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## Characterization of cardiolipins and their oxidation products by LC-MS analysis

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### Abstract

Cardiolipins, a class of mitochondria-specific lipid molecules, is one of the most unusual and ancient phospholipids found in essentially all living species. Typical of mammalian cells is the presence of vulnerable to oxidation polyunsaturated fatty acid residues in CL molecules. The overall role and involvement of cardiolipin oxidation (CLOx) products in major intracellular signaling as well as extracellular inflammatory and immune responses have been established. However, identification of individual peroxidized molecular species in the context of their ability to induce specific biological responses has not been yet achieved. This is due, at least in part, to technological difficulties in detection, identification, structural characterization and quantitation of CLOx associated with their very low abundance and exquisite diversification. This dictates the need for the development of new methodologies for reliable, sensitive and selective analysis of both CLOx. LC-MS-based oxidative lipidomics with high mass accuracy instrumentation as well as new software packages are promising in achieving the goals of expedited and reliable analysis of cardiolipin oxygenated species in biosamples.

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## Oxidizable polyunsaturated cardiolipins in eukaryotes

It is commonly accepted that life has originated on Earth ~three billion years ago and one of the major evolutionary transitions - eukaryogenesis - has occurred over one billion years ago (Koumandou et al., 2013). Prior to the Cambrian, very few metazoan body or trace fossils have been identified (Briggs and Fortey, 2005). This paucity of metazoan fossils in the strata of Earth has been broken by the sudden appearance of highly developed metazoan fossils in the Cambrian, a pattern referred to as the Cambrian evolutionary “explosion” (Conway Morris, 2006) about six hundred million years ago (Crawford et al., 2013). While the cause of this “explosion” still remains incompletely understood (Briggs and Fortey, 2005), one of the possible explanations is that the oxygen content in the atmosphere became sufficient for the maintenance of highly diversified aerobic life and its biochemical basis – enzymatic redox reactions (Crawford and Broadhurst, 2012). Mitochondria became the universal instrument of life in eukaryotic cells – from protozoan to mammals - as an organelle filled with machinery capable of oxygen-driven “burning” of different oxidizable substrates in a coupled enzymatic and electrochemical process involving highly effective transformation of chemical energy of ATP. In addition to their function as a powerhouse in cells, mitochondria are currently viewed as the major regulatory platform involved in numerous intra- and extracellular effects, from coordination of metabolism and cell death to immune responses whereby *phospholipids* are considered as important signaling molecules. One of the most unusual and ancient phospholipids found in essentially all living species are cardiolipins - (1,3-bis(sn-3'-phosphatidyl)-sn-glycerols) (CLs). Their general structure includes a unique dimeric phosphatidyl lipid moiety whereby two phosphatidylglycerols are connected via a glycerol backbone thus adding up to four acyl (fatty acid) chains and two negative charges of phosphate groups (Figure 1). Of note, this type of molecular organization with >15 fatty acids available for biosynthesis will lead to a remarkable diversification of CLs with the total theoretical number of possible isomers in excess of  $15^4$ . Is this potentially huge multiplicity of CL molecular species found in nature? CLs are predominantly distributed in bacterial plasma membranes and in eukaryotic mitochondrial inner membranes. This very specific confinement of CLs to the mitochondrial inner membranes (IMM) corresponds with the endosymbiotic theory- according to which mitochondria of eukaryotes evolved from free-living bacteria that were phagocytosed inside another cell as an endosymbiot (Yang et al., 1985). In spite of the potentially common evolutionary origin, both CL's biosynthetic pathways and molecular speciation are different. In bacteria, CLs are synthesized by CLS containing two phospholipase D (PLDc<sub>2</sub>) domains - CLS<sub>pld</sub>, while in eukaryotes, the reaction is catalyzed by CLS containing one CDP-alcohol phosphatidyltransferase (CAP) domain - CLS<sub>cap</sub>. (Tian et al., 2012). As far as molecular speciation is concerned, bacterial CLs exhibit shorter carbon chains with mostly saturated or mono-unsaturated fatty acids, while longer chain polyunsaturated fatty acids are predominant in eukaryotic CLs (Figure 2).

While a popular concept that polyunsaturated lipids can play an important structural role and they are essential for the maintenance of fluidity of numerous biological membranes. Although this popular concept has been enthusiastically perceived, even mono-enoic oleic acid (C18:1) residues are sufficient for keeping membranes fluid enough within the physiologically relevant ranges of temperatures. It is also known that polyunsaturated fatty

acids may act as precursors of signaling lipid molecules – lipid mediators – that can be formed only from polyunsaturated fatty acids through their oxygenation. In this pathway, different phospholipids with esterified polyunsaturated fatty acid residues undergo hydrolysis by phospholipase A<sub>2</sub> – a rate limiting enzymatic process that releases polyunsaturated fatty acids for the subsequent oxygenation steps catalyzed by one of several enzymes such as cyclooxygenases (COX) lipoxygenases (LOX), cytochromes P450 (P450) (Lukiw et al., 2005). Recently, polyunsaturated fatty acids-residues of CLs have been considered as a source of oxygenated lipid mediators thus suggesting the presence of highly diversified polyunsaturated fatty acid-CL species (Schlame et al., 2005). In addition to the role of CL's polyunsaturated fatty acids as precursors of lipid mediators, the signaling role of oxygenated CLs appears to be essential for several physiological processes in particular apoptosis (Kagan et al., 2005). Accumulation of oxidized CL may act as an apoptotic signal when the process of elimination of “bad” mitochondria fails (Kagan et al., 2005). We established that early during apoptosis CL and cytochrome c (cyt c) form a complex with peroxidase activity that utilizes reactive oxygen species (ROS) to induce oxidation of polyunsaturated fatty acids CLs in mitochondria (Kagan et al., 2005). Indeed, this event results in the release of cyt c from mitochondria, activation of caspases 3/7 and externalization of phosphatidylserine (PS) on the cell surface. Generation of oxygenated polyunsaturated fatty acids in CLs has been detected *in vitro* in different cell lines in response to a variety of apoptotic stimuli (Kriska et al., 2005; Tyurin et al., 2009) as well as *in vivo* under acute injury conditions such as brain trauma (Bayir et al., 2007; Ji et al., 2012), total body irradiation (Tyurina et al., 2008; Tyurina et al., 2011b), hyperoxia (Tyurina et al., 2010) and exposure to carbon nanotubes (Tyurina et al., 2011a). Such involvement of peroxidized CL species – in their direct esterified form or after hydrolysis of CLox species – in physiological functions and pathogenic mechanisms requires sensitive and reliable methods for their analysis in mitochondria, cells and tissues.

## Lipidomics/Oxidative lipidomics of cardiolipins

During recent years, the major technological innovations that have advanced lipid analysis were the development of “soft ionization” mass spectrometry (MS) techniques, such as electrospray ionization (Fenn, 2003) and high mass accuracy orbitrap-based instrumentation (eg, QExactive type of mass spectrometers with high resolving power (up to 140,000)). As a result of this, accurate mass detection of complex lipids, their identification and quantitation with high assurance as well as discrimination of co-eluting isobaric species became possible. Moreover, a combination of multiple capabilities of these instruments - such as all ion fragmentation, MS<sup>2</sup> analysis in a data dependent mode, inclusion and exclusion of exact m/z values - makes identification of numerous lipids and their oxidation products achievable. These technological advancements designated the emergence of two fields of research - lipidomics and oxidative lipidomics aimed at identification and structural characterization of lipids and oxidized lipids in model systems, complex mixtures from cells, tissues and biofluids as well as their physiological role and functions (Kagan et al., 2006; Meikle and Christopher, 2011; Sparvero et al., 2010). In spite of this remarkable progress in MS-technologies, analysis of highly diversified and low abundant molecular species of oxidized lipids still represents significant challenges. There are two major approaches commonly

used in lipidomics: direct infusion of lipid extracts (“shotgun lipidomics”) (Han et al., 2012; Schwudke et al., 2011) and separation of complex lipid mixtures by either HPTLC or HPLC coupled with MS. While shot-gun lipidomics is commonly employed for general characterization of multiple species of lipids (Han et al., 2012; Schwudke et al., 2011), detailed analysis of lipid oxidation products is usually based on chromatographic pre-separation of major lipid classes (Tyurina et al., 2013).

## Analysis of oxygenated CL in cells and tissues

Ordinarily, analysis of complex phospholipid mixtures includes consecutive utilization of either HPTLC (Figure 3a) or normal phase LC for separation of major classes of phospholipids followed by full-MS and MS<sup>n</sup> analysis (Figure 3b) of individual components within these classes. Identification and characterization of the majority of molecular species of CLs and their hydrolytic metabolites (mono-lyso-CLs and di-lyso-CLs) by using conventional normal phase LC/MS protocols are relatively straightforward. In contrast, analysis of CL oxidation products in cells and tissue requires more sophisticated protocols due to their high diversity (Figure 1), very low content as well as overlapping of numerous isobaric non-oxidized and oxidized CL species, and isotopic splitting of CL species. Normally, oxidized CL species are accountable for only a relatively small fraction of total CLs - less than 0.5 mol%. Given that CLs represent 1–3 mol% of all phospholipids in cells and tissues (Bayir et al., 2007; Tyurina et al., 2010), it is not surprising that reliable identification and quantitative assessments of individual oxidized CL (CLOx) distributed among dozens of their molecular species requires specific efforts. One of the effective techniques of oxidative lipidomics employs two-dimensional LC/MS (2D-LC/MS) and allows for physical separation of oxidized and non-oxidized CLs species (Kim et al., 2011; Minkler and Hoppel, 2010; Samhan-Arias et al., 2012). However, the major difficulties in quantitative analysis of oxygenated CL species by 2D-LC/MS still face up to a large diversity of oxygenated derivatives and lack of standards for their quantitative assessment. Direct detection of selected oxygenated molecular species of CLOx may be possible under specific circumstances as it has been demonstrated for human lymphocytes exposed to rotenone (Fig. 4), in traumatic brain injury (Bayir et al., 2007; Ji et al., 2012), hyperoxic lung injury (Tyurina et al., 2011b; Tyurina et al., 2010).

Lipid peroxidation occurs mainly in a few types of fatty acid residues esterified into phospholipids (Kagan, 1988), ie long-chain polyunsaturated fatty acids with two and more double bonds - C18:2, C18:3, C20:3, C20:4, C20:5, C22:5 and C22:6. Combinatorial numbers of possible peroxidized species of CLOx - with one or more out of four fatty acid residues containing from one to over 8–10 oxygens which are commonly presented by hydroxy-, hydroperoxy-, epoxy-, and oxo-functionalities (Figure 1) – may be very high. The formation of a wide number of oxygenated species with differences in their structures makes the detection and identification of each CLOx molecular species more difficult. This huge diversification of CLOx species can be significantly reduced by enzymatic hydrolysis of oxidized CLs by a mixture of phospholipases A<sub>1</sub> plus A<sub>2</sub> to yield a limited number of readily identifiable hydrolysis products – oxygenated and non-oxygenated fatty acids and lyso-CLs. This, along with the commercial availability of standards for most of oxygenated fatty acids, makes detection of oxidatively modified CL feasible. It is therefore frequently

advisable to begin analysis of CL peroxidation from this simplified protocol (Tyurina et al., 2013). A mixture of commercially available phospholipases A - phospholipase A<sub>1</sub>, (Thermomycescandidae) plus porcine pancreatic phospholipase A<sub>2</sub> – in relatively high concentrations are effective in catalyzing the hydrolysis of CLs and CLox at *sn*-1 and *sn*-2 positions, respectively (Buckland et al., 1998; Pete and Exton, 1995). One of the complications of this protocol is still associated with high abundance of non-oxygenated fatty acids vs orders of magnitude lower concentrations of oxygenated fatty acids. This can be avoided by a more selective liberation of oxygenated fatty acids from CLox using low-density lipoprotein-associated phospholipase A<sub>2</sub> (LpPLA<sub>2</sub>VIIA or PAF-Acetylhydrolase) (Davis et al., 2008; Tyurin et al., 2012). While in major classes of phospholipids, oxidizable polyunsaturated fatty acids occupy predominantly the *sn*-2 position, in CLs they can be located in both the *sn*-1 and *sn*-2 positions. Therefore, enzymatic treatment of oxidized CL can yield a mixture of oxygenated and non-oxygenated fatty acids as well as oxygenated and non-oxygenated mono-lyso-CL. MS<sup>n</sup> analysis with collision induced dissociation (CID) technique (also called collisionally activated decomposition (CAD)) is a common method of fragmentation. MS<sup>n</sup> is sufficient for identification and characterization of hydrolyzed fatty acid residues. Analysis of CLox molecular species and mono-lyso-CLs can be also achieved by MS<sup>n</sup> but in the ion trap mass spectrometer, full characterization could be compromised due to a low mass cut off at 28% of the precursor *m/z*. This limitation can be overcome and the structure of CLox and mono-lyso-CLs can be confirmed by employing pulsed-Q dissociation technique (PQD) that allows for analysis of low molecular weight fragment ions with no low mass cut off. Coupling of enzymatic hydrolysis of CLs and CLox with MS<sup>n</sup> analysis provides: i) complete and accurate characterization of the molecular structure of oxygenated fatty acids, ii) valuable information on positional distribution (*sn*-1 or *sn*-2) of oxidized epitopes in phospholipids and iii) quantitative assessments of CLox species (Tyurina et al., 2013). Further, Lp-PLA<sub>2</sub>-based oxidative epitope targeted enzymatic digestion of CLox is a promising protocol for simplified detection and characterization of stereo-specificity of oxygenated fatty acid residues in CLox – that may be useful in the overall workflow of CLox analysis in cells and tissues (Figure 5).

Oxidized phospholipids have been identified as important signals in several pathological conditions (da Silva et al., 2012; Greig et al., 2012; Thomas and O'Donnell, 2012), thus necessitating detailed studies of tissue-, cell- and organelle-specific biomarkers of phospholipid oxidation. Notably, changes in the CL content, composition (Chicco and Sparagna, 2007; Lesnefsky and Hoppel, 2008; Paradies et al., 1993) as well as its oxidation levels (Ferreira et al., 2013; Paradies et al., 2009; Tyurina et al., 2013), have been associated with mitochondrial dysfunction potentially pointing to their role as informative biomarkers. For example, using enzymatic digestion of CLs and CLox followed by MS analysis, we were able to identify, characterize and quantitatively assess major oxygenated molecular species of CL in dysfunctional mitochondria from human lymphocytes exposed to rotenone (Figure 6a). Linoleic acid, located in both *sn*-1 and *sn*-2 positions of CL, was the major oxidation substrate for rotenone-triggered mechanisms in lymphocytes (Figure 6b) whereby hydroxy- and hydroperoxy-functionalities represented the major oxidative modifications (Tyurina et al., 2013). Unexpectedly, oxygenated products were detected predominantly in C<sub>18:2</sub> acyls of CLs in injured brain (Figure 7) and lung in spite of the presence of C<sub>20:4</sub> and

C22:6 polyunsaturated residues known to be “more oxidizable” (Tyurina et al., 2011a; Tyurina et al., 2010). The mechanisms of this unusual specificity towards C18:2 species of CLs remain to be elucidated; however *a priori* they may be dissociated from random non-enzymatic free radical pathways.

## CL oxidation in model systems

Model systems are widely used to mimic complex cell environments. Information and pattern of structural modification of particular compounds obtained in simple models give us a clue to possible mechanisms and pathways involved. The MS analytical strategies are based on the assignment of specific fragmentation fingerprints of each CLox molecule. This methodology helps to predict and verify the pathways of CL oxidative modifications, which might be subsequently searched in *in vivo* systems using a lipidomic approach. The detection of the oxidation products in biological samples requires a point targeted lipidomic analysis that can be performed by quantification of specific modified phospholipids using both single and multiple reaction monitoring (SRM and MRM) protocols. The design of such protocols requires preliminary unambiguous identification of accurate masses and types of product ions for each modified phospholipid species. Although these protocols have not been commonly applied to CLox analysis they are widely used for quantification of other phospholipids and their oxygenated species (O'Donnell, 2011).

In particular, questions related to apparent specificity of CL peroxidation can be tested in simple model chemical or biochemical systems. As has been noted above, the non-random character of CL oxidation is one of its most prominent features detectable *in vivo* and in cell cultures suggesting the involvement of enzymatic catalytic pathways. Several studies indicate that TLCL oxidation in model systems is also non-random. While the TLCL molecule is symmetric and has four equivalent C18:2 fatty acyl chains, their oxidation catalyzed by oxygen radicals and singlet oxygen displayed specificity. In multi-oxygenated TLCLox species with over 4–8 oxygen atoms, there are fatty acid residues with two hydroperoxy-groups while the other acyls in the same molecule remain non-oxidized (Kim et al., 2011; Kim et al., 2010; Maciel et al., 2011b). While numerous *in vitro* studies have implicated transition metals and their complexes as well as metalloproteins (particularly hemoproteins) as potential catalysts of CL peroxidation (Kim et al., 2011; Losito et al., 2011; Maciel et al., 2011a) their role in mitochondrial enzymatic CL oxidative metabolism *in vivo* remains ambiguous (Belikova et al., 2006; Kagan et al., 2005; Kapralov et al., 2007; Kapralov et al., 2011). In a series of studies, similarity between cyt *c*-catalyzed peroxidation of CLs in model biochemical systems and in cyt *c*<sup>+/+</sup> mouse embryonic cells has been documented (Belikova et al., 2006; Kagan et al., 2005; Kapralov et al., 2007; Kapralov et al., 2011). Notably, the cells lacking cyt *c* were incapable of catalyzing accumulation of CLox and also displayed resistance to pro-apoptotic stimuli (Kagan et al., 2005). Importantly, accumulation of the same types of CL oxidation products have been established in mitochondria of acutely damaged tissues – brain, lung, small intestine – associated with massive apoptotic cell death (Bayir et al., 2007; Ji et al., 2012; Tyurina et al., 2008; Tyurina et al., 2011b; Tyurina et al., 2010). Based on this, a peroxidase model of cyt *c*/CL complexes with a competence and selectivity towards CL peroxidation has been proposed (Kagan et al., 2005). It is possible that transmembrane redistribution and externalization of CLs on the

mitochondrial surface during mitophagy and apoptosis (Chu et al., 2013; Kagan et al., 2005) may create conditions for CL interactions with other tentative catalysts, whose identity is yet to be ascertained.

## Outlook and perspectives

Lipidomics has repeatedly conquered frontier positions among other omics approaches, particularly metabolomics (German et al., 2007; Griffiths et al., 2011; Hu et al., 2009). However, this triumph of lipidomics has been substantially more modest for a highly diversified group of mitochondrial phospholipids - CLs, and particularly CLox. The major reasons for this are very low abundance and exquisite diversification of CL molecular species leading to overwhelming difficulties in their detection, identification, structural characterization and quantitation. In spite of this, optimistic prognosis can be made with regards to future studies of CLs and CLox. This is mainly due to the emerging discoveries and growing understanding of their roles in major intracellular signaling (mitophagy, apoptosis) (Atkinson et al., 2011; Chu et al., 2013; Kagan et al., 2009) as well as extracellular inflammatory and immune responses. This dictates the need for the development of new methodologies for reliable, sensitive and selective analysis of CLs and CLox. With this in mind, new generations of high mass accuracy instrumentation along with the newly developed software packages for the identification (eg, LipidSearch) and quantification (TracerFinder) of MS data are very promising in achieving the goals of expedited and reliable analysis of CLs and, most importantly, CLox in biosamples.

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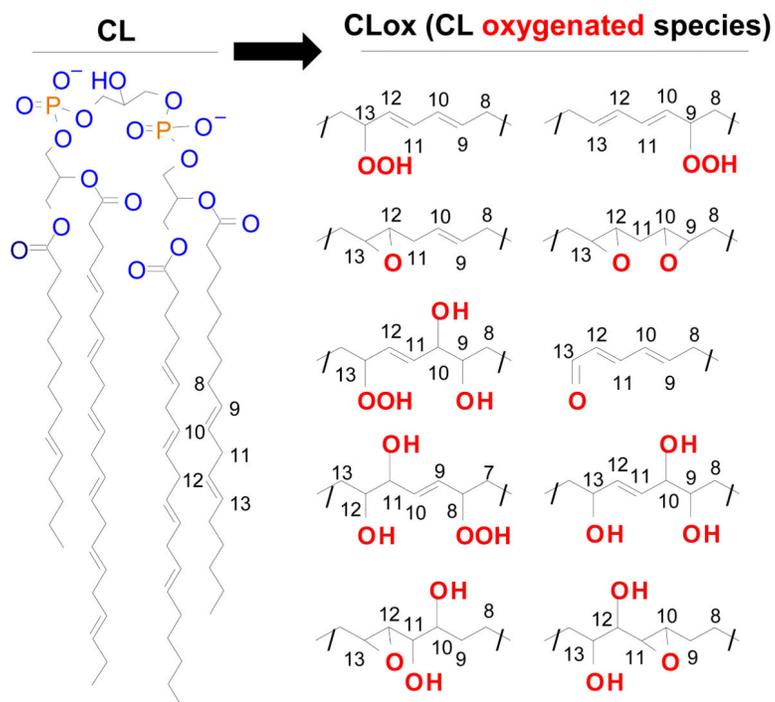
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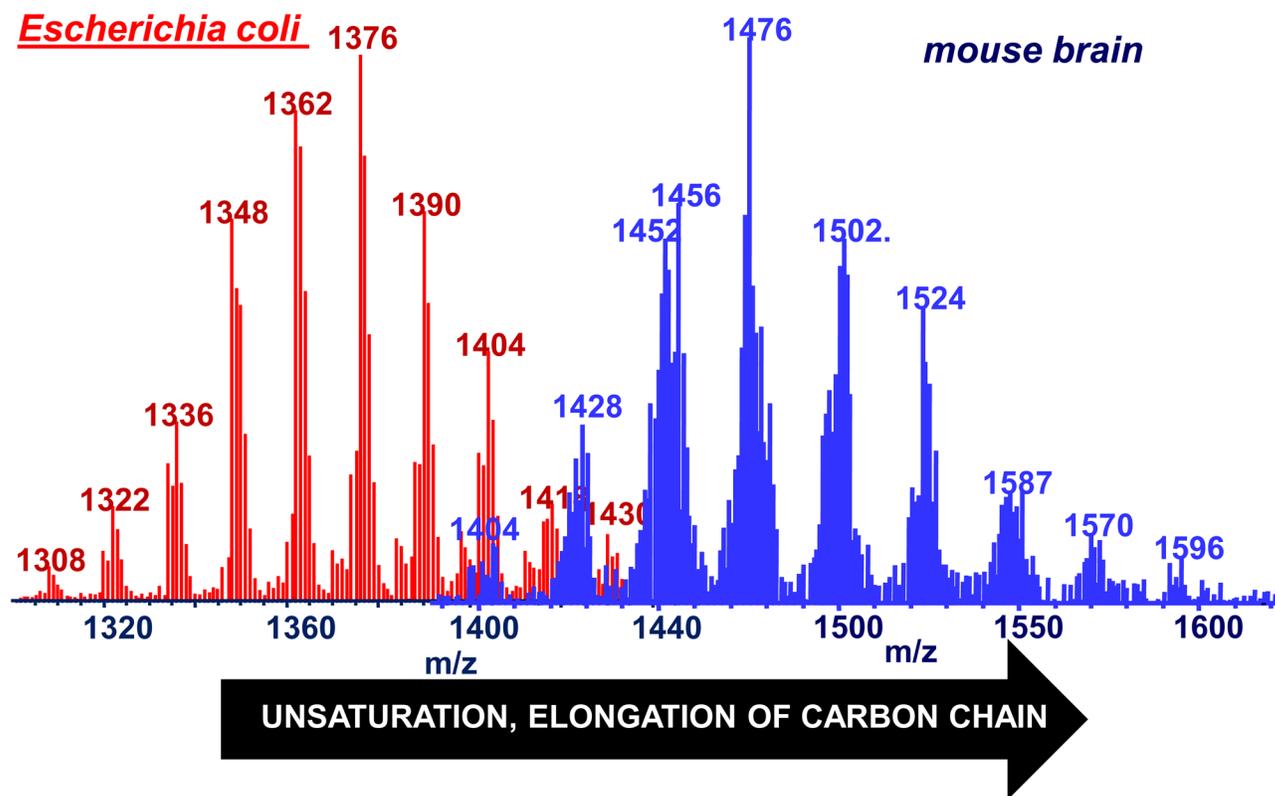
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- Oxidizable polyunsaturated cardiolipins in eukaryotes
- Lipidomics/Oxidative lipidomics of cardiolipins
- Analysis of oxygenated CL in cells and tissues
- CL oxidation in model systems

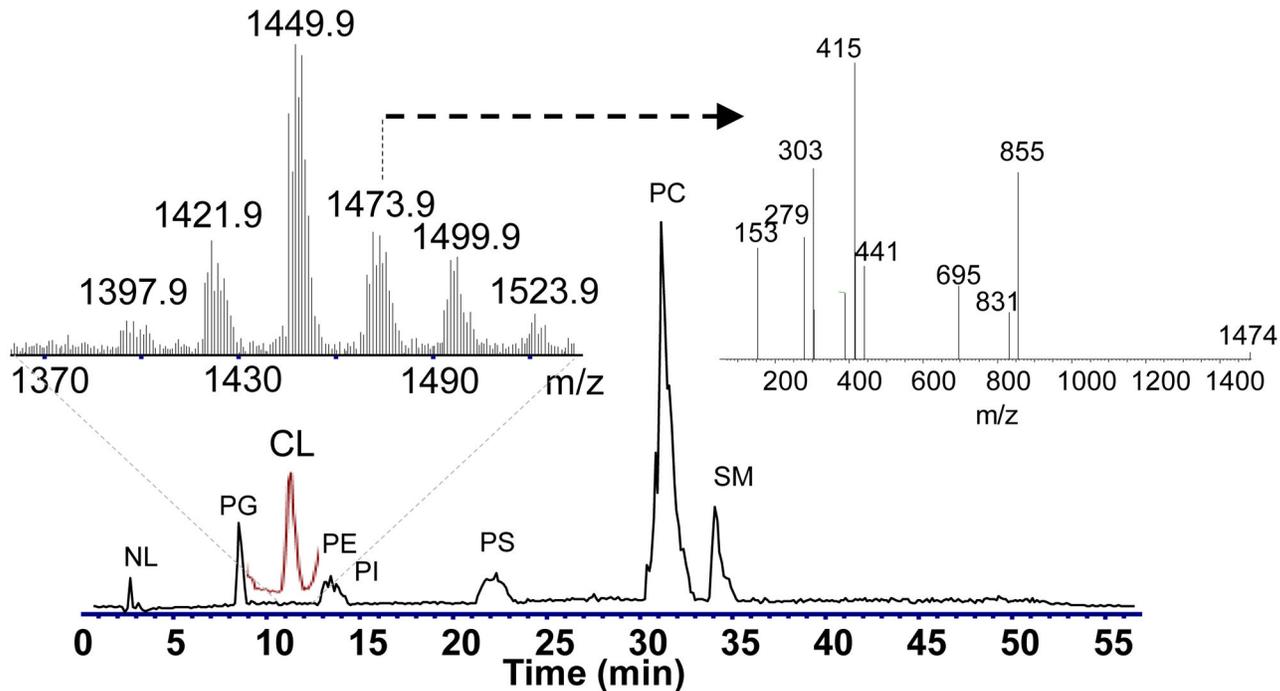
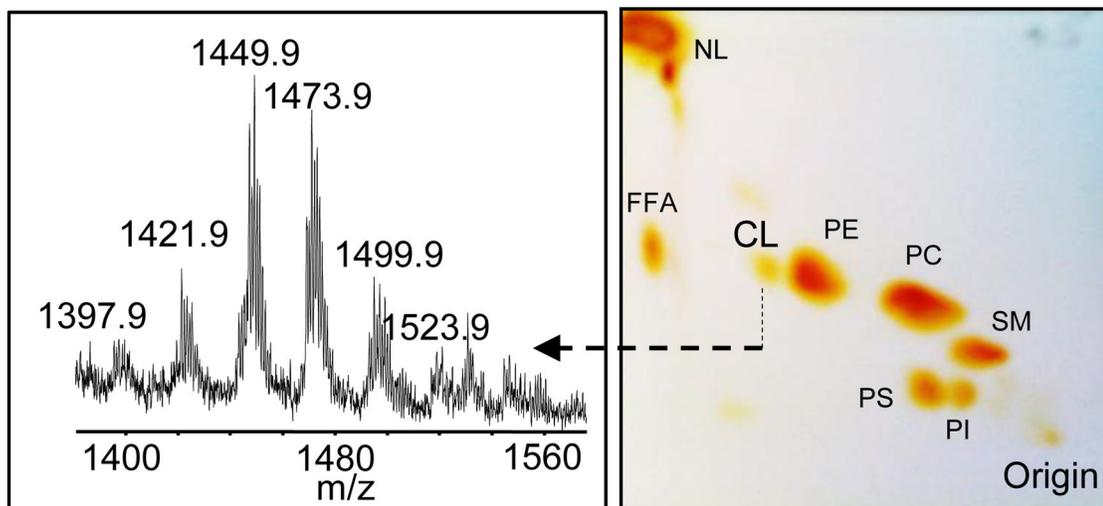


**Figure 1.**

Structure and oxidation products of CL. Left panel: Structural formula of a prototypical CL with four different fatty acid residues: sn-1-monounsaturated oleic acid (C<sub>18:1</sub>), and polyunsaturated sn-2-arachidonic acid (C<sub>20:4</sub>), sn-2'-docosahexaenoic acid (C<sub>22:6</sub>) and sn-1'-linoleic acid (C<sub>18:2</sub>). Right panel - major oxygen-containing functionalities in oxidized CL (CLOx) exemplified by oxidation of C<sub>18:2</sub> residues.



**Figure 2.** Mass spectra of caldiolipins isolated from *E. coli* (red) and mouse brain (blue).

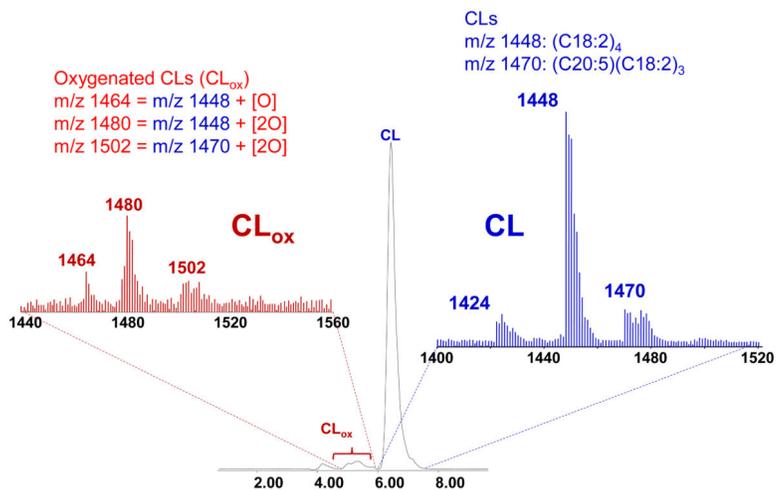


**Figure 3.**

MS analysis of cardiolipin isolated from mouse lung.

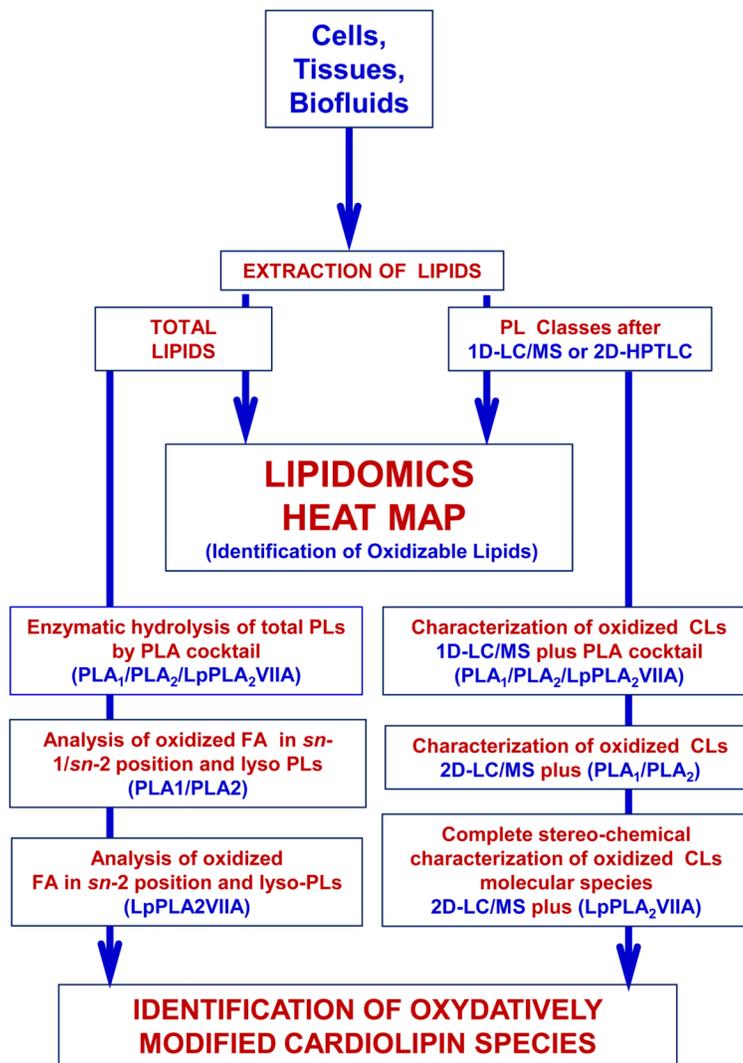
a). 2D-HPTLC of phospholipids isolated from mouse lung. CL spot was scraped, extracted and subjected to MS analysis by direct infusion. Full scan ESI-MS analysis in negative mode was employed for CL. b). Detection and identification of CL by LC/MS. Typical LC base profile (bottom) and full mass spectrum of CL (top left). MS<sup>2</sup> spectrum of CL molecular species with m/z 1473.9 (top right). LXQ Ion trap mass spectrometer (Thermo-Fisher, San Jose, CA) was employed. NL, neutral lipids, CL, cardiolipin; PE,

phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; FFA, free fatty acids

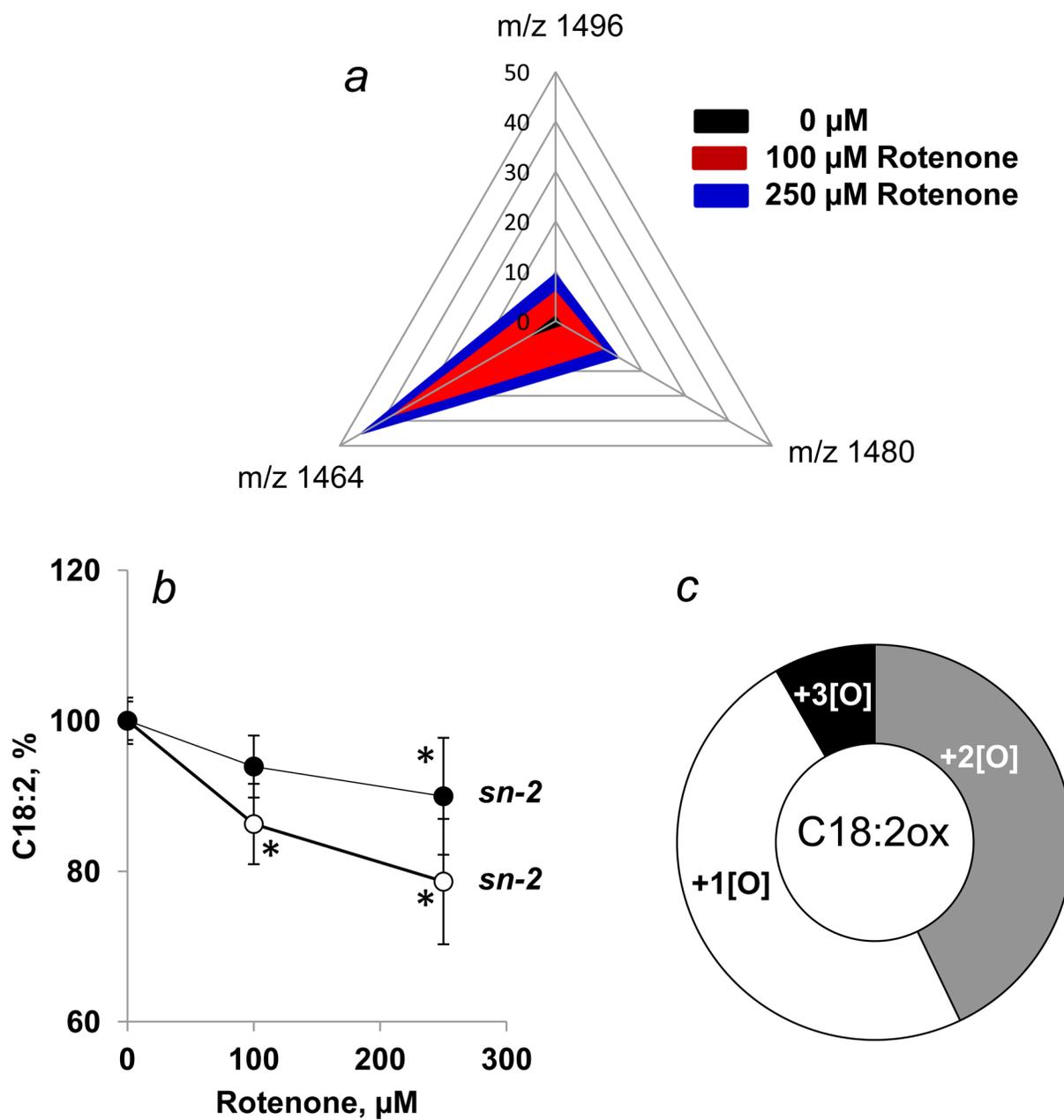


**Figure 4.**

2D-LC/MS detection of CL oxygenated molecular species in human lymphocytes after rotenone exposure. Typical 2D-HPLC profile and MS spectra of CL (blue) and oxidized CL (red) after treatment of lymphocytes with 250  $\mu$ M rotenone (18h). CL was isolated by normal phase LC/MS analysis. The CL fraction was then subjected to reverse phase LC/MS analysis (shown here) using a C8 column (4.6 mm  $\times$  15 cm). CL and CL-ox were separated using an isocratic solvent system consisting of propanol:water:triethylamine:acetic acid (450:50:2.5:2.5) at a flow rate of 0.4ml/min. Under these conditions, CL-ox eluted prior to CL. Molecular species of CL containing C<sub>18:2</sub>-OH (m/z 1464), C<sub>18:2</sub>-OOH or C<sub>18:2</sub>-(di-OH) (m/z 1480) from CL parent ion with m/z 1448 and C<sub>18:2</sub>-OOH or C<sub>18:2</sub>-(di-OH) (m/z 1502) from CL parent ion with m/z 1470 were detected in the samples. Molecular species of CL were identified based on m/z ratios.



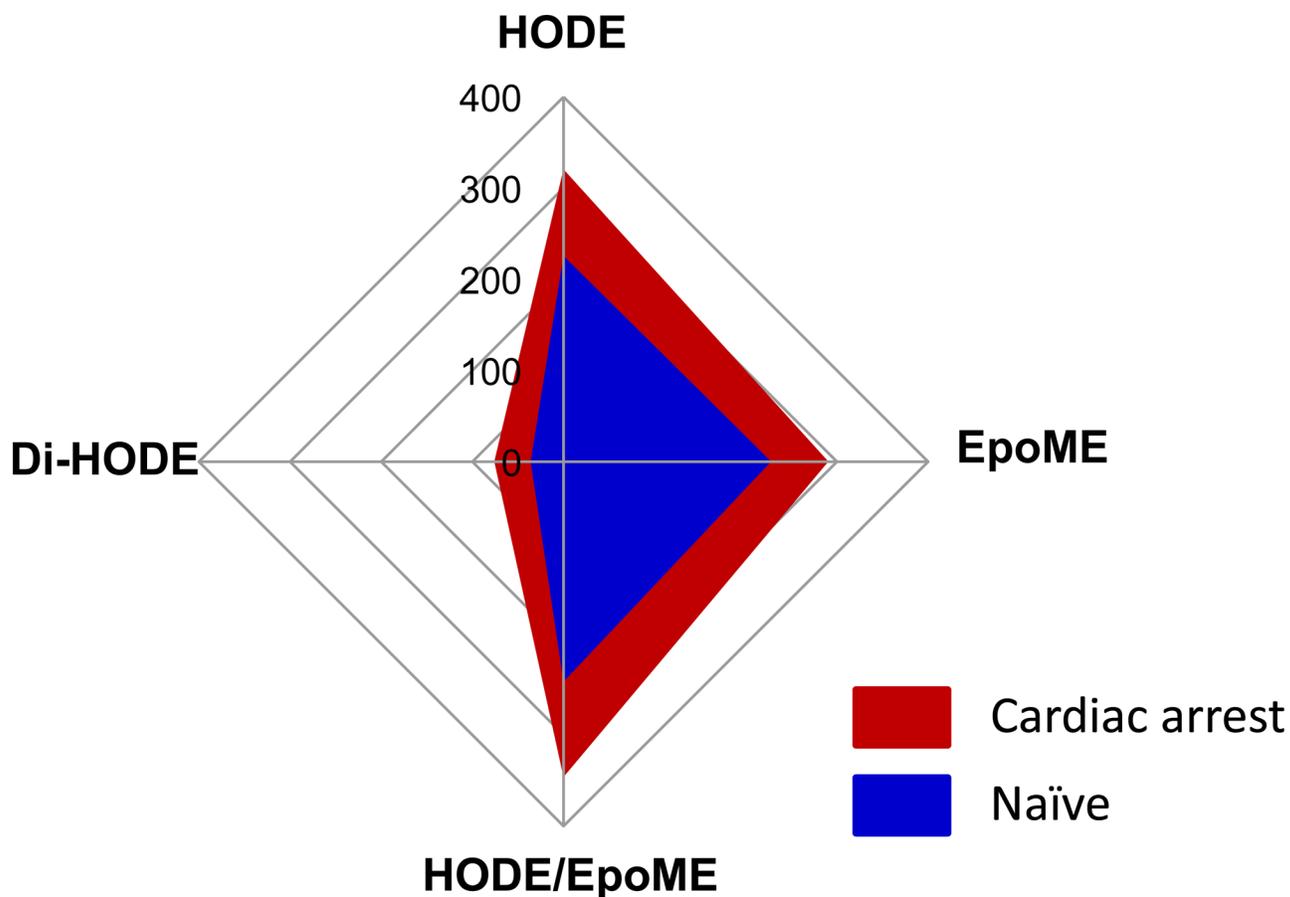
**Figure 5.** Flow-chart for complete identification of oxidatively modifies species of cardiolipins in cells and tissues.



**Figure 6.**

Detection and identification of CL oxygenated molecular species formed in human lymphocytes exposed to rotenone by using enzymatic approach coupled with MS analysis. a.) Quantitative assessment of oxygenated molecular species of CL in rotenone treated lymphocytes. Molecular species of CL containing oxygenated linoleic acid such as  $\text{C}_{18:2}\text{-OH}$  (m/z 1464),  $\text{C}_{18:2}\text{-OOH}$  (m/z 1480),  $\text{C}_{18:2}\text{-OOH}/\text{C}_{18:2}\text{-OH}$  (m/z 1496) were detected. Data are presented as pmol of  $\text{CL}_{\text{ox}}$ /nmol of CL. b.) Rotenone-induced oxidation of linoleic acid in CL molecule. CL was separated by 2D-HPLC, hydrolyzed either with phospholipase A<sub>1</sub> or phospholipase A<sub>2</sub> and liberated fatty acids were analyzed by LC/MS. c.) Oxygenated

species of C18:2 formed in lymphocytes exposed to rotenone (250  $\mu$ M). Data are mean  $\pm$  S.D., n=5, \*p<0.05 vs non-treated lymphocytes.



**Figure 7.**

Quantitative assessment of oxygenated molecular species of C18:2 liberated by a mixture of phospholipase A<sub>1</sub> and A<sub>2</sub> from phospholipids isolated from brains of naïve rats and rats after cardiac arrest (ACA). Sprague Dawley PND 17 male rats underwent asphyxial cardiac arrest (ACA) as previously described (Fink et al., 2004)(Fink et al., 2004). Animals were sacrificed at 24 h after resuscitation. Brains were removed and stored at  $-80^{\circ}\text{C}$  until use. To identify oxidized fatty acid (FA<sub>ox</sub>), CLs were isolated from total lipids by normal phase LC/MS and treated with phospholipase A<sub>1</sub> from *Thermomyces lanuginosus* (10  $\mu\text{l}/\mu\text{mol}$  CL) and phospholipase A<sub>2</sub> from porcine pancreatic (10U/ $\mu\text{mol}$  of CL) as described (Tyurina et al., 2013). Liberated FA<sub>ox</sub> were extracted by Folch procedure and analyzed by reverse phase (C18 column) LC/MS as described before (Tyurina et al., 2011). This study was approved by the institutional Animal Care and Use Committee at the University of Pittsburgh. Data are presented as pmol of FA<sub>ox</sub>/mg protein. N=4. HODE – hydroxy-species of C18:2; EpoME- epoxy-species of C18:2; HODE/EpoME- species of C18:2 containing both epoxy and hydroxyl-groups; diHODE - di-hydroxy-species of C18:2.