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Mass-Spectrometry Based Oxidative Lipidomics and Lipid Imaging: Applications in Traumatic Brain Injury

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Abstract

Lipids, particularly phospholipids, are fundamental to central nervous system (CNS) tissue architecture and function. Endogenous polyunsaturated fatty acid chains of phospholipids possess cis-double bonds each separated by one methylene group. These phospholipids are very susceptible to free-radical attack and oxidative modifications. A combination of analytical methods including different versions of chromatography and mass spectrometry allows obtaining detailed information on the content and distribution of lipids and their oxidation products thus constituting the newly emerging field of oxidative lipidomics. It is becoming evident that specific oxidative modifications of lipids are critical to a number of cellular functions, disease states and responses to oxidative stresses. Oxidative lipidomics is beginning to provide new mechanistic insights into traumatic brain injury (TBI) which may have significant translational potential for development of therapies in acute CNS insults. In particular, selective oxidation of a mitochondria-specific phospholipid, cardiolipin, has been associated with the initiation and progression of apoptosis in injured neurons thus indicating new drug discovery targets. Further, imaging mass-spectrometry represents an exciting new opportunity for correlating maps of lipid profiles and their oxidation products with structure and neuropathology. This review is focused on these most recent advancements in the field of lipidomics and oxidative lipidomics based on the applications of mass-spectrometry and imaging mass-spectrometry as they relate to studies of phospholipids in TBI.

Lipids, particularly phospholipids, are indispensable parts of cell membranes where they play the major structural role as parts of non-raft-organized bilayer and protein annulus zones, and as precursors of diverse regulators of intra- and extracellular metabolism. The distinction of polyunsaturated fatty acid residues from less unsaturated fatty acids – that significantly defines their roles in membranes – is the presence of a repeating =CH-CH(2)-CH= unit that produces an extremely flexible structure rapidly isomerizing through conformational states (Wassall and Stillwell 2008). This essentiality of polyunsaturated phospholipids is also associated with the vulnerability of membranes to oxidative modifications and damage. The development of contemporary mass spectrometry (MS) designated the major breakthroughs in our understanding of structure-activity relationships of different membrane lipids. More specifically, the technological advancements facilitated the emergence of a new field of research and knowledge – lipidomics – opening remarkable opportunities for sensitive quantitative and structural analysis of individual molecular species of phospholipids and their role in cellular metabolism. This review is focused on the most recent advancements in the field of lipidomics and oxidative lipidomics based on the

applications of mass-spectrometry and imaging mass-spectrometry as it relates to studies of phospholipids in traumatic brain injury (TBI).

Diversity of Brain Lipids

Lipids are fundamental to central nervous system (CNS) tissue architecture and function. This is evident on a gross level based on lipid content and tissue dry weight, where CNS tissue has the highest lipid content next to adipose tissue (Han 2007) and is further supported by the fact that brain lipids constitute more than half of the dry weight in human brain (Piomelli et al. 2007). Brain lipid composition and metabolism change during development and these qualities can vary with anatomical region (Rouser et al. 1971). CNS tissue contains a diverse variety of complex lipids including neutral lipids (such as cholesterol and acylglycerols), glycolipids (such as galactosylceramide and gangliosides) and phospholipids (such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingolipids, etc). Due to the fact that phospholipids are the major building blocks of plasma and intracellular membranes, they account for approximately 25% of the dry weight of the adult rat brain (Yusuf 1992). Phospholipids are precursors of important signaling molecules such as neuroprotectins and resolvins, which are formed by multistage oxygenation of docosahexaenoic acid and eicosapentaenoic acid (Marcheselli et al. 2003). In addition to their role in membrane architecture and signaling, phospholipids also play a critical role in sub-cellular organelle function.

Phospholipids consist of a glycerol backbone, fatty acid chains, and a phosphoester-connected headgroup (Figure 1). Because of the hydrophobic nature of their fatty acid chains, most phospholipids are found in various cellular and sub-cellular membranes with their hydrophilic headgroups exposed to the aqueous environment. Different headgroups define each phospholipid class and their properties (<http://www.lipidmaps.org/>). Different classes of phospholipids will have various combinations of fatty acid chains that can be esterified to the sn-1 and sn-2 positions on the glycerol backbone. Fatty acids can be released by phospholipase A from phospholipids and have important roles in cell signaling and metabolism as shown in Figure 2. There are several fatty acid residues that are more common than others in brain phospholipids (Table1) (Fahy et al. 2005; Fahy et al. 2009). Just among the eight most common fatty acid chains in brain, this gives $8^2=64$ possible molecular species for each class. Cardiolipin (CL), a mitochondria specific phospholipid, on the other hand, has four fatty acid chains which allows for the formation of a diverse number of possible molecular species, usually in excess of one hundred (Bayir et al. 2007; Cheng et al. 2008; Tyurin et al. 2008b).

Phospholipids contain a mixture of polyunsaturated, monounsaturated and saturated fatty acid chains. Usually, polyunsaturated fatty acid residues are located in the sn-2 position whereas saturated ones are in the sn-1 position of phospholipids. Under physiological conditions monounsaturated and saturated fatty acids do not undergo oxidation. Endogenous polyunsaturated chains on sn-2 position possess cis-double bonds each separated by one CH_2 group. These hydrogens are very reactive to free-radical attack, making polyunsaturated fatty acids subject to oxidative modification (Huvaere et al. 2010). While the proportion of highly oxidizable polyunsaturated sn-2 fatty acid residues with 4-, 5-, and 6-double bonds in the brain varies according to class, these highly oxidizable substrates are always in abundance compared to the actual presence of peroxidized phospholipids under normal physiological conditions and even after TBI or chronic disease conditions (Tyurin et al.; Benjamins et al. 2006; Bayir et al. 2007; Tyurin et al. 2008a). This availability of peroxidation substrates is not usually a limiting factor that determines specificity of phospholipid peroxidation (Bayir et al. 2007; Tyurin et al. 2008b).

The distribution and molecular species of phospholipids in the brain varies depending on the developmental stage, anatomical, cellular and subcellular localization. For example, the major species of phospholipids in synaptosomes in postnatal day (PND) 17 rat consist of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Bayir et al. 2007) followed by phosphatidylserine (PS) and phosphatidylinositol (PI). The major molecular species of PC in turn are PC(16:0/16:0), PC(16:0/18:1), and PC(16:1/20:3) (Tyurin et al.). Imaging mass spectrometry (IMS) studies further show that PC(18:0/18:1) localizes mostly to the gray matter while PC(16:0/16:0) localizes mostly to the white matter (Wang et al. 2007; Wang et al. 2008).

In addition to the anatomical (both cellular and sub-cellular) and developmental differences, phospholipid class differences, differences in diversity within each class (especially in the case of CL) and susceptibility to oxidation need to be considered and assessed. Therefore, the demands for detailed analysis of lipid molecular species in brain tissue are daunting. Subtle differences in fatty acid chains can impart drastically different oxidative properties to phospholipids and only certain species of phospholipids appear to play a role in oxidative signaling (Greenberg et al. 2006; Hazen 2008). Therefore, a technique that provides both molecular and spatial information of phospholipid species becomes mandatory.

Lipidomics and Mass Spectrometry

Lipidomics is a “systems-based study of all lipids, the molecules with which they interact, and their function within the cell” (Watson 2006). Lipidomics integrates various “front end” liquid chromatography (LC) methods used for the separation of lipids and multiple “back end” technologies such as MS for their detection, identification and characterization. The field of lipidomics has experienced a heightened interest in the last several years due to its critical role in health and disease (Han and Gross 2003; Tyurin et al. 2008a).

Mass spectrometry – the most powerful contemporary analytical approach in lipidomics - is based on the detection of ions (as charged lipid molecules) after their separation within an electromagnetic field. Typically, MS instruments consist of an ion source, a mass analyzer and a detector. MS as a scientific technique has existed at least since J.J. Thompson's discovery of isotopes at the turn of the 20th century, and plays a prominent role in diverse chemical fields such as petroleum, nuclear, environmental, forensic and archeological chemistry (Dass 2007; Hillenkamp and Peter-Katalinic 2007; Ekman 2009). It was not until the development of soft ionization techniques in the late 1980's that the field of biological MS blossomed (Griffiths 2008). Soft ionization allowed the direct examination of intact biomolecules without splitting them into multiple fragments. Gas chromatography-MS (GC-MS) has also been used for many years in the field of lipidomics. However, GC-MS usually requires chemical derivatization for phospholipid analyses which prevents detailed structural information from being determined (Peterson and Cummings 2006). Soft ionization techniques provide information about molecular identity, composition, and oxidation state. The two most prominent ionization techniques are Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption Ionization (MALDI) (Karas and Hillenkamp 1988; Fenn et al. 1989). They can detect intact peptides, proteins, oligonucleotides, and phospholipids *in situ* without requiring labeling or chemical derivatization. With tandem instruments and analyses (MS/MS), structural information by selective fragmentation can also be obtained (Domingues et al. 2008). Through IMS, information about the spatial mapping of specific molecular species of phospholipids directly from tissue slices can be realized (Hillenkamp and Peter-Katalinic 2007). Given the remarkable opportunities offered by different versions of MS analysis in neuro-lipidomics, the major emphasis of this review will be placed on the major principles and new developments in this area.

ESI and MALDI

Electrospray requires the sample to be in a liquid state. The sample is sent into the mass analyzer from a heated capillary as a steady stream of charged droplets. Solvent molecules are evaporated during inlet, leaving charged analyte molecules to enter the instrument in the gas phase. During inlet, the sample rapidly transits from ambient pressure and high temperature to high vacuum. This could present a problem with the analysis of oxidized lipids (see below). This method of sample inlet is most often coupled to “continuous scanning” analyzers such as ion traps and/or quadrupoles. It is essential to remove excess salts and buffers prior to ESI analysis, although most methods of lipid extraction include a step that performs this and thus the extract can be analyzed directly by ESI (Han and Gross 2003; Pulfer and Murphy 2003). Frequently, the effluent from a LC column is directly coupled to a mass spectrometer containing an ESI source allowing continuous analysis by LC-MS.

MALDI analysis requires the sample to be dried or otherwise turned into a condensed state. A “matrix” compound, which is usually a small organic molecule exhibiting a strong absorption at a laser light wavelength, is added in large excess to the analyte of interest. DHB (2,5-dihydroxybenzoic acid) has often been used as a matrix for phospholipid analysis (Schiller et al. 2004; Fuchs et al. 2007; Fuchs and Schiller 2008). The matrix is either mixed with the sample (“dried droplet”) or deposited over the sample (“overspotting”). The laser (usually in the UV range) is then pulsed across the sample. With each pulse, the matrix absorbs the laser light. This added energy desorbs it and nearby analyte molecules off the surface in a plume. The laser intensity is regulated to be high enough to excite the matrix compound but low enough to avoid damaging the sample. Rapid charge-transfer reactions take place during or immediately after desorption (Karas et al. 2000). Since the ions are formed in discrete packets with each laser pulse, MALDI is most often coupled to a Time-of-flight (TOF) analyzer. A distinct and significant advantage of MALDI in analysis of oxidatively modified lipids is that the sample is kept at ambient temperature and under high vacuum during analysis, minimizing oxidative artifacts. A disadvantage is that matrix clusters will sometimes interfere with the detection of lower-mass phospholipids, which limits the range of low mass ions that can routinely be detected. MALDI is also used as a rapid “pre-screening” technique prior to the more extensive ESI-LC-MS. A simple MALDI-MS analysis can take as little as a few seconds of instrument time, compared to minutes or hours for full ESI-LC-MS analyses. However, chromatographic separation of lipid classes is sometimes required for complete analysis of mixtures (see below) (Fuchs et al. 2007).

Mass Accuracy, resolution and sensitivity

If different ionization methods determine what samples can be examined, then different mass analyzers determine how well minimal differences in mass-values can be distinguished and identified as individual species of phospholipids (Table 2). For example, the Bruker Daltonik Apex FT-ICR (Ion Cyclotron Resonance) analyzer with a MALDI source can differentiate a mono-oxidation state (+15.9959 Da exact mass) from a structure containing the addition of one carbon and four hydrogens (+16.0313 Da) (Ishida et al. 2004). In theory MALDI and ESI can be used with any analyzer, although most instruments are coupled to one type for their lifetime.

All soft ionization techniques depend on charge transfer, and different analytes have different proton affinities. Acidic phospholipids will tend to take negative charges, and basic ones positive charges. Both MALDI and ESI can analyze either positively or negatively charged ions (positive and negative modes), and the resulting lipid spectra look drastically different in different modes (Han and Gross 2003; Schiller et al. 2007; Fuchs et al. 2009). Therefore, direct quantitative comparisons between different classes of lipids based on

signal intensities are not practical. However, relative changes in abundances are easily detectable, and thus comparisons between different samples can be performed. This is seen in “shotgun lipidomics” for identification and relative quantification of multiple molecular species (Han et al. 2006; Han et al. 2007). Addition of an internal standard of the same lipid class (which will thus ionize with similar efficiency) allows for adequate quantification.

Lipid mixtures can give signal suppression, especially with MALDI analyses. If one analyte is in high excess over another, then competition for, or suppression of, available charges may take place. This will reduce or eliminate the signal from the less abundant analyte. This is important for two reasons: first, oxidized lipids are in relatively low abundance versus non-oxidized (see below), and second, anionic lipids that may be key targets of oxidative damage, such as CL and PS, are usually in lower abundance than zwitter-ionic lipids (like PC).

Suppression of anionic lipids by PC in MALDI has been known for many years, and hampers direct analysis of anionic lipids in mixtures (Petkovic et al. 2001; Schiller et al. 2002). Off-line separation and collection of the lipids by TLC or LC prior to analysis is one alternative, although the time and effort required to do this eliminates the advantage of speed that MALDI offers over ESI (Fuchs et al. 2009). Suppression can become a problem when performing IMS for anionic lipids in tissue, since prior chromatographic separation is not possible. However, there are several MALDI-based approaches that do not use off-line separation and collection that can be utilized. One approach is the use of disposable pipette-tip solid phase columns (Emerson et al. 2010). This allows PC to be selectively removed in a simple and rapid way. Another is on-target washing to selectively remove some phospholipids, although this has mainly been used for IMS applications (Seeley et al. 2008). Yet another approach directly combines MALDI and TLC. The lipids are separated on the TLC plate, which is then sprayed with matrix and directly analyzed by MALDI (Fuchs et al. 2007; Stübiger et al. 2009). This not only eliminates sample loss and the time consuming step of sample collection, but also utilizes software developed for IMS to detect subtle differences in mobility resulting from different fatty acyl chains (Goto-Inoue et al. 2009). A final approach is to optimize the matrix for detection of different classes of phospholipids (Sun et al. 2008; Teuber et al. 2010).

Oxidative Lipidomics

Oxidative lipidomics is a part of lipidomics and includes: identification and structural characterization of oxidatively modified lipids by MS, quantitative analysis of their content and identification of the mechanisms and pathways through which they interact with other molecules within the cell and contribute to cellular functions. Membranes require polyunsaturated lipids for their structural organization and optimal function. Consequently, membrane structure/function can be compromised when vulnerable lipids are subjected to oxidative modification and degradation. As shown in Figure 3, propagation of free radical lipid peroxidation occurs through abstraction of hydrogen from a lipid molecule. This reaction happens much more readily in polyunsaturated lipids vs. saturated or monounsaturated lipids. In other words, free radical lipid oxidation is specific towards the degree of polyunsaturation of lipids rather than chemical nature of their polar headgroups. With the newly emerging field of oxidative lipidomics, specific oxidative modifications of lipids now appear to be critical to a number of cellular functions, disease states and responses to oxidative stresses. However, the field has been somewhat hampered with regard to the assessment of specific oxidized species of lipids, due to the difficulty in their detection. There are several main reasons for this:

Abundance

Lipid hydroperoxides exist in low abundance. Model systems produce large amounts of lipid hydroperoxides in comparison to what is usually seen within biological systems. These manipulations are usually done with individual (single) lipid species, where the mole percents of oxidized species produced are abundant and easily detected. However, in biological systems, the insults (ie. those causing the production of reactive oxygen species (ROS) which can attack and damage lipids), usually result in minor but very critical amounts of oxidized lipid products.

Stability

The general consensus is that lipid hydroperoxides have some degree of chemical and thermal instability, making the low abundance of these species in biological systems even more difficult to detect. Chemical instability may include the reaction of peroxides with trace metals in one's "system", thus promoting further oxidation and degradation. One way around this problem is to chemically derivatize the oxidized species. However, as with any manipulation, this may result in significant loss of the oxidized species in question.

Oxidative propagation

Once formed, lipid hydroperoxides can act as propagating molecules for further oxidations. Other fates of peroxy radicals include the formation of cyclic peroxides and endoperoxides. In addition, peroxidation of PUFA may produce mixtures of shorter fatty acids with aldehyde, keto, hydroxy or epoxy functional groups. Existence of these species will increase lipid diversity and reduce the likelihood of detecting intact peroxide species at any given moment in time.

Inherent complexity of brain cardiolipin

Unlike many tissues where only a few major species of cardiolipin exist, brain tissue cardiolipin is extremely diverse with respect to the number of CL species as mentioned above. A typical CL spectrum of murine brain exhibits approximately 8–10 major CL clusters, most of which have 8–10 individual CL species associated with them (Bayir et al. 2007; Kiebish et al. 2008c). Figure 4 shows typical spectra of brain CL obtained by ESI and MALDI-MS in comparison to lung and heart CL. Because of this structural diversity, detecting hydroperoxide species of CL by MS is challenging, since masses associated with oxidized species are likely to overlap the natural brain CL species present (Figure 5). While CL plays a critical role in mitochondrial membrane architecture, membrane fluidity and electron transport chain function, these functions, by themselves, cannot account for all of the CL species present in brain tissue. The high degree of diversity seen in brain CL species most likely represents/suggests additional signaling/biological and other yet to be identified roles for brain CL. Interestingly, decreased number of molecular species of CL was observed in brain tumors compared to normal tissue (Kiebish et al. 2008b; Kiebish et al. 2008c; Kiebish et al. 2009). Shotgun lipidomics studies have revealed that synaptic mitochondria had lower levels of CL than nonsynaptic mitochondria (Kiebish et al. 2008a) however there is insufficient information on specific localization (mapping) and alterations of this particular phospholipid species in brain tissue has been reported, especially after acute brain injury. Thus, assessing the alterations in CL species and their associated oxidative events becomes necessary.

Application of oxidative lipidomics approach to TBI

Mass spectrometry has been employed by our group and a number of other groups to detect and analyze oxidized lipid products. However, these studies have been met with some difficulty due to the points mentioned above. In addition, the ability to detect the loss of a

hydroperoxy or hydroxy group by various mass spectrometric scanning/fragmentation techniques has been met with limited success.

This problem is actually two fold. First, the species of interest must be present in sufficient concentration to perform any type of fragmentation. As stated above, peroxidized lipids exist in low abundance to begin with. Second, the group of interest must be able to be fragmented at the expense of all other fragmentations, that is, it must be one of the only groups lost. Since it requires more energy to “dissociate” an OH or OOH group than it does to fragment the remainder of the phospholipid in question, one cannot scan for losses of 16 or 32 Da very easily as other fragmentations within the phospholipid structure will also occur. Taking all of the above points together, detection of brain lipid hydroperoxides becomes an extremely challenging task.

Lipid peroxidation has been widely studied in TBI (Lewen et al. 2000; Roof and Hall 2000; Bazan 2006). A number of lipid peroxidation markers such as F₂-isoprostane and malondialdehyde, have been shown to increase in brain tissue, serum and cerebrospinal fluid (CSF) after experimental and clinical TBI (Hoffman et al. 1996; Tyurin et al. 2000; Kasprzak et al. 2001; Bayir et al. 2002; Singh et al. 2006; Seifman et al. 2008). Several studies suggest that lipid oxidation markers might be associated with outcome after clinical TBI. Increased levels of highly oxidizable PUFA in CSF were associated with worse clinical outcome in adults with severe TBI (Pilitsis et al. 2003). Increased levels of thiobarbituric acid reactive substances (TBARS) in CSF correlate with TBI severity in adults with contusion (Kasprzak et al. 2001). Although these markers give an idea about the scale of lipid peroxidation they do not indicate the source of lipid being oxidized. The latter is important for design of targeted therapeutic interventions.

We applied oxidative lipidomics and analyzed oxidation of lipids in brain after TBI and in neurons exposed to proapoptotic agents. Unexpectedly, the profiles of lipid oxidation did not correlate with the abundance of PUFA residues in them. Highly abundant phospholipids, such as PC and PE, were not substrates for oxidation whereas minor anionic phospholipids were the preferred substrates for oxidation (Bayir et al. 2007; Tyurin et al. 2008a). In particular, mitochondrial specific CL and extramitochondrial PS accumulated most of the oxygenated fatty acid residues. This suggests that TBI-induced phospholipid oxidation does not follow the “polyunsaturation rule” hence likely does not occur via random free radical mechanisms. So what then may be the mechanisms catalyzing peroxidation of CL and PS? Based on previous studies, it is possible that cell damage and execution of an apoptotic program are the major causes for lipid peroxidation (Tyurin et al. 2008a). If so, it is logical to expect that CL and PS will be involved in oxidation reactions proceeding as a part of the apoptotic program. As it has been shown earlier, cytochrome c acts as the catalyst of CL peroxidation early in mitochondria-dependent apoptosis (Kagan et al. 2005). Subsequently cytochrome c released into the cytosol participates in the catalysis of PS peroxidation. TBI is accompanied by significant neuronal apoptosis: thus it is likely that nonrandom peroxidation of phospholipids in the injured brain reflects the peroxidation processes occurring in apoptotic neurons. In support of this interpretation are the data demonstrating that selective oxidation of CL and PS in neurons is triggered during staurosporine-induced apoptosis (Tyurin et al. 2008b) (Figure 6a). The significance of these findings is that CL peroxidation is essential for the release of proapoptotic factors from mitochondria into the cytosol (Kagan et al. 2005). Moreover PS peroxidation is a part of the cascade of events ultimately leading to PS oxidation and externalization—signals required for recognition of apoptotic cells by professional phagocytes such as macrophages and microglia in the brain.

Cardiolipin cytochrome c interactions and apoptosis

If the hypothesis that nonrandom oxidation of phospholipids is true, then one can assume that inhibition of CL peroxidation may lead to suppression of apoptosis and protection against TBI. Doubly negatively charged CL with its four fatty acid residues anchoring it to the hydrophobic core of the lipid bilayer, is located exclusively in the inner mitochondrial membrane (Hatch 1998; McMillin and Dowhan 2002). In the inner mitochondrial membrane, CLs interact and are essential for normal functioning of many intrinsic proteins, including major electron transport complexes. Formation of respiratory super-complexes, “respirasomes”, requires the presence of CLs. In particular, the formation of respiratory supercomplexes III–IV is dependent on “gluing” by CLs (Schagger 2002). Our previous work demonstrated that cytochrome c forms a high-affinity complex with CL; the complex exerts strong peroxidase activity towards bound CL and causes its selective peroxidation. Early in apoptosis, translocation of CL takes place resulting in its appearance in the outer mitochondrial membrane (Kagan et al. 2005). Consequently, significant amounts of CL become available for the interactions with cytochrome c, one of the major proteins of the intermembrane space. In contrast to many other hemoproteins, normally, all six of the coordination positions in heme iron of cytochrome c are occupied, thus preventing its interactions with small ligands such as hydrogen peroxide (H_2O_2), and nitric oxide ($NO\bullet$) (Stellwagen 1968). By contrast, cytochrome c bound to CL exerts an entirely different conformation, with partial unfolding of the protein and a weakened/ruptured Fe-Met80 bond (Tuominen et al. 2002; Bernad et al. 2004). The heme site of CL-modified cytochrome c allows access to H_2O_2 and small organic peroxides, conferring catalytic peroxidase competence on the protein (Theorell and Åkesson 1941; Stellwagen 1968). Most importantly, thus formed mitochondrial complex of cytochrome c with CL acts as a potent CL-specific peroxidase and generates CL hydroperoxides (CL-OOH). The catalytic mechanisms of CL oxidation include the formation of protein-derived (tyrosyl) radicals detectable by low-temperature electron paramagnetic resonance (EPR) spectroscopy as well as by immuno-spin trapping.

The mechanisms of CL transmembrane redistribution and exposure at the contact sites between inner and outer mitochondrial membranes are not well understood. Several proteins have been implicated with the apoptotic transmigration of CL such as truncated Bid (tBid), one of the BH3-only members of the Bcl-2 family of proteins, phospholipid scramblase-3 (PLS-3), and mitochondrial isoforms of creatine kinase (MtCK) and nucleoside diphosphate kinase (NDPK-D) (Liu et al. 2003; Gonzalvez et al. 2005; Tyurin et al. 2007; Schlattner et al. 2009). tBid has a CL binding domain and in tBid treated mitochondria, CL redistributes from inner leaflet to other leaflet of inner mitochondrial membrane and also appears in the outer mitochondrial membrane (Gonzalvez et al. 2005; Tyurin et al. 2007). This in turn causes Bak/Bax oligomerization and cytochrome c release. Moreover, CL acts as an activation platform for the caspase-8 that is responsible for cleavage of Bid to form tBid (Schug and Gottlieb 2009). Activation of caspase-8 and cleavage of Bid has been reported in neurons and glia after experimental and clinical TBI (Franz et al. 2002; Zhang et al. 2003). Recently it has been shown that Caspase-8 translocates to mitochondria where it oligomerizes and gets activated (Gonzalvez et al. 2008). Furthermore, this association of caspase-8 with mitochondria depends on the presence of CL. Whether caspase-8 also interacts with CL-OOH is not known.

Several small molecule inhibitors have been proposed as potential regulators of cytochrome c/CL peroxidase activity. Their action may be directed towards different stages of the peroxidase reaction such as *i*) production of H_2O_2 as a source of oxidation equivalents *ii*) formation of cytochrome c/CL complexes and *iii*) inhibition of the peroxidase activity of cytochrome c in the complex. Among small molecule inhibitors of the first category, we explored the potential protective effects of a mitochondria-targeted stable nitroxyl radical -

2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO). While oxygen radical-scavenging substances such as nitroxides are well known to reduce the intracellular ROS levels and have been shown to confer neuroprotection after TBI (Deng-Bryant et al. 2008), the high millimolar concentrations required for their significant therapeutic effects limit their clinical applications. We discovered that it is possible to engineer bacterial membrane targeting antibiotic gramicidin to deliver 4-amino TEMPO (GS-TEMPO) to the mitochondrial membrane and markedly increase 4-amino TEMPO's potency (Wipf et al. 2005). One of these compounds, XJB-5-131, has been shown to inhibit CL peroxidation and improve survival after lethal hemorrhagic shock and irradiation (Macias et al. 2007; Jiang et al. 2008). We have recently shown that GS-TEMPO is preferentially partitioned in neuronal mitochondria and displays neuroprotective effects *in vitro* and *in vivo* (Ji et al. 2009).

There are also endogenous regulators that inhibit cytochrome c mediated neuronal apoptosis. We have shown that alpha synuclein (Syn), an abundant lipid-binding protein of synaptic terminals, interacts with anionic lipids (including CL) and cytochrome c to form a triple complex, Syn/CL/cytochrome c, which acts as a catalytically competent peroxidase that covalently cross-links Syn with cytochrome c into hetero-oligomers (Bayir et al. 2009). The covalent conjugation of Syn with cytochrome c into aggregates prevents death signaling effects of the cytochrome c in the cytosol (Figure 6b). In line with this, accumulation of Syn has been described in axonal swellings and neuronal cytoplasm in the traumatized brain in mice and in humans (Newell et al. 1999; Uryu et al. 2003; Ikonovic et al. 2004). Recent reports indicate that Syn can translocate to mitochondria, especially during oxidative or metabolic stress conditions (Cole et al. 2008). Corroborating this, accumulation of co-localized Syn-cyt c hetero-oligomers in mitochondria of cells exposed to oxidative stress was observed (Bayir et al. 2009). Since Syn has CL binding capacity, mice lacking Syn show reduced brain CL content with increased saturated fatty acids in CL molecular species and decreased activity of linked complex I/III in mitochondria (Ellis et al. 2005; Barcelo-Coblijn et al. 2007). There has not been a study evaluating deficiency of Syn in TBI. Given the fact that mitochondrial damage and impairment of bioenergetics occurs after injury (Singh et al. 2006; Pandya et al. 2007), Syn deficiency may worsen outcome after TBI.

Imaging lipids in the brain

In spite of the innovative nature of biochemical MS techniques described above, topographical evaluations of lipids and their oxidation events in cells and tissues remain obscure. Extracts of whole-brain tissue will, of course, not have spatial resolution. Since a small amount of tissue is required to be extracted for LC-MS, lipid extractions from progressively finer cuts of tissue could give regional localization. Also, laser microdissection has been used for a few non-lipid analytes, giving high spatial resolution. With the advent of IMS, studies of the topography of proteins and particularly small molecules, such as drugs and lipids, are now possible (McDonnell and Heeren 2007; Heeren et al. 2009) (Figure 6). Recently, this topic has been also reviewed in special. In our view, MALDI has the best combination of sensitivity and spatial resolution for oxidatively modified lipids, but other techniques such as Desorption Electrospray Ionisation (DESI), Secondary-Ion MS (SIMS) and Matrix-Enhanced SIMS (ME-SIMS) have advantages over MALDI in some areas (Table 2). Proper tissue preparation is essential for subsequent imaging mass spectrometry. Unfortunately, most archived tissues are not usable for IMS (see below), but ongoing work is being devoted to determine just what information can be obtained with them (Nirmalan et al. 2008; Mange et al. 2009).

DESI involves sputtering a stream of charged solvent droplets onto a surface, desorbing and ionizing. Unlike SIMS or MALDI, it is conducted at atmospheric pressure under ambient conditions, and requires no sample preparation (Takáts et al. 2004; Manicke et al. 2008;

Girod et al. 2010). Its spatial resolution of approximately 200 microns is worse than SIMS or MALDI, but it is a much milder ionization method and has also been used for lipid imaging of the rat brain (Manicke et al. 2008; Daiki et al. 2010).

SIMS involves “sputtering” of a tightly focused beam of high-energy ions onto a surface, and analyzing the resulting secondary ions generated from the surface (Jungnickel et al. 2005). MALDI has a practical limitation in spatial resolution to the width of the laser beam (10–30 microns), while SIMS can give sub-micron resolution (Benabdellah et al. 2010). This means that MALDI can image lipids in cells or small groups of cells, while SIMS can image at the sub-cellular level. However, SIMS tends to produce uncontrolled fragmentation of lipids above 1000 Da, which makes it very difficult to obtain information on mammalian CL (Benabdellah et al. 2010). A modification of this method is Matrix-Enhanced SIMS (ME-SIMS), where a matrix is applied to the tissue as in MALDI. This results in less fragmentation of lipids, but at the expense of poorer spatial resolution, making it an intriguing mix of the best features of MALDI and SIMS (McDonnell et al. 2005; Fitzgerald et al. 2010).

MALDI-based IMS was originally developed for detection of peptides and proteins in intact tissue slices by Richard Caprioli and Pierre Chaurand (Caprioli et al. 1997). Several MS laboratories now perform MALDI-IMS, although preparation of the tissue prior to analysis is just as important as the MS instrumentation (McDonnell and Heeren 2007). One requirement for this technique is that the tissue must be fresh frozen, and neither formalin fixed nor optimal cutting temperature polymer (OCT)-embedded. MALDI-MS techniques optimized to detect lipids will also detect OCT, and the latter signal will overwhelm the spectrum (Puolitaival et al. 2008; Burnum et al. 2009; Ridenour et al. 2010). Care must be taken during tissue cutting that only minimal OCT is used – just enough to fix the sample to the cryostat block – and that the blade never touches OCT. Washing with alcohol as part of the standard tissue fixation for IMS of proteins will de-lipidate the tissue, preventing IMS of lipids (Chaurand et al. 2008). Therefore, most archived histology samples (embedded blocks of fixed tissue and stained slides) are not able to be subsequently analyzed by IMS for lipids. There is ongoing work in the field to determine what information can be obtained out of archived samples, although success with protein analysis may be more likely than with lipids (Nirmalan et al. 2008; Mange et al. 2009). However, selective washing which removes salts or some lipids is an intriguing possibility for improving IMS analysis of other, particularly less abundant, lipid species (Puolitaival et al. 2008).

Early IMS was performed on tissue slides placed on stainless steel or gold MALDI sample holders (Caprioli et al. 1997). However, special glass histology slides that have a very fine coating of Indium-Tin Oxide (ITO) can be used (Chaurand et al. 2004; Altelaar et al. 2007; Vidova et al. 2010). The ITO provides a conductive surface (essential for MALDI), but the thin coating is transparent to light, and thus the tissue can be subsequently stained and examined by light microscopy after IMS. Of course, serial or near-serial sections can also be analyzed with immunohistochemical techniques for comparison with IMS data.

The MALDI matrix needs to be deposited uniformly onto the tissue slice to prevent “hot spots” of matrix clusters or analyte delocalization. The ultimate resolution of IMS depends on several factors including matrix crystal size. Therefore, small homogeneous crystals of matrix are desired (Werner et al. 2010). Methods commonly employed for IMS matrix deposition vary from climate-controlled aerosol chambers (“Image Prep” by Bruker Daltonik) to manual application with TLC sprayers or even commercial airbrushes (Chaurand et al. 2004). Other methods that have been used include sublimation, dry-powder application, and electro-blotting with a modified inkjet printer (Mange et al. 2009). In the

end, the “best” method is the one that consistently yields reproducible results for the lipids of interest.

IMS has progressed to include detection of other biomolecules including phospholipids (Schwartz et al. 2003; Caldwell and Caprioli 2005). Recent developments in MALDI have enabled direct detection of lipids as intact molecular species present within cellular membranes. Abundant lipid-related ions are produced from the direct analysis of thin tissue slices (10 μm) when sequential spectra are acquired across a tissue surface that has been coated with a MALDI matrix. With high resolution mass analyzers, the lipid derived ions can often be distinguished from other biomolecules because of cumulative and significant mass defects resulting from the larger proportion of hydrogen (1.007825 Da) present in the fatty acyl chains of lipids (Murphy et al. 2009).

Imaging in lipidomics has been used to visualize intact molecular distributions and determine head group identity, acyl chain length, and degrees of unsaturation on single cell surfaces (Heeren et al. 2009). In our preliminary experiments, we utilized MALDI-IMS in PND 17 rat brain showing spatial distributions of polyunsaturated oxidizable [834.6 m/z (PS(40:6), red)] and monounsaturated non-oxidizable [788.6 m/z (PS(36:1), green)] molecular species of PS in addition to ganglioside, GD1 (18:1/18:0) as potassium adduct at 1874 m/z and sulphatide, ST(24:0) at 888.8 m/z (Figure 7). IMS assessments of changes in oxidizable molecular species of phospholipids can be utilized as indirect measures of their likely oxidative modifications caused by brain injury or disease. As can be seen in Figure 8, signal intensity for polyunsaturated oxidizable molecular species of PS [834.6 m/z (PS(40:6), red)] and PI [885.7 m/z PI(38:4), green)] were decreased in the ipsilateral hemisphere compared to contralateral hemisphere after controlled cortical impact in PND 17 rat. Clearly, methodological advancements, particularly with regards to detection of low abundance lipids, will make direct IMS of peroxidized phospholipids possible. In particular, the diversity of CL molecular species and relatively low abundance of each of the CL species in the brain makes the task of their IMS challenging. Development of new methods based on selective photo-sensitization and ionization of CL molecules or products of their light-induced decay may be very promising in this respect. Notably, MS imaging has recently been successfully utilized for mapping of not only abundant species of PC but also its minor lyso-derivatives, lyso-PCs (Koizumi et al.; Hayasaka et al. 2008). Moreover, a recent study showed that several lyso-PCs (mostly lyso-PC(16:0) and (18:0)) were elevated with a decrease in several polyunsaturated PC species such as PC(36:4) in the rat cortex after focal cerebral ischemia (Wang et al.). This suggests that low abundance species of phospholipids such as peroxidized phospholipids, including oxidized CL and PS - are promising candidates for MS imaging.

Conclusions

Lipid oxidation products play essential regulatory and signaling roles in the CNS. Detailed information on the content and distribution of lipids and their oxidation products can be obtained by a combination of analytical methods including chromatography and MS. Oxidative lipidomics is beginning to provide new mechanistic insights into TBI which may have significant translational potential for development of therapies in acute CNS insults. Finally, lipid imaging represents an exciting new opportunity for correlating maps of lipid profiles and their oxidation products with structure and neuropathology.

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Figure 1.

Glycerophospholipid structure. **(a)** Fatty acyl chains in the sn-1 (R1) and sn-2 (R2) positions attach directly to the glycerol backbone, while the head group (X) attaches through the phosphate group. Although fatty acyl linkages are shown, both ether and plasmalogen (vinyl ether) bonds can occur in the sn-1 position. **(b)** Several common glycerophospholipid headgroups. **(c)** Cardiolipin (CL) structure. Four fatty acyl chains (sn-1, sn-2, sn-1', sn-2') can provide much molecular diversity.

Figure 2.

Fatty acids can be released by phospholipase A from phospholipids and have important roles in cell signaling and metabolism.

Figure 3.
Peroxidation of membrane phospholipids.

Figure 4.

MS Analysis of CL from brain, lung and heart. (a) LC-ESI-MS analysis of CL from murine brain. A murine brain lipid extract was separated by normal-phase HPLC to assess phospholipid classes. CL, which eluted with the 10–12min retention time window, displayed 8 to 10 major clusters of mass ions. Each cluster contains approximately 8–10 different species of CL. For simplicity, fatty acyl chain analysis is shown for only one of the major mass ions in each cluster in panels 4a–4d as determined by MS/MS analysis. Other fatty acyl chain combinations are present. In some cases, multiple fatty acyl combinations were detected for CL species of the same mass. Two additional CL clusters (m/z 1404 and 1592) were detected by the LC-ESI-MS method as compared to MALDI analysis. (b) MALDI

analysis of murine brain CL. A murine brain lipid extract was analyzed by negative ion mode MALDI using DHB as a matrix compound. A series of mass ions with a similar degree of complexity is seen for CL using this platform. **(c)** MALDI analysis of CL species from murine lung in negative ion mode. DHB was utilized as the matrix. A mitochondria enriched fraction from murine lung was extracted for CL analysis. A reduced number of CL species exists as compared to brain tissue. In the absence of any LC separation, sodium adducts are also present. TLCL and TLCL + sodium adducts are the major species. **(d)** MALDI analysis of CL from bovine heart in negative ion mode. DHB was utilized as the matrix. TLCL dominates as the major CL species running as the $(M-H)^-$ ion as well as its sodium adduct. Other minor CL species are also present.

Figure 5. MALDI-TOF MS (negative mode) of pure CL(18:2)₄ oxidized in vitro. Starting from one molecular species (tetralinoleoyl-cardiolipin (TLCL), 1448.9 m/z) will produce many combinations of hydroxy- (+16 Da) and hydroperoxy- (+32 Da) products.

Figure 6.

(a) Comparison of the abundance of major phospholipid (PL) classes with their oxidation. Profiles of PL and PL-hydroperoxides in control and apoptotic primary cortical neurons. PL content is expressed as percentage of total PL and shown in green scale. PL-hydroperoxides are presented as percentage of PL and shown in purple scale. Highly abundant PL, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were not substrates for oxidation whereas minor anionic PL, cardiolipin (CL) and phosphatidylserine (PS) were the preferred substrates for oxidation in neurons triggered to undergo staurosporine-induced apoptosis. PI: phosphatidylinositol. (b) CL oxidation and cytochrome c release during neuronal apoptosis. Cytochrome c forms a high-affinity complex with CL; the complex

exerts strong peroxidase activity towards bound CL and causes its peroxidation. Accumulation of CL oxidation products leads to the release of proapoptotic factors, including cytochrome c into cytosol. Alpha synuclein (Syn), an abundant lipid-binding protein of synaptic terminals, interacts with anionic lipids (including CL) and cytochrome c to form a triple complex, Syn/CL/cytochrome c, which acts as a catalytically competent peroxidase that covalently cross-links Syn with cytochrome c into hetero-oligomers. This prevents death signaling effects of the cytochrome c in the cytosol.

Figure 7.

(a) MALDI-IMS of brain showing different spatial distributions of different compounds. (Left) Red: Ganglioside GD1(d18:1/18:0) as potassium adduct at 1874 m/z, Blue: PS(36:1) at 788.6 m/z, Green: PI(38:4) at 885.6 m/z. (Right) Red: PS(40:6) at 834.7m/z, Blue: ST(24:0) at 888.8 m/z, Green: PI(38:4)at 885.7 m/z (green). Hippocampus is marked by a rectangle. (b) Negative mode MALDI-IMS (left panel) of 834.6 m/z [PS(40:6), red] and 788.6m/z [PS(36:1), green] showing different localization for different molecular species of PS. This is compared to a serial H&E stain (right panel), showing that fine features of brain architecture can be correlated between MALDI imaging and histology staining at 50 micron resolution. (c) Zoom in on hippocampus.

Figure 8.
(Left) Optical image of injured brain coated with DHB matrix prior to MALDI imaging of highlighted region. Controlled cortical impact was applied to the hemisphere marked.
(Right) Imaged areas across hippocampal region of PS(40:6) at 834.7m/z, (red), PI(38:4)at 885.7 m/z (green) along with overlaid area.

Table 1

Common endogenous fatty acid chains and their nomenclature (with oxidizable marked)

12:0	Dodecanoic	Lauroyl	saturated	non-oxidizable
14:0	Tetradecanoic	Myristoyl	saturated	non-oxidizable
16:0	Hexadecanoic	Palmitoyl	saturated	non-oxidizable
18:0	Octadecanoic	Stearoyl	saturated	non-oxidizable
18:1 (n-9)	9-Octadecenoic	Oleoyl	monounsaturated	non-oxidizable
18:2 (n-6)	9,12-Octadecadienoic	Linoleoyl	polyunsaturated	oxidizable
20:4 (n-6)	5,8,11,14-Eicosatetraenoic	Arachidonoyl	polyunsaturated	oxidizable
22:5	7,10,13,16,19-Docosapentaenoic	Docosapentaenoyl	polyunsaturated	oxidizable
22:6 (n-3)	4,7,10,13,16,19-Docosahexenoic	Docosahexaenoyl	polyunsaturated	oxidizable

The shorthand notation is given by number of carbons followed by number of double bonds. Thus “PC(16:0/16:0)” is dipalmitoyl phosphatidylcholine, and “PC(32:0)” is a combination of any two saturated chains that add up to 32 carbons. All the chains that possess two or more double bonds form conjugated systems and thus are potential targets for oxidative modification.

Table 2

Comparison of Mass Spectrometry instrumentation

Ionization Method	Resolution for Imaging MS	Mass Range
ESI	Not possible	Less than 40 kDa
DESI	100–200 microns	Less than 40 kDa
MALDI	10–30 microns	400 Da – 200+ kDa(2)
ME-SIMS	1–10 microns	Less than 2500 Da
SIMS	sub-micron	Less than 1000 Da

Analyzer	Mass Resolution(1)	Structural Information
FT-ICR, Orbitrap	0.01 – 0.05 Da	Best (MS ⁿ)
Linear Ion Trap	0.5 – 1.0 Da	Very Good (MS ⁿ)
Triple Quadrupole	0.5 – 1.0 Da	Good (MS/MS with precursor and neutral loss scans)
TOF (reflector, TOF/TOF)	0.1 – 0.2 Da	Good (MS/MS by PSD and CID)
TOF (linear)	1.0 – 3.0Da (but much worse at high masses)	Poor (prompt decay only)

These values are taken from multiple examples in the literature (see text for exact citations) and are based on what a typical laboratory can routinely achieve. They are meant as a rough guide only. Labs that are experts in a given technique probably can achieve better. Different samples will give different quality of results, and also there is variation in performance within each equipment classification depending on model and manufacturer. (ESI: electrospray ionization, DESI: desorption electrospray ionization, MALDI: matrix assisted laser desorption ionization, SIMS: secondary-ion mass Spectrometry, ME-SIMS: matrix-enhanced SIMS, FT-ICR: Fourier-transform ion cyclotron resonance mass spectrometry, TOF: time of flight, PSD: post source decay, CID: collision induced dissociation).

1: Assumed for a typical 750 Da Phospholipid, different masses will give different resolutions

2: Matrix clusters tend to put a lower limit on MALDI, very much dependent on choice of matrix and amount used