FORMATION OF 4-HYDROXYNONENAL FROM CARDIOLIPIN OXIDATION: INTRAMOLECULAR PEROXYL RADICAL ADDITION AND DECOMPOSITION

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Abstract

We report herein that oxidation of a mitochondria-specific phospholipid tetralinoleoyl cardiolipin (L_4CL) by cytochrome c and H_2O_2 leads to the formation of 4-hydroxy-2-nonenal (4-HNE) *via* a novel chemical mechanism which involves cross-chain peroxyl radical addition and decomposition. As one of the most bioactive lipid electrophiles, 4-HNE possesses diverse biological activities ranging from modulation of multiple signal transduction pathways to the induction of intrinsic apoptosis. However, where and how 4-HNE is formed *in vivo* is much less understood. Recently a novel chemical mechanism has been proposed that involves inter-molecular dimerization of fatty acids by peroxyl bond formation; but the biological relevance of this mechanism is unknown because a majority of the fatty acids are esterified in phospholipids in the cellular membrane. We hypothesize that oxidation of cardiolipins, especially L_4CL, may lead to the formation of 4-HNE *via* this novel mechanism. We employed L_4CL and di-linoleoyl-phosphatidylcholine (DLPC) as model compounds to test this hypothesis. Indeed, in experiments designed to assess the intramolecular mechanism, more 4-HNE is formed from L_4CL and DLPC oxidation than 1-palmitoyl-2-linoleoyl-phosphatydylcholine (PLPC). The key products and intermediates that are consistent with this proposed mechanism of 4-HNE formation have been identified using liquid chromatography – mass spectrometry (LC-MS) methods. Identical products from cardiolipin oxidation were identified *in vivo* in rat liver tissue after carbon tetrachloride treatment. Our studies provide the first evidence *in vitro* and *in vivo* for the formation 4-HNE from cardiolipin oxidation *via* cross-chain peroxyl radical addition and decomposition, which may have implications in apoptosis and other biological activities of 4-HNE.

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Keywords

4-hydroxy-2-nonenal (4-HNE); cardiolipin; cytochrome c; LC-MS; lipid peroxidation; free radicals; mitochondria; apoptosis

Introduction

Free radical-induced oxidation products of polyunsaturated fatty acids (PUFAs) have been implicated as a major upstream component in signal transduction cascades in many cellular functions including apoptosis, proliferation, inflammatory responses, stimulating adhesion molecules, and chemoattractant production [1–3]. Fatty acid hydroperoxides are the primary products in enzymatic and non-enzymatic oxidation of fatty acids. These lipid hydroperoxides not only have potent biological activities related to human physiology and pathophysiology, but also can serve as precursors to form other highly oxidized lipid oxidation products or decompose to generate an array of reactive lipid electrophiles [4]. Over the past two decades, 4-hydroxy-2-nonenal (4-HNE) has become one of the most studied reactive lipid electrophiles [5–9]. As a highly reactive α, β-unsaturated aldehyde, 4-HNE exhibits a variety of biological activities including inhibition of protein and DNA synthesis and inactivation of enzymes. 4-HNE can also form protein adducts with amino acid residues such as cysteine, histidine, arginine, and lysine due to its strong electrophilic character [10–12]. Moreover, the presence of these protein adducts can serve as biomarkers for lipid peroxidation and oxidative stress [5]. 4-HNE can also trigger multiple signaling events in a physiological context [8].

In contrast to the biology of 4-HNE, the mechanisms for its formation are much less understood [13–15]. Several mechanisms have been proposed that may account for the formation of 4-HNE, which includes free radical-induced decomposition of lipid hydroperoxides such as 13-hydroperoxyoctadecadienoic acid (13-HpODE) or 15-hydroxyeicosatetraenoic acid (15-HETE) [16,17]. Ferrous iron (Fe^{2+}) or vitamin C induced decomposition of lipid hydroperoxides have also been proposed [18,19]. Recently, an alternative mechanism was suggested, which involves an intermolecular cross-linking of a peroxyl radical [13,17]. This mechanism has precedence in styrene-oxygen polymerization and depolymerization [20]. There is also evidence to support the decomposition of these cross-linked peroxides of linoleic acid as initiators of free radical lipid oxidation and formation of reactive lipid aldehydes *in vitro* [21–25]. It should be noted that all these studies were carried out using linoleic acid or methyl ester as models and thus the biological relevance of this mechanism remains unclear because a majority of linoleic acids are esterified on phospholipids in cellular membranes.

Cardiolipin (CL) is a unique class of phospholipids containing four fatty acyl side chains and three glycerol moieties (Figure 1A). In most mammalian tissues the predominant form of CL is tetralinoleoyl CL (L_4CL), and typically the distribution of linoleate mitochondrial CL is around 85–90 % [26–28]. Recently, cardiolipin oxidation has attracted much attention due to the fact that it is involved in regulation of programmed cell death (apoptosis) initiated in mitochondria [29]. During apoptosis, CL interacts with cytochrome c to form a peroxidase complex that catalyzes CL oxidation and accumulating evidence indicates that the oxidation products of CL play a critical role in the mitochondrial stage of the execution of the cell death program [30, 31].

We hypothesize that L_4CL is the best model compound with biological relevance to test the idea of inter-chain peroxyl radical addition and decomposition to form 4-HNE. In this case, the addition of a peroxyl radical on one linoleic acid to another fatty acid side chain occurs

intra-molecularly in L_4CL instead of *inter*-molecular addition in the model systems that have been studied previously. Moreover, the *intra*-molecular addition of a peroxyl radical in L_4CL is favored kinetically over the *inter*-molecular addition. Due to the complexity of the peroxidation of L_4CL, we first use DLPC as a simplified model to test this idea. A number of mass spectrometry (MS) - based techniques have been employed to study the formation of 4-HNE from free radical oxidation of DLPC and L_4CL, decomposition products and their postulated dimeric precursor have also been identified using state-of–the-art MS methods. Furthermore, we identified the key products with epoxyalcohol on one side chain and truncated aldehyde on the adjacent side chains *in vivo* in rat liver tissue after treatment with carbon tetrachloride. Our data are consistent with the concept that intramolecular cross-chain peroxyl radical addition and decomposition may contribute significantly to the enhanced peroxiditon of CL and 4-HNE formation. These findings link CL oxidation and 4-HNE formation to mitochondrial dysfunction, apoptosis, and other biological activities in mitochondria caused by oxidative stress and free radical lipid peroxidation.

EXPERIMENTAL PROCEDURES

Reagents

Phospholipids, 1-palmitoyl-2-oleoyl-3-phosphatidylcholine (POPC), 1-palmitoyl-2-linoleoyl-3-phosphatidylcholine (PLPC), 1,2-dilinoleoyl-3-phosphatidylcholine (DLPC), tetraoleoyl cardiolipin (O_4CL) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Bovine heart cardiolipin was purchased as a chloroform solution and purified by HPLC method described below. Bovine heart cytochrome c, diethylenetriaminepentaacetic acid (DTPA), dinitrophenylhydrazine (DNPH), and soy bean lipoxygenase were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). HPLC quality solvents, such as methanol, water, 2-propanol, and acetonitrile were purchased from either Fisher Chemical (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). The free radical azo initiator 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN) and C-0 (VA-044) were generously donated by Wako Chemicals USA Inc. (Richmond, VA). 4-HNE, 4-oxo-2-nonenal (4-ONE), 4-hydroperoxy-2-nonenal (4-HPNE), and 4-HNE-d_3 were purchased from Cayman Chemicals (Ann Arbor, MI) and used without further purification. All animal procedures were performed in accordance with institutional guidelines after approval from the Animal Care and Use Committee of Vanderbilt University.

Purification of L_4CL from bovine heart cardiolipin

Bovine heart cardiolipin contains about 80% L_4CL. HPLC was carried out on a Waters Model 600E pump with a Waters 996 Photodiode array detector. Millenium32 chromatography software (Waters Corp., Milford, MA) was used to control the array detector and to collect and process data. Purification of L_4CL was performed on a Phenomenex (Torrence, CA) Luna C8 column (3μm, 4.6×150 mm) with a flow rate of 1.0 ml/min. Mobile phase A contained MeOH: CH_3CN: NH_4Ac (2mM) = 60:20:20 (v:v:v) while mobile phase B was methanol. The gradient started with 100% A and increased to 100% B in 15 min and held for 5 min before it returned to 100% A. L_4CL eluted from 18 to 20 min under this condition. The purity of L_4CL was analyzed by LC-MS method described below. The stocked solution of the purified L_4CL was stored at −80ºC under argon.

Oxidation of PLPC, DLPC, and L_4CL in liposomes with free radical azo initiator C-0

PLPC, DLPC, L_4CL, and POPC were dissolved in chloroform as stock solutions. Appropriate volumes of the stock solution were added into separate glass vials to make final solution of 0.6, 0.3, and 0.15 mM of PLPC, DLPC and L_4CL respectively. A constant concentration of 14.4 mM of POPC was added to each vial before the chloroform solutions

were evaporated by a stream of nitrogen. PBS (50 mM, pH 7.4) with 100 μM of DTPA was added to make the above concentrations for the phospholipids. After the lipid mixture was vortexed and sonicated for 10 min under nitrogen in water, 0.06 mM of C-0 in PBS was added to the mixture immediately to initiate oxidation. The reaction mixture was incubated at 37 °C under air.

Oxidation of L$_4$CL with cytochrome c and hydrogen peroxide

Oxidation of CL by cytochrome c and H_2O_2 was carried out based on a published protocol [29]. CL and POPC stored in chloroform were added to a glass vial and the solvent was removed by a flow of nitrogen. PBS (50 mM, pH 7.4) with 100μM DTPA was added and the lipid mixture was vortexed and sonicated for 10 min under nitrogen in water. Then to the lipid mixture were added cytochrome c and H_2O_2 at 37°C under air. H_2O_2 was added four times (every 15 min) during the incubation period. The final reaction mixture contained 250 μM CL, 250 μM POPC, 20 μM cytochrome c, and 100 μM H_2O_2. Oxidation of PLPC and DLPC using the cyt c/H_2O_2 system was carried out in the presence of O$_4$CL.

Synthesis of phospholipid hydroperoxides

A previously published protocol was used to synthesize PLPC and DLPC hydroperoxides [32]. Soybean lipoxygenase (20 mg) was dissolved in 30 ml of borate buffer that contained 10 mM deoxycholate at pH = 9.0. PLPC (25 mg) or DLPC (20 mg) was taken up in 3 ml of the same buffer and added to the enzyme solution. The reaction was stirred at room temperature for 30–60 min. The reaction was monitored by measuring UV absorbance at 234 nm. The reaction mixtures were extracted with chloroform: methanol (2:1, v/v) three times. The combined organic phase was evaporated and reconstituted with methanol. The hydroperoxide was further purified by reverse phase HPLC using a Discovery C18 HPLC column (Supelco, Bellefonte, PA; 25 cm × 21.2 mm, 5 μm) equilibrated with methanol/water (90/10) at a flow rate of 15 ml/min. The detection was monitored by the absorbance at 234 nm.

Mass Spectrometry

Oxidized phospholipids were separated online by UPLC using a Waters Acquity UPLC system (Waters Corp., Milford, MA) with a Phenomenex Luna 3μ C8 column (150 × 4.6 mm) at a flow rate of 1.0 ml/min and a flow splitter which sent approximately 20% of the mobile phase to the mass spectrometer. LC separation was performed using the same gradient that was used to purify L$_4$CL from bovine heart cardiolipin. Mass spectrometry analysis was carried out on Finnigan LTQ Iontrap mass spectrometer or Thermo Quantum Ultra triple quadrupole mass spectrometer. The ESI source was fitted with a stainless steel capillary (100 μm inner diameter). Mass spectrometer was operated in the negative ion mode for structural identification while quantitative analysis was carried out using selective reaction monitoring (SRM) in positive mode for some oxidized PC. Analysis of cardiolipins was performed in the negative ion mode. Nitrogen was used as the sheath gas at 38 p.s.i. The capillary temperature was 350 °C. The spray voltage was 4.5 kV, and the tube lens voltage was 100 V. Spectra were displayed by averaging scans across chromatographic peaks. Data acquisition and analysis were performed using Xcaliber software, version 2.0.

Analysis of 4-HNE, 4-HPNE, and 4-ONE by LC-MS method

Analysis of 4-HNE, 4-HPNE, and 4-ONE was carried out using a modified literature method [33]. Briefly, phospholipid oxidation mixtures were extracted by CHCl$_3$/MeOH (2:1, v/v) and 4-HNE-d$_3$ was added as the internal standard. After the solvent was removed by a flow of nitrogen, 0.5 ml of saturated DNPH solution containing 1N HCl was added to the residue and stored in the dark at room temperature for 2 h. DNPH derivatives were extracted twice

from the mixture with CH_2Cl_2 and evaporated to dryness under a stream of nitrogen. The sample was reconstituted with MeOH/water (3:1, v/v). LC-MS/MS was conducted using a Thermofinnigan TSQ Quantum Ultra equipped with a Finnigan Surveyor Autosampler Plus. Reverse phase HPLC was performed using a Phenomenex Luna C18 column (250×4.6 mm, 5 μm) with an isocratic elution of MeOH/water (3:1, v/v) at a flow rate of 1 ml/min. The MS was operated in the negative ion mode using electrospray ionization (ESI) in the selective reaction monitoring (SRM) mode. MS parameters were optimized for 4-HNE-DNPH derivative and were as follows: auxiliary gas pressure was set at 45 psi, sheath gas pressure was 50 psi, utilizing nitrogen for both. The vaporizer temperature was set at 400 °C. Collision induced dissociation (CID) for the 4-HNE-DNPH, 4-ONE-DNPH and 4-HpNE-DNPH were optimized at 25, 24 and 24 eV respectively under 1.0 mTorr of argon. Data acquisition and analysis were performed using Xcaliber software, version 2.0 (San Jose, CA).

Identification of cadiolipin oxidation products that are consistent with this novel pathway in vivo

After intragastric administration of CCl_4 (2 mg/kg) in corn oil to Sprague-Dawley rats for two hours, the animals were anesthetized with pentobarbital (60 mg/kg) intraperitoneally and sacrificed, and the livers were harvested. Around 1 gram of liver tissue was homogenized in a mixture of chloroform and methanol (2:1) with triphenylphosphine and BHT. The organic phase was evaporated and reconstituted in methanol for LC-MS analysis.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using analysis of variance followed by Student's t test. Differences were considered significant when $p < 0.05$.

RESULTS

Proposed mechanism of 4-HNE formation: Peroxyl radical addition to conjugated dienes and peroxide decomposition

Despite the substantial efforts to study the biological activities of 4-HNE, there is a lack of understanding of the mechanisms of its formation *in vivo*. Evidence provided here suggests that free radical-induced 4-HNE formation from PUFA may be due to peroxyl radical addition to a conjugated diene followed by the decomposition of the cross-linked peroxides due to the instability of these intermediates [17]. As shown in Figure 1B, linoleic acid **1a** is one of the major fatty acids that serve as the precursor for 4-HNE. 13-HPODE **1b** and 9-HPODE **1c** are the two primary oxidation products formed under free radical conditions. In this mechanism, a peroxyl radical from one of the hydroperoxides adds to the conjugated double bond of another hydroperoxide to form compound **1e** after addition of O_2 and hydrogen atom abstraction. This dimeric hydroperoxide has limited stability and is proposed to decompose to give 9-oxononanoic acid **1f** and 4-HPNE **1g**; the latter can be reduced to form 4-HNE **1h** or dehydrated to generate 4-ONE **1i**. The epoxide hydroperoxide **1j** is also formed on the other fatty acid in this mechanism. It is of some interest that the linoleic acid-containing L_4CL may be the best source for the 4-HNE formation by this mechanism because this CL contains four linoleic acids in the same molecule. The peroxyl radical addition reaction is therefore ***intra***-molecular in this instance since it occurs between pendant chains of the caridolipin.

In order to simplify a model study, we first compared oxidation of DLPC, where peroxyl radical addition could occur ***intra***molecularly with PLPC, a compound that requires that addition occur ***inter***molecularly. These studies were carried out in liposomes diluted with an

inert phospholipid such that the concentration of linoleate in the liposome was comparable for the two substrates. That is, the mole fraction of PLPC used in these test reactions was twice that of DLPC used in parallel experiments.

More 4-HNE is formed from the oxidation of DLPC and L_4CL than PLPC

Based on the mechanism illustrated in Figure 1B, more 4-HNE is expected to be formed from DLPC and L_4CL than from PLPC in the oxidation of liposomes containing equivalent mole fractions of linoleate ester. We developed an LC-MS method to simultaneously quantify the levels of 4-HNE, 4-HPNE and 4-oxononenal (4-ONE) from the oxidation of different phospholipids. As shown in supplement Figure S1, SRM experiments were carried out on the derivatives of 4-ONE, 4-HPNE and 4-HNE after derivatization with 2,6-dintrophenylhydrazine (DNPH). A deuterated internal standard of 4-HNE was used to quantify these compounds. As shown in Figure 2, significantly higher levels of 4-HNE are formed in the oxidation of DLPC and L_4CL than from PLPC using a free radical initiator. Similar results are obtained for the formation of 4-HPNE and 4-ONE (data not shown). It is noteworthy that when the oxidation was carried out with either an azo-free radical initiator or cytochrome c/H_2O_2 systems, similar results were observed, i.e., the amounts of 4-HNE, L_4CL > DLPC > PLPC. These data suggest that phospholipids containing two or more linoleate fatty acids in the same molecule generate more 4-HNE and related compounds under free radical conditions, consistent with the mechanism proposed in Figure 1B.

DLPC dihydroperoxide is a valid model to identify the key products and precursors for 4-HNE formation

In addition to the formation of 4-HNE and related compounds, epoxy-alcohol or epoxyhydroperoxides are predicted products from the decomposition of the dimeric intermediates [17]. We propose that DLPC dihydroperoxides **3a** (Figure 3) can be used as a simplified model to test this mechanism. As shown in Figure 3A, dihydroperoxides DLPC **3a** form a cross-linked peroxide **3b** which readily decomposes to form two alkoxyl radicals. The alkoxyl radical on *sn*-1 cyclizes to form epoxy-hydroperoxide or hydroxide **3c** whereas the alkoxyl radical on *sn*-2 undergoes β-cleavage to form an aldehyde on C9 and 4-HPNE. It should be noted that other isomers can be formed during the process, but only the structures that are relevant to the proposed mechanism are illustrated for simplicity.

In order to show that DLPC dihydroperoxide **3a** can be used as a valid model to test this mechanism, we synthesized DLPC dihydroperoxide **3a** and PLPC hydroperoxide **3e** by the use of soybean lipoxygenase. These two precursor hydroperoxides were diluted in an unoxidizable phospholipid POPC (2% for DLPC dihydroperoxide and 4% for PLPC hydroperoxides respectively) and decomposed as a thin film in the presence of an azo free radical initiator MeOAMVN. Time-dependent formation of 4-HNE was observed and significantly more 4-HNE was generated from decomposition of DLPC dihydroperoxide **3a** than was formed from PLPC hydroperoxide **3e** (Figure 3C). Similar results were obtained for 4-HPNE and 4-ONE (Figure S2). Even though the hydrogen atoms in OOH are more easily abstracted than those at the bis-allylic positions of un-oxidized phospholipids, the data shown in Figure 3c suggest that DLPC-dihydroperoxide 3a offers a valid model for study of the proposed mechanism when the levels of 4-HNE from these two hydroperoxides are compared.

Identification of the critical decomposition products epoxy-alcohol and epoxy-hydroperoxide 3c from DLPC dihydroperoxide

The reaction mixture of DLPC dihydroperoxide taken at the 7hr time point was treated with PPh_3 and analyzed by LC-Iontrap MS. PC species are readily ionized in the positive ion mode MS but only limited structural information can be obtained in this way because the

fragmentation from CID only leads to a prominent ion of the choline head group with *m/z* 184. Thus, the acetate (Ac⁻) adduct of compound **3c** (*m/z* 764) was subjected to CID in the negative ion mode. The results are illustrated in Figure 4. MS[2] of *m/z* 764 gives a fragment of *m/z* 704 which represents the loss of acetic acid whereas a fragment of *m/z* 690 corresponds to the further loss of a methyl group on the choline head group [34,35]. A characteristic fragment of *m/z* 311 is consistent with the carboxylate of the epoxyalcohol side chain. Dehydration of *m/z* 311 gives rise to m/z 293. MS[3] of *m/z* 311 is shown in Figure 4B. The fragments with *m/z* 171 and 139 are indicative of the epoxyalcohol moiety in compound **3c**. Taken together, these data support the notion that decomposition of DLPC dihydroperoxide **3a** generates the key product epoxyalcohol **3c**, a conclusion that is consistent with the formation 4-HNE through the dimerization mechanism.

Identification of the intact cross-linked peroxides 3b

In order to identify the cross-linked peroxide precursors **3b,** we fractionated the reaction mixture derived from oxidation of DLPC dihydroperoxide by HPLC. The separated fractions were evaporated and let stand as a thin film for two hours to determine whether they contained 4-HNE precursors. Six fractions were collected and the levels of 4-HNE and epoxyalcohol **3c** were analyzed before and after the two-hour incubation. The results are summarized in supplement Figure S3. Two fractions had elevated levels of 4-HNE and **3c** after further decomposition, which suggested that cross-linked peroxides **3b** eluted in these fractions. Structural elucidation of this compound was carried out by LC-ESI-Iontrap MS experiments. The analyses are summarized in Figure 5 and the proposed fragmentation pathways are shown in Figure 6. Multi-stage CID experiments were employed to characterize the proposed structure **3b** in the negative mode of MS. MS[2] carried out on the acetate adduct of **3b** with *m/z* 936 and further MS[3] experiments were performed on the two major fragments of *m/z* 862 and 791 respectively. Based on the MS data, the intact cross-linked peroxide **3b** was unambiguously identified by the LC-MS technique.

Derivatization of the intact cross-linked peroxides 3b and characterization of the resulting products

Due to the limited stability of the cross-linked peroxides, characterization of these precursors proved to be difficult. Reaction of the peroxides **3b** with PPh₃ led to compounds that are more stable and can be characterized by MS methods. Reduction of the cross-linked peroxide **3b** leads to compound **7a** as shown Figure 7. Successful conversion of **3b** to **7a** was confirmed by MS analysis and the data were summarized in supplement Figure S4.

Characterization of compound **7a** was performed by Iontrap MS in the negative ion mode and the results are shown in Figure 7. MS[2] of the acetate adduct of compound **7a** with *m/z* 906 (Figure 7B) produced two major fragments: a peak with *m/z* 832 was consistent with the loss of acetic acid and a methyl group on the choline head group; the fragment of m/z 761 represented the loss of choline head group. MS[3] on *m/z* 832 clearly indicated the two acyl chains with m/z 329 and *m/z* 295 respectively (Figure 7C). The unambiguous structural support of these two oxidized acyl chains was obtained in the MS[4] spectra (Figure 7D). Taking these data together, the chemical structure of compound **7a** was unambiguously confirmed by the Iontrap MS.

Formation of 4-HNE from L₄CL via the similar mechanism that involves decomposition of intramolecular cross-linked peroxide

Using DLPC as a model compound, we have provided evidence that 4-HNE and other reactive aldehydes are derived from the novel chemical mechanism, and we show here that L₄CL is the precursor for these reactive aldehydes *in vivo* with biological relevance.

The proposed key products consistent with the peroxyl radical addition mechanism are epoxyalcohol on one acyl side chain and an aldehyde at C9 on the other side chain. The putative structure as shown in Figure 8 has m/z 1371 and it lacks a conjugated diene structure as shown in supplementary Figure S5. Cardiolipin is an anionic phospholipid which is readily detected in the negative ion mode MS. The two oxidized side chains can be on the same (**8a**) or different (**8b**) glycerol backbone. As shown in MS^2 of m/z 1371.6 (Figure 8A), in addition to the common fragments of m/z 1217 and 1091 for these different regioisomers, fragments with m/z 619 and 695 are consistent with structure **8a** whereas m/z 727 and 587 are indicative of the structure **8b**. The assignment of these two critical fragments m/z 619 and 695 was further supported by respective MS^3 experiments. MS^3 on m/z 1371.6 to 619 generated characteristic fragments with m/z 311, 307, 447 and 293 (Figure 8B). A simpler fragmentation pattern of MS^3 on m/z 1371.6 to 695 was obtained as shown in Figure 8C. Fragments with m/z 279, 415, and 433 were consistent with the two intact linoleoyl side chains on the same glycerol backbone. The presence of the key epoxy-alcohol in the structure was evident in MS^4 of m/z 1371.6 to 619.3 to 311.2 (Figure 8D). In addition to the fragments of m/z 293 and 275, two fragments with m/z 171 and 211 were consistent with the presence of epoxyalcohol moiety in this compound. MS^n experiments were carried out for ions m/z 727 and 587 and the data are consistent with the proposed structure **8b** (data not shown).

We also attempted to identify the cross-linked peroxide precursors that would lead to 4-HNE. The stabilized precursor by PPh_3 reduction had an alcohol moiety on one side chain and three hydroxyl groups on the neighboring acyl chain. The LC-MS data of the reduced oxidation mixture are summarized in Figure 9. As shown in Figure 9A, the two oxidized side chains can be on the same (**9a**) or different (**9b**) glycerol bones. MS^2 of m/z 1514 showed two characteristic fragments of m/z 761 and 695 for **9a** while m/z 711 and 745 are derived from **9b**. Further structural characterization was performed by MS^3 and MS^4. MS^3 on m/z 1514 to 761 generated m/z 465 and 295. In the same way, fragments with m/z 431 and 329 were consistent with the presence of three hydroxyls on the other side chain. MS^3 on m/z 1514 to 695 demonstrated that the compound had two intact linoleate side chains on the same glycerol moiety because of the fragments with m/z 279, 415 and 433. MS^4 on m/z 1514 to 761 to 329 clearly showed the proposed structure due to the presence of the characteristic fragment m/z 229. MS^4 on m/z 1514 to 761 to 295 indicated that there was a mixture of regioisomeric hydroxide on one of the side chain: m/z 171 resulted from 9-HODEs while m/z 195 was indicative of 13-HODE. Similar data were obtained for structure **9b**. Therefore, the precursor with cross-chain peroxides is unambiguously identified based on MS data.

Taken together, these data indicate that the oxidation of L_4CL generates 4-HNE through a mechanism similar to that of DLPC based on the presence of the key products as well as the putative cross-linked peroxide precursors.

Identification of the key products of cardiolipin oxidation via the novel mechanism in vivo

Carbon tetrachloride-induced liver injury is a well-established animal model for *in vivo* free radical generation and oxidative stress [36]. Elevated levels of lipid peroxidation products, such as F_2-IsoPs, malondialdehde (MDA), TBARS, and 4-HNE, have been detected in this model. We performed experiments to identify the key products of cardiolipin oxidation *in vivo* in rat liver tissue after carbon tetrachloride treatment. The results were shown in Figure 10. We identified characteristic fragments for compound **8a** in CID experiments on m/z 1371.8 (singly charged ions) or m/z 685.6 (doubly charged ion) from *in vitro* oxidation of L_4CL and extracts of rat liver tissue (data not shown). A peak at 16.2 min in Figure 10A and 10B demonstrated the formation of this key product *in vitro* and *in vivo*. It is noted that unoxidized L_4CL and the major oxidation product with addition of one oxygen atom were

also detected. These data indicate that the novel mechanism for 4-HNE also operates *in vivo* and it may play an important role in 4-HNE formation *in vivo*.

DISCUSSION

Free radical-induced oxidation of PUFAs has been extensively studied for more than half a century while 4-HNE was first discovered *in vivo* almost three decades ago [37]. Investigation of the toxicological and physiological actions of this reactive lipid electrophile has been extensive, but the mechanism for its formation is much less understood. A novel mechanism for the formation of 4-HNE by intermolecular peroxyl radical addition and decomposition was recently proposed [13,17]. We provide evidence here that this mechanism may play an important role for the oxidation of the mitochondria-specific phospholipid cardiolipin, especially L_4CL. Formation of 4-HNE and other reactive aldehydes from this process may have significant implications in apoptosis and other functions of cardiolipins in mitochondria.

Substantial efforts have been made to understand the mechanism of 4-HNE formation *in vivo* since the discovery of 4-HNE [14,15]. PUFAs containing ω-6 chains, such as linoleic acid (C18:2) and arachidonic acid (C20:4), are precursors for 4-HNE and much attention has been paid to the decomposition of lipid hydroperoxides to generate 4-HNE and related lipid electrophiles. We have utilized different lipid hydroperoxides as precursors to study the formation of 4-HNE. Our previous studies using 15-HETE and 15-HPETE identified epoxyalcohol and epoxyhydroperoxide derivatives as major products during the formation of 4-HNE from these precursors [17]. These results prompted us to propose a novel mechanism of 4-HNE formation *via* intermolecular addition of a peroxyl radical to form a dimeric peroxide, followed by decomposition of this unstable precursor (Figure 1B). There is precedence for the dimerization of linoleic acid during the free radical oxidation *in vitro* and these resulting peroxides can be used as initiators for further free radical processes [21]. Successful separation and characterization of several dimeric lipid hydroperoxides were carried out and their decomposition gave rise to a number of reactive aldehydes. These studies also discovered the instability of these peroxide dimers [22–25,38]. However, the biological relevance of the intermolecular cross reaction mechanism is unknown because a majority of the fatty acids are esterified on phospholipids in cellular membranes and the linoleate acyl chain is often separated by saturated acyl side chains. The data we presented here suggest that CL is one of the major sources for 4-HNE because it has four linoleate acyl chains in the same molecule and the formation of these important peroxyl dimers occurs ***intra*** molecularly. Thus, it is this unique chemical structure of CL that is responsible for the enhanced oxidation of CL (Figure S1B) and formation of 4-HNE under free radical conditions (Figure 2). It is noted that ***intra*** molecular peroxyl radical addition to a conjugated diene is favored kinetically over the ***inter*** molecular addition. Similar chemistry has been reported *in vitro* in other lipids that have two or more linoleate side chains in the same molecule. For example, kinetic studies showed that triglycerides containing two or more linoleate side chains underwent enhanced autooxidation due to the arm-to-arm hydrogen atom abstraction under free radical conditions [39,40].

We also detected the characteristic product compound **8a** that are postulated to be formed *via* this novel mechanism *in vivo* in rat liver tissue after carbon tetrachloride treatment. As a well-established rodent model for *in vivo* oxidative stress, CCl_4 treatment induces free radical generation in the liver that leads to massive lipid peroxidation, protein modification, and DNA damage [36]. Recent studies also demonstrate that CCl_4 treatment inhibits hepatic mitochondrial aldehyde dehydrogenases through JNK-mediated phosphorylation and further exacerbates the liver damage by 4-HNE and other reactive lipid aldehydes [41].

The MS techniques that we employed here have proved indispensible to characterize the proposed oxidation products of phospholipids [42–44]. Free radical oxidation of PUFA-containing phospholipids generates an array of oxidation products and most of them are unstable peroxides. It is extremely difficult to isolate these oxidation products and characterize them by the conventional techniques such as NMR. MS coupled with LC separation has advantages in providing the molecular weight and structural information for the proposed compounds. Iontrap MS has been widely employed for this purpose due to the capability of multi-stage CID [45–47]. In the negative ion mode, the chemical structures that are consistent with the proposed mechanism were unambiguously identified using consecutive CID experiments. On the other hand, the iontrap technique has limitations in efficient trapping of the ions with lower m/z ratios. We also employed triple quadruple MS to conform the formation of these characteristic smaller fragments that did not show in MS^2 of iontrap instruments (data now shown). These two complimentary techniques enable us to identify the key products that are consistent with our proposed mechanisms from a complex oxidation mixture of phospholipids.

The mechanism presented here links 4-HNE formation to CL oxidation in mitochondria where substantial amount of ROS, such as super oxide anion and hydrogen peroxides, are generated. Lipid peroxidation and subsequent formation of 4-HNE in mitochondria have been suggested to play an important role in mitochondrial dysfunction [48]. Paradies et al showed a direct correlation between lipid peroxidation, decreased CL and decreased cytochome c oxidase activity when they exposed rat heart mitochondria to t-butyl hydroperoxides [49]. Sen et al oxidized rat brain mitochondria using iron-ascorbate and found that increased lipid peroxidation as measured by MDA/TBARS correlated with decreased levels of cardiolipin [50]. A seminal work carried out by Kagan et al demonstrated that oxidation of CL by the peroxidase function of cytochrome c due to its association with CL was required to trigger intrinsic apoptotic pathways [29,31]. Using a state-of-the-art oxidative lipidomics approach they showed that CLs in mitochondria were preferentially oxidized in the presence of other highly oxidizable phospholipids, such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Previous studies also established that hydroperoxides of CL were important in the induction of apoptotic events [51–53]. Our studies suggest that 4-HNE formation from these CL-OOHs may also play a significant role in these events because 4-HNE has been shown to induce apoptosis [8,54].

This novel mechanism for 4-HNE formation from CL oxidation in mitochondria may potentially have some physiological and pathophysiological significance. Generation of 4-HNE *in vivo* has been implicated in cardiovascular diseases, such as atherosclerosis [55], and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease [5,56–60]. L$_4$CL is the major CL species in the mitochondria of most mammalian tissues and it constitutes more than 70% in the heart [61]. Thus formation of 4-HNE *via* this novel mechanisms is likely important. 4-HNE alters multiple essential functions of brain mitochondria, which plays a pivotal role in initiation and progression of neurodegenerative diseases [62]. 4-HNE also induces mitochondrial dysfunction and aberrant axonal outgrowth in adult sensory neurons that mimics features of diabetic neuropathy [63].

Modulation of the formation of 4-HNE and other lipid electrophiles from CL oxidation in mitochondria may lead to novel approaches to control the cytotoxic activities of these compounds. A number of strategies have been explored to modulate the oxidation of CL in mitochondria [64]. The peroxidase activity of cytochrome c and CL can be activated by hydrogen peroxide or lipid hydroperoxides [65]. Thus disruption of this interaction by scavenging H$_2$O$_2$ or reducing lipid hydroperoxides may lead to the inhibition of apoptosis. Overexpression of phospholipid hydroperoxide glutathione peroxidase (PHGPX) in

mitochondria of RBL2H3 cells prevented the release of cyt c, activation of caspase-3, and apoptosis caused by 2-deoxyglucose (2DG), whereas cells overexpressing nonmitochondrial PHGPx were prone to apoptosis [51,66]. Furthermore, liver apoptosis and cytochrome c release were suppressed in transgenic mice overexpression of this enzyme when mice were treated with diquat to induce apoptosis. Levels of cardiolipin oxidation products in these transgenic animals was also suppressed compared to the wild type animals [67]. Reduction of mitochondrial hydrogen peroxide by overexpressing mitochondria specific peroxiredoxin 3 (Prdx3/Prx3) led to the reduced levels of F_2-IsoPs, a widely accepted standard for oxidative stress *in vivo* [68], and 4-HNE [69]. Lastly, some antioxidants may also have inhibitory effects on the formation of these reactive lipid aldehydes if they can be incorporated into the cytochrome c/CL peroxidase complex and compete with the intramolecular hydrogen atom abstraction or addition to the double bonds.

Alternative approach to mitigate the detrimental effects of 4-HNE and related compounds may involve in stimulation of the deactivation mechanism for these compounds [70]. Aldehyde dehydrogenase-2 (ALDH2) is a mitochondria-specific enzyme that is involved in oxidation of 4-HNE to its acid analogue. A recent study demonstrated that ischemic damage to the heart was significantly reduced by activation of ALDH2 using a small molecule agonist Alda-1 [71,72]. Moreover, increasing evidence suggested that mitochondrial detoxification pathways for 4-HNE play a significant role in delay of the development of AD and other neurodegenerative diseases [62].

In summary, we have provided evidence that 4-HNE and related reactive lipid electrophiles can be generated through a novel chemical mechanism of cardiolipin oxidation under free radical conditions *in vitro* and *in vivo*. This mechanism links 4-HNE generation to cardiolipin oxidation in mitochondria where abundant ROS are generated. Biological studies are under way to investigate the role of CL oxidation and 4-HNE formation in mitochondria function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ALDH	aldehyde dehydrogenase
AD	Alzheimer's disease
C-0	a water soluble azo initiator
CID	collision-induced dissociation
CL	cardiolipin
DHA	docosahexaenoic acid
DLPC	1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine
DNPH	2,6-dinitrophenylhydrazine
EPA	eicosapentaenoic acid
ESI	electrospray

GST	glutathione S-transferase
4-HNE	4-hydroxy-2-nonenal
4-HPNE	4-hydroperoxy-2-nonenal
HPLC	high performance liquid chromatography
HODE	hydroxyoctadecadienoic acid
HPODE	hydroperoxyoctadecadienoic acid
HETE	hydroxyeicosatetraenoic acid
IsoP	isoprostanes
L$_4$CL	tetralinoleoyl cardiolipin
MeOAMVN 2	2′-azobis(4-methoxy-2,4-dimethylvaleronitrile
MS	mass spectrometry
O$_4$CL	tetraoleoyl cardiolipin
4-ONE	4-oxo-2-nonenal
PLPC	1-palmitoyl-2-linoleoyl-phosphatidylcholine
POPC	1-palmitoyl-2-oleoyl-phosphatidylcholine
PC	phosphatidylcholine
PE	phosphotidylethanolamine
PHGPX	phospholipid hydroperoxide glutathione peroxidase
Prdx	peroxiredoxin
PD	Parkinson disease
PPh$_3$	triphenylphosphine
ROS	reactive oxygen species
SRM	selective reaction monitoring
UPLC	ultra pressure liquid chromatography

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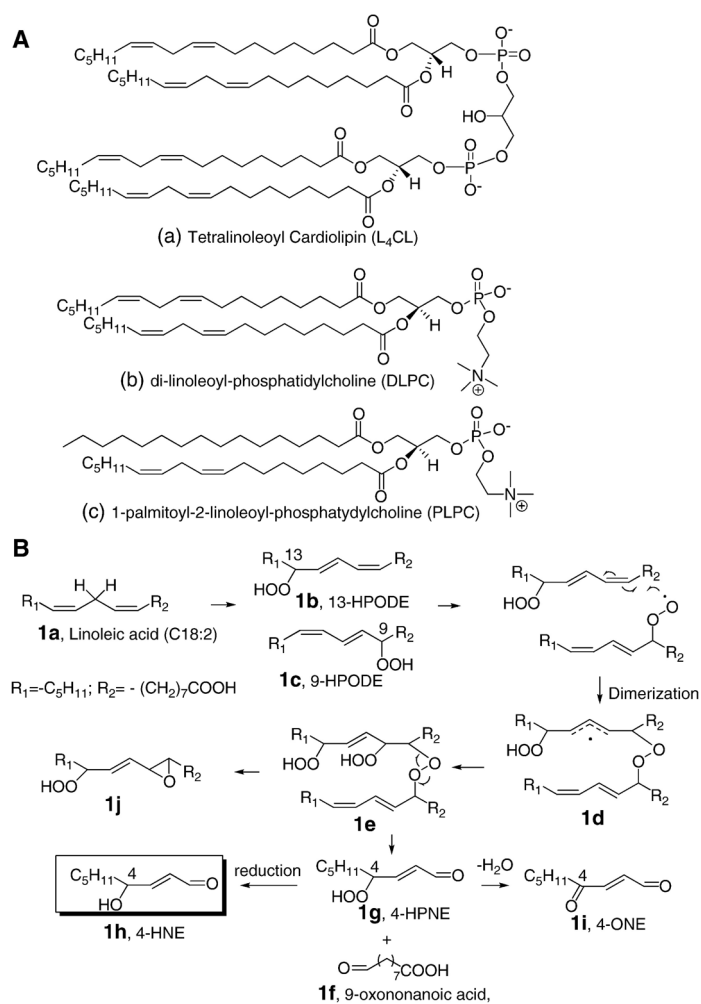
Fig. 1. Chemical structures of linoleoyl-phospholipids and proposed chemical mechanism for 4-HNE formation

(A) Chemical structures of L₄CL, DLPC, and PLPC. (B) Formation of 4-HNE from dimeric peroxides of two linoleic acids under free radical conditions. Note: other isomers of **1e** can be formed during the process, but only the structures that are relevant to the proposed mechanism are illustrated for simplicity.
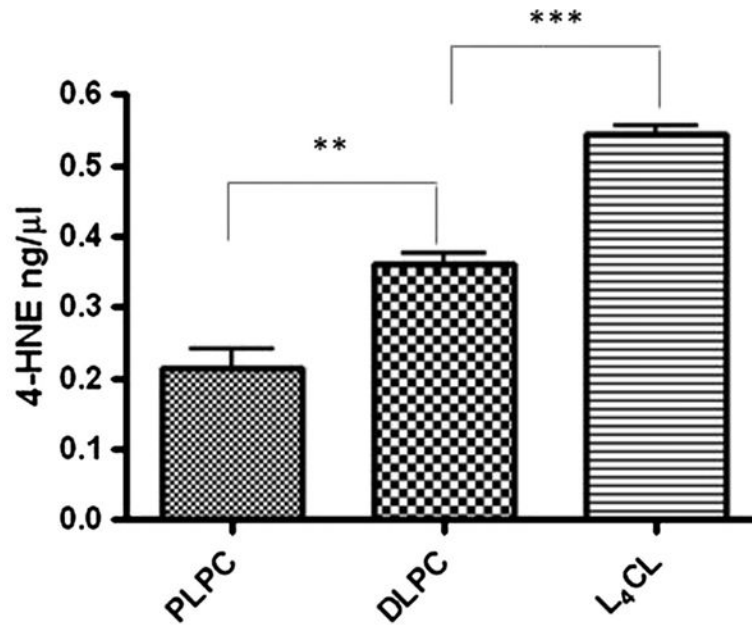
Fig. 2. Levels of 4-HNE from free radical-induced oxidation of PLPC, DLPC and L₄CL
The reaction was carried out with 0.15, 0.3, 0.6 mM of L_4CL, DLPC and PLPC respectively in 14.4 mM of POPC. Analysis of 4-HNE was performed by LC-MS after derivatization with DNPH. **, $p< 0.01$; ***, $p< 0.001$. The phospholipids were oxidized using free radical initiator but similar results were observed when the oxidation was carried out using cytochrome c and H_2O_2.
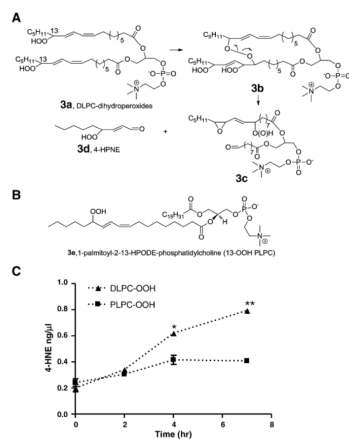
Fig 3. Formation of 4-HNE from phospholipid hydroperoxides
(A) Pathways for 4-HNE formation from oxidation of DLPC hydroperoxides *via* cross-linked two side chains. (B) Chemical structure of 13-HPODE-PLPC. (C) Time course of 4-HNE formation from decomposition of DLPC and PLPC hydroperoxides. *, p< 0.05; **, p< 0.01, relative amounts of 4-HNE from DLPC vs PLPC. The phospholipid hydroperoxides were synthesized by soy bean lipoxygenase and further oxidation was carried out using free radical initiator.
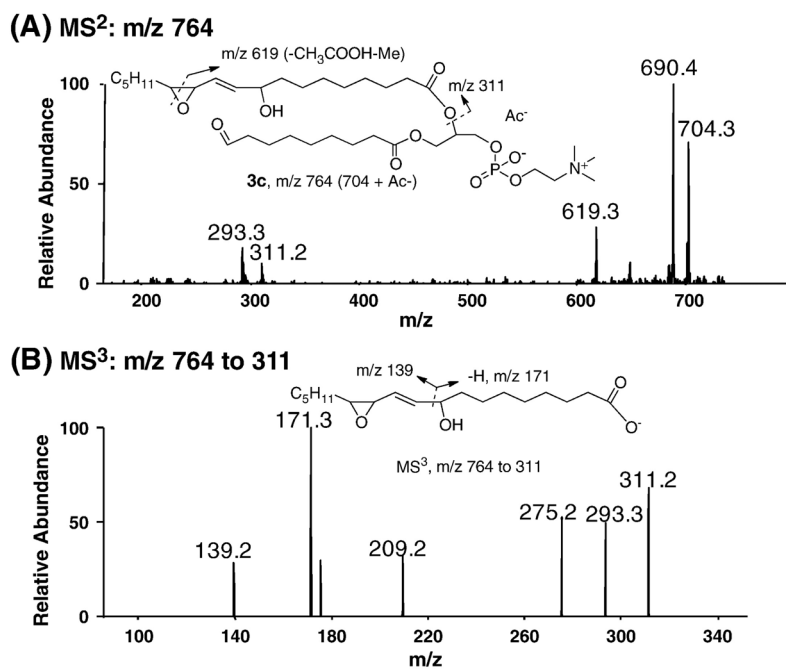
Fig 4. Mass spectra of epoxyalcohol 3c from decomposition of DLPC hydroperoxides analyzed by LC-Iontrap MS

(A) MS^2 of acetate adduct of compound **3c**, m/z 764. (B) MS^3 of m/z 764 to 311. The oxidation mixture used was from the experiment described in Figure 3.
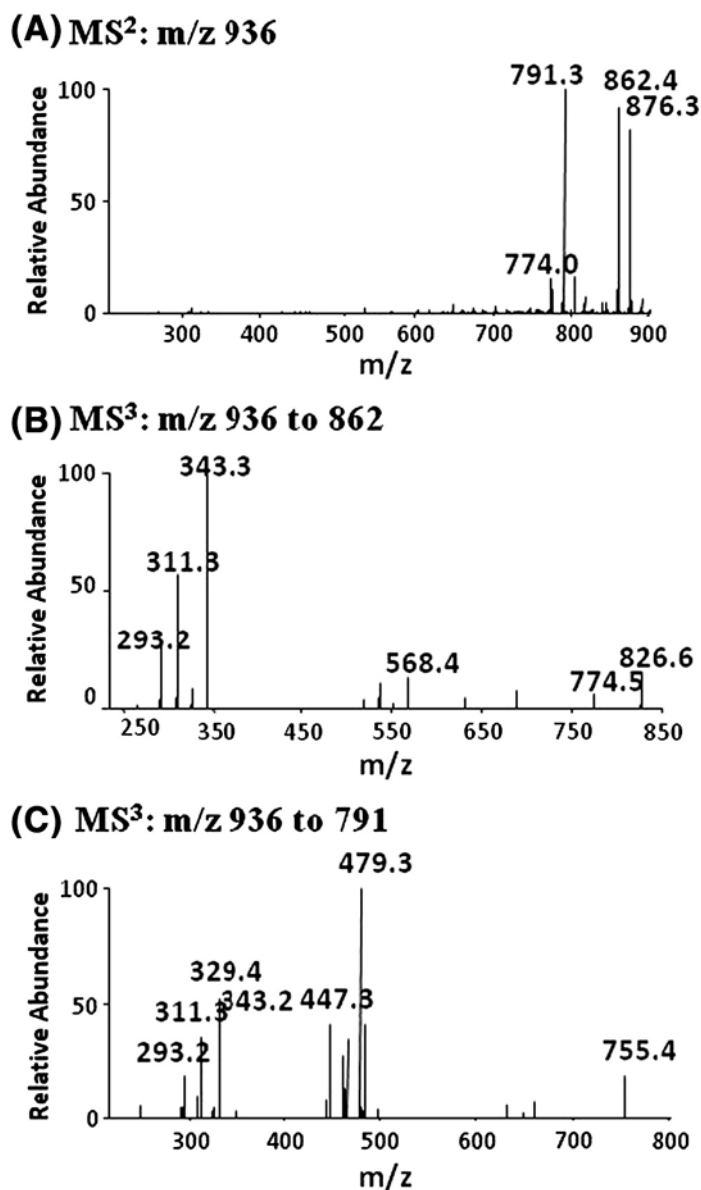
Fig. 5. Mass spectra of intact cross-linked peroxide 3b analyzed by LC-Iontrap MS
(A) MS^2 of acetate adduct of compound **3b**, m/z 936. (B) MS^3 of m/z 936 to 862. (C) MS^3 of m/z 936 to 791. The oxidation mixture used was from the experiment described in Figure 3.
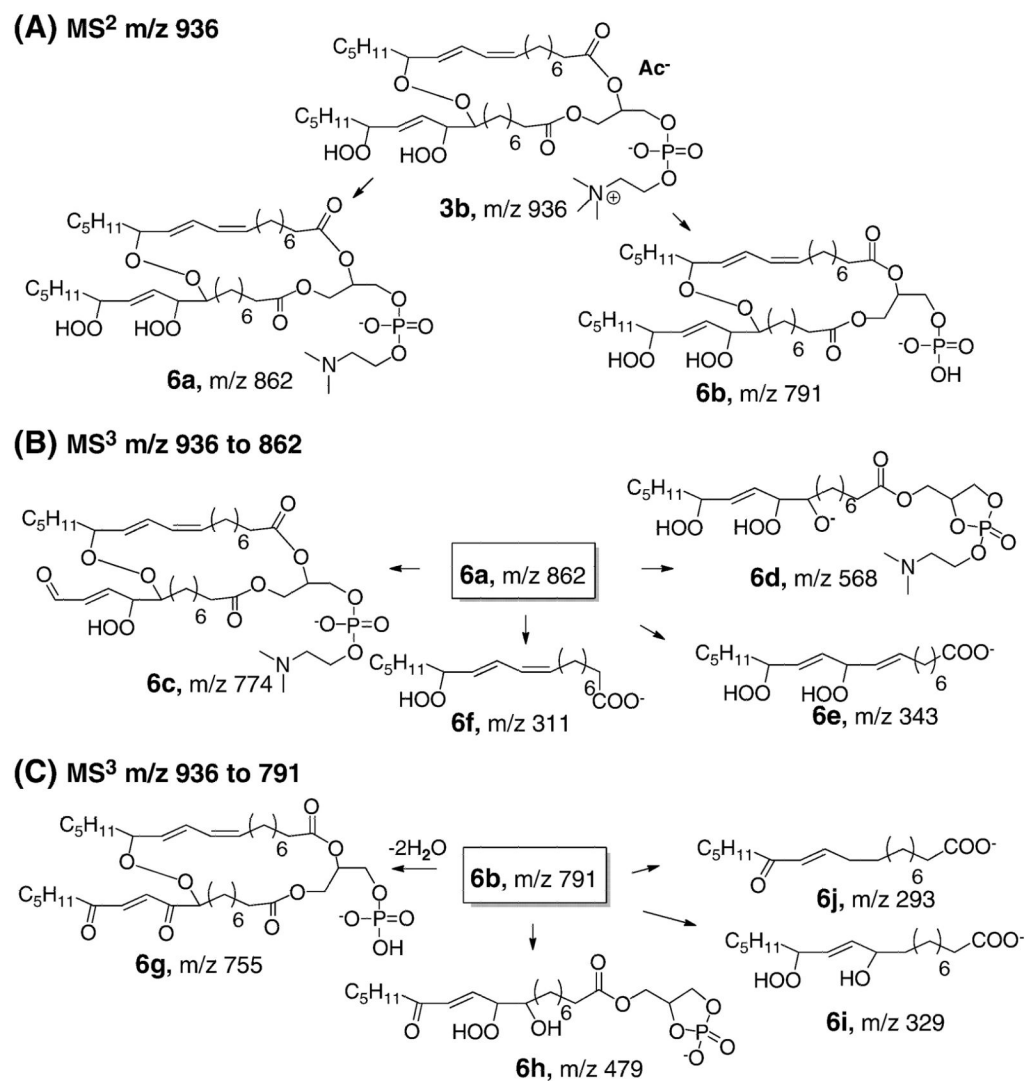
(A) MS² m/z 936

3b, m/z 936

6a, m/z 862

6b, m/z 791

(B) MS³ m/z 936 to 862

6a, m/z 862

6c, m/z 774

6d, m/z 568

6f, m/z 311

6e, m/z 343

(C) MS³ m/z 936 to 791

6b, m/z 791

6g, m/z 755

6h, m/z 479

6j, m/z 293

6i, m/z 329

Fig 6. Fragmentation pathways of multi-stage CID experiments for the intact cross-chain peroxide 3b

(A) MS² of m/z 936: m/z 876 represents the loss of acetic acid whereas fragments with m/z 862 and 791 are further loss of methyl group on the choline head group (**6a**) and complete loss of the head group (**6b**) respectively. **(B) MS³ of m/z 862**: m/z 774 (**6c**) is derived from the cleavage of the tail end of the fatty acid whereas a peak with m/z 568 (**6d**) is consistent with a fragment having one of the side chain and head group attached; m/z 343 (**6e**) and m/z 311(**6f**) are consistent with the cleavage of the cross-linked peroxyl bond. **(C) MS³ on m/z 936 to 791**: m/z 755 may be due to the loss of two molecules of water from **6b**; m/z 479 (**6h**) is derived from the loss of one of the side chain when the phosphate anion cyclizes and removes one of the side chains followed by dehydration; m/z 329 and 293 are indicative of the proposed side chains. The oxidation mixture used was from the experiment described in Figure 3.
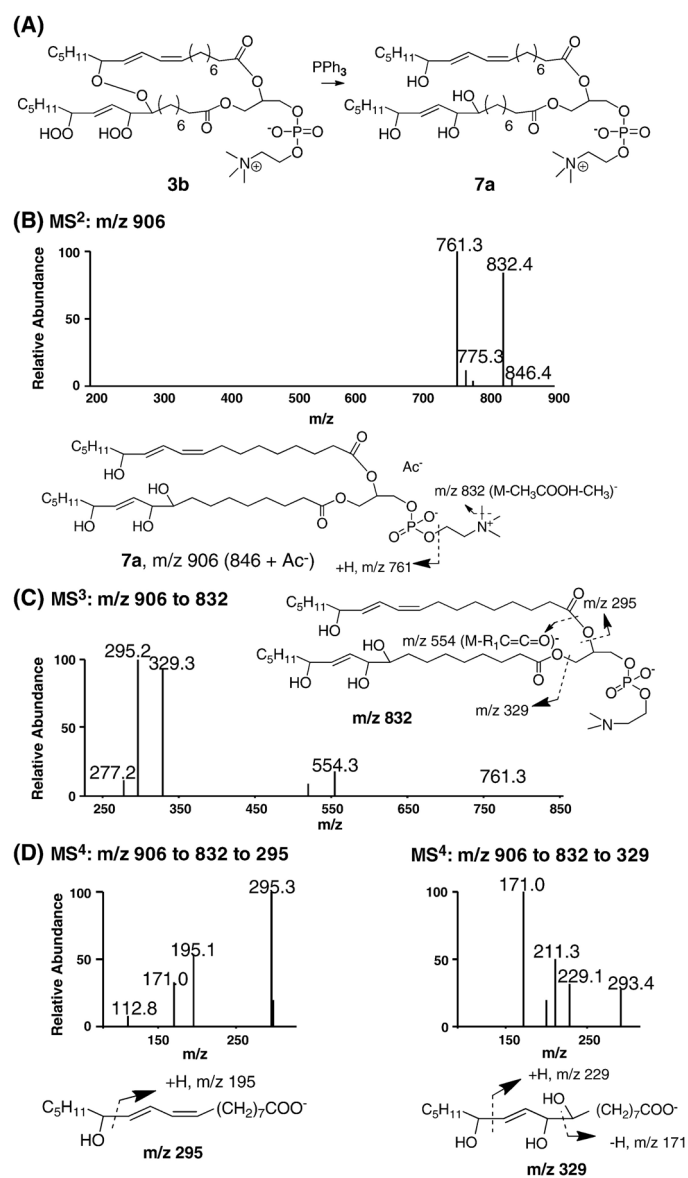
Fig. 7. Characterization of the reduced cross-chain peroxides 7a by LC-Iontrap MS
(A) Reduction of compound **3b** by PPh₃ leads to a more stable compound **7a**. (B) MS^2 of acetate adduct of compound **7a**, m/z 906. (C) MS^3 of m/z 906 to 832. (D) MS^4 of m/z 906 to 832 to 295; MS^4 of m/z 906 to 832 to 329. In addition to the characteristic fragments of m/z 229 and 171, m/z 293 represents loss of two molecules of water from m/z 329 while m/z 211 is from dehydration of m/z 229. The oxidation mixture used was from the experiment described in Figure 3.
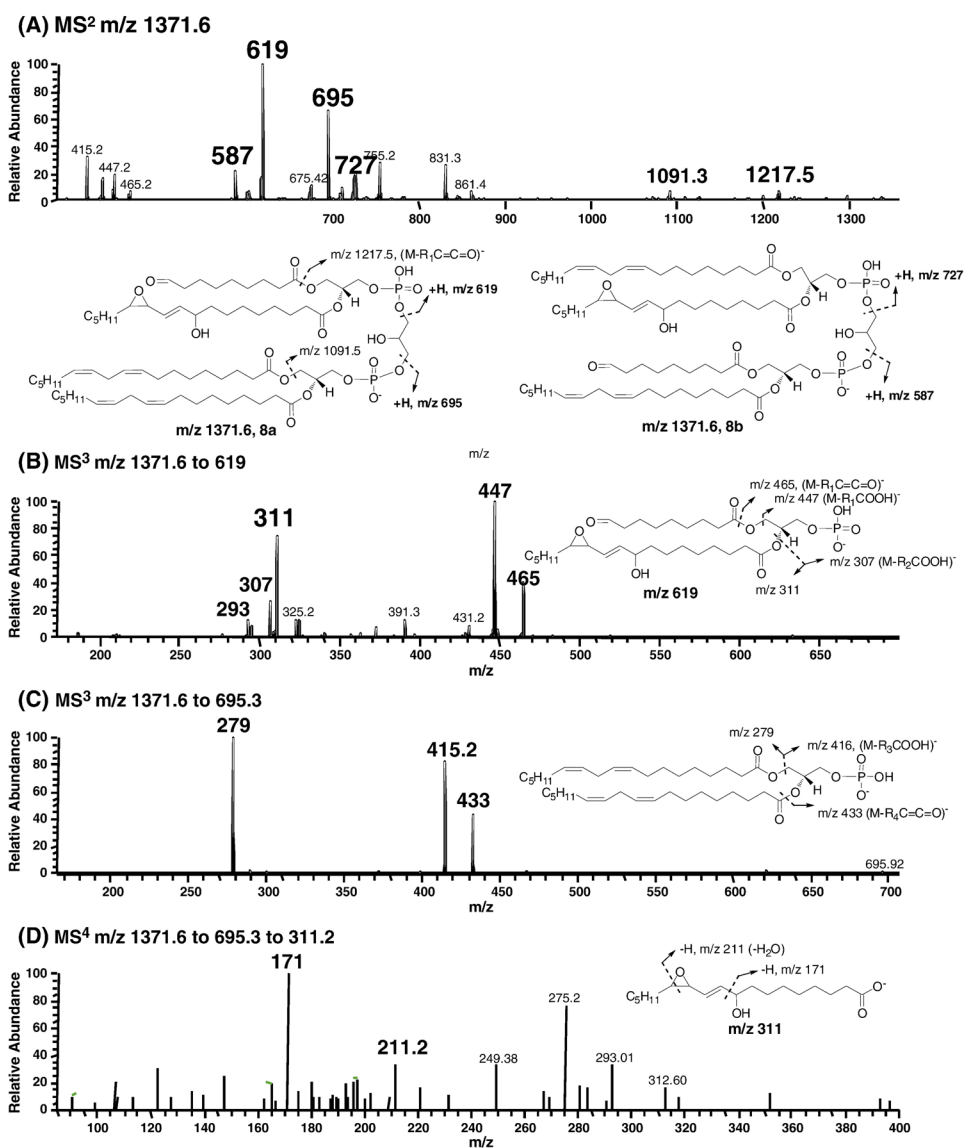
Fig. 8. Characterization of the key product of epoxyalcohol from L$_4$CL oxidation by LC-Iontrap MS

(A) MS^2 of m/z 1371.6. The oxidized two side chains can be on the same glycerol backbone (**8a**) or different glycerol backbone (**8b**). Besides the common fragments such as m/z 1217 and 1091, fragments m/z 619 and 695 are characteristic of **8a** while m/z 587 and 727 are from **8b**. (B) MS^3 of m/z 1371.6 to 619. (C) MS^3 of m/z 1371.6 to 695. (D) MS^4 of m/z 1371.6 to 619 to 311. L$_4$CL was oxidized using cytochrome c and H$_2$O$_2$ as described in details in the experimental section.
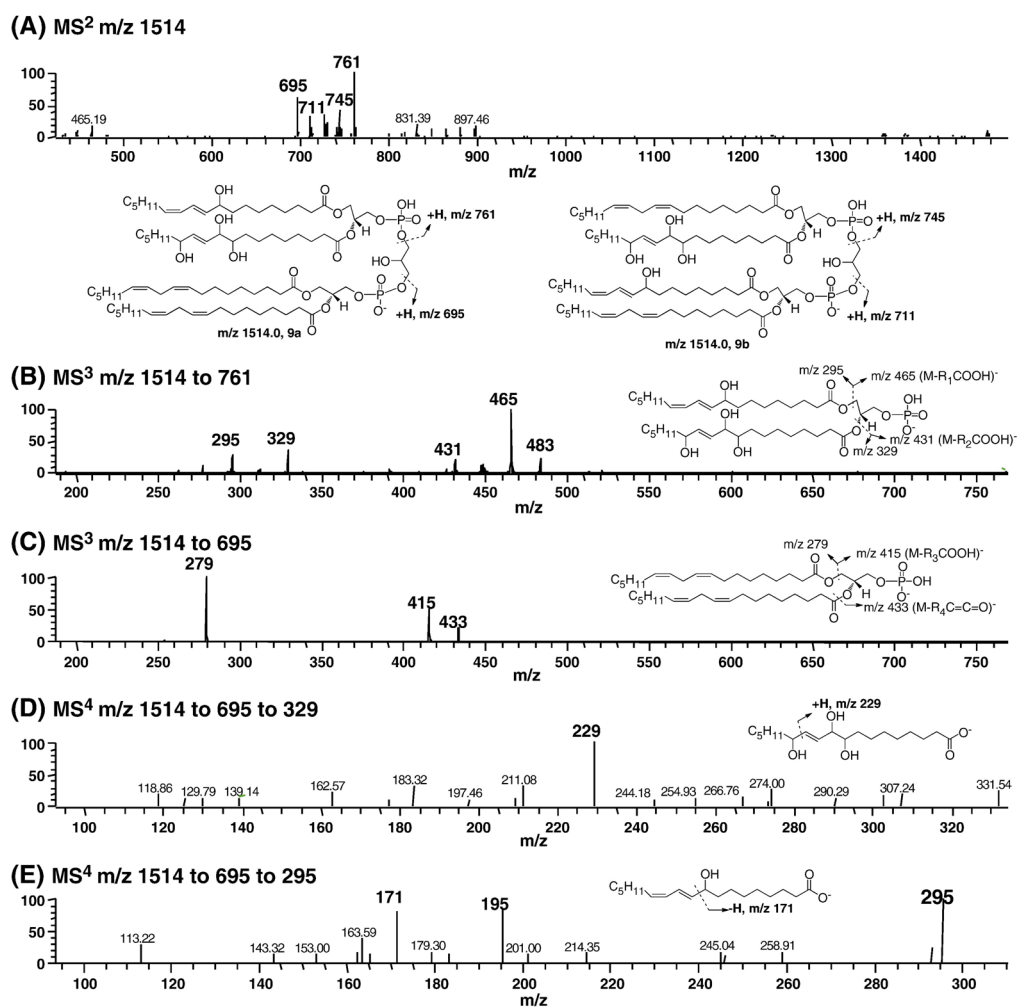
Fig. 9. Characterization of reduced cross-chain peroxide derived from oxidation of L$_4$CL by LC-Iontrap MS

(A) MS^2 of m/z 1514. The oxidized two side chains can be on the same glycerol backbone (**9a**) or different glycerol backbone (**9b**). Fragments m/z 761 and 695 are characteristic of **9a** while m/z 711 and 745 are from **9b**. (B) MS^3 of m/z 1514 to 761, the fragments of m/z 465, 431, 295, and 329 indicate the presence of a hydroxyl group on one side chain and three hydroxyl groups on a neighboring side chain. (C) MS^3 of m/z 1514 to 695, the fragments with m/z 279, 415, and 433 suggest two intact linoleoyl side chains. (D) MS^4 of m/z 1514 to 761 to 329. (E) MS^4 of m/z 1514 to 761 to 295, m/z 171 and 195 are characteristic of 9- and 13-HODE respectively. L$_4$CL was oxidized using cytochrome c and H$_2$O$_2$ as described in details in the experimental section.
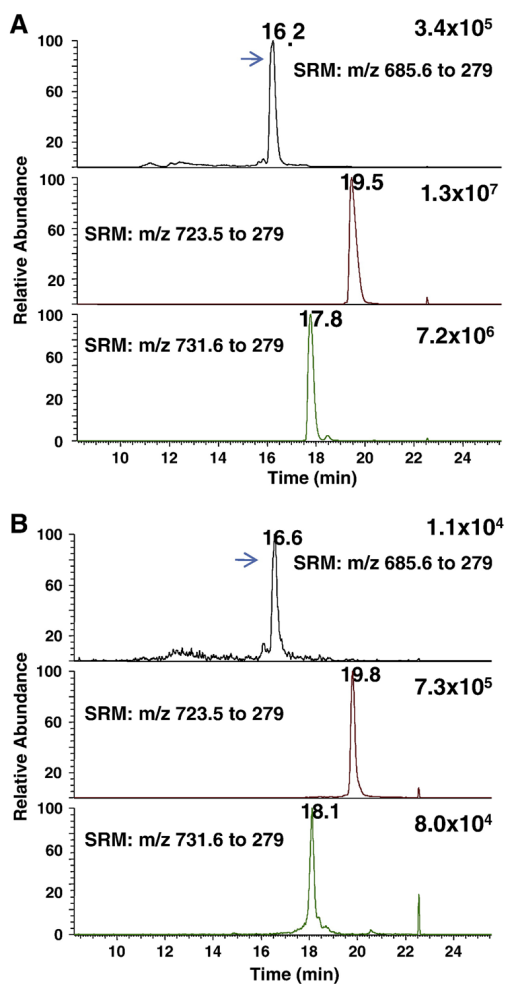
Fig. 10. Identification of the key oxidation product (compound 8a) of cardiolipin from the novel mechanism *in vivo*

(A) In vitro oxidation of L_4CL by cytochrome c/H_2O_2. (B) Rat liver extracts after CCl_4 treatment. SRM transitions: m/z 685.6 to 279, Compound **8a**; L_4CL, m/z 723.5 to 279; primary oxidation product with the addition of one oxygen, m/z 731.6 to 279. The experiments were performed on a triple quadruple MS in the negative ion mode coupled to reverse phase LC separation. The arrows indicate compound **8a**. See experimental section for details.