-isoprostanes are most often analyzed by stable isotope dilution gas chromatography negative chemical ionization-mass spectrometry of the PFB esters (93) or by commercially available ELISA. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) using MRM has also been used to analyze isoprostanes with some success (94). Most isoprostanes are produced by oxidation of esterified arachidonic acid, so base hydrolysis is required prior to mass spectrometric analysis. Measurements can be made from urine, plasma, and other bodily fluids, and their abundance correlates well with the oxidative state in vivo. Elevated levels of isoprostanes are associated with many diseases in which free radicals have been implicated, including, carbon tetrachloride hepatotoxicity (94), diabetes mellitus (95), Alzheimer's disease (96), tobacco abuse (97), renal failure (98), heart failure (99), and atherosclerosis (100, 101).

Another group of lipids that appear to contribute to cardiovascular disease in several ways are oxidized phospholipids (102). Products of phospholipids containing PUFAs at the *sn*-2 position are potent stimulators of endothelial activation (103). Using laborious lipidomic techniques including ESI-MS/MS with and without derivatization, deuterium exchange experiments, and ^{1}H-NMR, three biologically active oxidation products, 1-palmitoyl-2(5)-oxyvaleryl-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-gluteroyl-*sn*-glycero-3-phosphocholine, and 1-palmitoy-2-(5,6)-epoxyisoprostanoyl-*sn*-glycero-3-phosphocholine, were found to be present in mildly oxidized LDLs (Ox-LDLs) and atherosclerotic lesions (71, 104). A different group of oxidized phospholipids present in Ox-LDL were identified by liquid chromatography-ESI-MS/MS. These species are recognized by scavenger receptors on macrophages and may be involved in the uptake of Ox-LDL by macrophages (105).

Finally, oxidized phospholipids appear to have a fundamental role in innate immunity. The modification of proteins by oxidized phospholipids, such as POVPC, appears to produce a neo-epitope, which is recognized by the immune system as a pathogen-associated molecular pattern (106, 107). Oxidized phospholipids, as determined by immunoreactivity with the E06 monoclonal antibody, are significantly correlated with the presence and extent of carotid and femoral atherosclerosis, development of new lesions, and risk of cardiovascular events (108). In the blood, they seem to form a close association with lipoprotein [a] and are released from atherosclerotic lesions during angioplasty (109, 110).

CONCLUSIONS

The implications of systems biology for the future of disease treatment and prevention are vast (1). The study of lipids and the profound influence they have on systems biology is gaining attention in large part because of the availability of mass spectrometry to characterize a large number of lipids with minimal sample preparation. Consortia and centers have been established with the sole purpose of lipidome analysis. One of these groups, Lipid Metabolites and Pathways Strategy, is a large consortium with the goal of determining the complete lipidome of the mouse macrophage in response to various stimuli such as oxidized lipids and lipopolysaccharides. As the field of lipidomics advances, the ways in which lipids affect pathologic states will become clearer, and therapeutics aimed at interfering with this process will become more focused. A major challenge in the future will be the bioinformatics side of lipidomics, the ability to integrate lipidomic data with genetic, proteomic, and metabolomic data, thereby generating new modeling paradigms.

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