Research Paper

Formation of electrophilic oxidation products from mitochondrial cardiolipin in vitro and in vivo in the context of apoptosis and atherosclerosis

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Emerging evidence indicates that mitochondrial cardiolipins (CL) are prone to free radical oxidation and this process appears to be intimately associated with multiple biological functions of mitochondria. Our previous work demonstrated that a significant amount of potent lipid electrophiles including 4-hydroxy-nonenal (4-HNE) was generated from CL oxidation through a novel chemical mechanism. Here we provide further evidence that a characteristic class of CL oxidation products, epoxyalcohol-aldehyde-CL (EAA-CL), is formed through this novel mechanism in isolated mice liver mitochondria when treated with the pro-apoptotic protein t-Bid to induce cyt c release. Generation of these oxidation products are dose-dependently attenuated by a peroxidase inhibitor acetaminophen (ApAP). Using a mouse model of atherosclerosis, we detected significant amount of these CL oxidation products in liver tissue of low density lipoprotein receptor knockout (LDLR−/−) mice after Western diet feeding. Our studies highlight the importance of lipid electrophiles formation from CL oxidation in the settings of apoptosis and atherosclerosis as inhibition of CL oxidation and lipid electrophiles formation may have potential therapeutic value in diseases linked to oxidant stress and mitochondrial dysfunctions.

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Introduction

Cardiolipin (CL) is a class of phospholipids that contain four fatty acid side chains and three glycerol backbones in the same molecule. CLs are a structurally unique class of phospholipids as phospholipids commonly have two fatty acids and one glycerol unit. CLs primarily reside in the inner membrane of mitochondria and are critical for maintaining the structural integrity of mitochondrial membranes and functions of multiple protein complexes in the electron transport chain (ETC) [1]. In most mammalian tissues, a majority of CLs contain four linoleic acid (LA, 18:2, ω-6) chains (tetralinoleoyl CL, L4CL). The fatty acid composition of CL appears to be an important factor for maintaining mitochondrial function [2]. Furthermore, mitochondrion is the major cellular source for reactive oxygen species (ROS) including superoxide and hydrogen peroxide (H2O2). Incorporation of four LA side chains in CLs and their association with mitochondria render CL to be readily oxidized by ROS [3]. The mechanisms for this seemingly paradoxical combination remain to be elucidated. Cardiolipin oxidation has attracted increased research attention in recent years and emerging evidence shows that it is critically involved in regulation of apoptosis [4], mitochondrial dysfunction, mitophagy [5], and several human diseases [6]. Upon apoptotic stimulation, CL interacts with cytochrome c (cyt c) to form a peroxidase complex that catalyzes CL oxidation. Accumulating
evidence indicates that these oxidation products of CL play an important role in the mitochondrial stage of the cell death program [1,7].

Oxidative stress-induced lipid peroxidation has been linked to numerous human diseases including atherosclerosis [8,9]. Polyunsaturated fatty acids (PUFAs) such as LA in cell membranes are the primary targets for free radicals attack [8]. Lipid peroxidation generates an array of oxidation products and one class of these products, reactive lipid electrophiles, is increasingly recognized as an important lipid mediator due to its potential to alter protein structure and functions by covalent modification of certain critical nucleophilic amino acid residues. 4-Hydroxy-2-nonenal (4-HNE) is one of the most studied reactive lipid electrophiles and it can trigger multiple signaling pathways in different biological processes [10]. Elevated levels of 4-HNE were observed in atherosclerotic lesions and liver tissues and were positively correlated with cell death in these tissues [11,12]. In contrast to the well-studied pleiotropic biology of 4-HNE, mechanisms responsible for 4-HNE formation in vivo are much less understood [13,14]. Our previous in vitro work demonstrated that oxidation of L4CL by cyt c and H2O2 led to the formation of 4-HNE and other oxidation products via a novel chemical mechanism that involved cross-chain peroxy radical addition and decomposition [16]. As shown in Fig. 1, oxidation of L4CL by the peroxidase activity of cyt c and CL complex in the presence of H2O2 results in the formation of hydroperoxyoctadecadienoic acid (HpODE). HpODE can be reduced to form hydroxyoctadecadienoic acid (HOODE) or dehydrated to form keto-octadecadienoic acid (KODE). During this process, through intra-molecular peroxy radical addition and decomposition of an unstable intermediate, several reactive aldehydes are produced including epoxyalcohol-aldehyde-CL (EAA-CL) (1 d), 4-HNE (1e), and 4-oxo-2-nonenal (4-ONE) (1f). 4-HNE and 4-ONE are diffusible electrophiles that can reach protein targets far away from the generation sites but EAA-CL most likely targets only closely associated proteins. Moreover, the formation of this unique EAA-CL can serve as a footprint for this novel chemical mechanism in vivo. However, the biological relevance of these lipid electrophiles EAA-CL from CL oxidation remains to be defined.

In this study, we provide mass spectrometry (MS) evidence for this class of characteristic bioactive lipid electrophiles EAA-CL from cardiolipin oxidation in the context of apoptosis using t-Bid induced cyt c release in isolated mice liver mitochondria. Interestingly, we observed dose-dependent inhibition of CL oxidation and formation of EAA-CL by a widely used analgesic drug acetaminophen (ApAP) that also served as a peroxidase inhibitor [15]. Furthermore, the very mechanism appeared to operate in vivo in the settings of atherosclerosis in a mice model of Western diet-induced atherosclerosis using low density lipoprotein receptor knockout (LDLR −/−) mice. We found significant amounts of EAA-CL present in the mice liver after Western diet feeding. Taking these data together, our current study shed light on CL oxidation and lipid electrophiles production relevant to apoptosis and atherosclerosis, with inhibition of this process appearing to be a viable strategy for preventing cyt c release and apoptosis.

Methods and materials

Materials

Phospholipids, tetralinoleoyl cardiolipin (L4CL) and tetramyristeoyl cardiolipin (M4CL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. All other chemicals were purchased from Sigma Aldrich Chemical Company (Milwaukee, WI). HPLC quality solvents, such as methanol, water, 2-propanol, and acetonitrile were purchased from Fisher Chemical (Phillipsburg, NJ) or EM Science (Gibbstown, NJ).

Animals and diets

LDLR −/− mice were originally purchased from Jackson Laboratory. At 2–3 month of age, these mice were fed a Western diet (TD 88,137, Harlan Teklad, Madison, WI) containing 0.2% cholesterol and 21.2% fat or normal chow diet for 16 weeks. At the end of 16 weeks, the mice were food-deprived for 5 h and sacrificed by an overdose of isoflurane followed by cervical dislocation. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Shanghai Institutes for Biological Sciences.

Oil Red O staining of liver sections

Oil red O staining of liver section was performed according to a literature procedure [16]. Briefly, liver samples were embedded in an optimal cutting temperature compound (Tissue-Tek, and were frozen at −20 °C. Liver sections were cut and stained with Oil Red O for 4 h. Sections were then counterstained with hematoxylin for 3 min. Images were captured using a Q-Imaging Micropublisher camera mounted on an Olympus upright microscope.

Quantification of oxidation products of CL by liquid chromatography–mass spectrometry (LC–MS)

The MS analysis of oxidized CL was performed according to our previously published methods [15,17]. After addition of 0.75% NaCl to the liver mitochondria pellet or homogenized liver tissue, the total lipids were extracted with chloroform and methanol (2:1, v:v) containing 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM triphenylphosphine. The separated organic phase was evaporated, red-suspended in methanol:acetonitrile:H2O (60/20/20, v/v/v) and stored at −80 °C until analysis by LC–MS. The extracted lipid fraction was separated online by UPLC using a Waters Acquity UPLC system (Waters Corp., Milford, MA). Mass spectrometry analysis was performed on a Thermo Quantum Ultra or Vantage triple quadrupole mass spectrometer (Thermo Scientific Inc., San Jose, CA, USA). The mass spectrometer was operated in the negative ion mode using selective reaction monitoring (SRM). Nitrogen was used as the sheath gas and auxiliary gas. The capillary temperature was set at 350 °C. The spray voltage was 4.5 kV, and the tube lens voltage was 100 V. The following

![Fig. 1. Proposed chemical mechanism for lipid electrophiles formation from L4CL oxidation. Only one regioisomer at carbon 13 (C13) is shown in the figure for simplicity.](image-url)
transitions were monitored in SRM mode: M₄CL, m/z 619.6–227.2; L₄CL, m/z 723.6–279.2; L₅OCL, m/z 724.6–279.2; HODE-L₄CL, m/z 731.6–279.2, 295.2; HODE-L₅OCL, m/z 732.6–279.2, 295.2; KODE-L₄CL, m/z 730.6–279.2, 293.2; KODE-L₅OCL, m/z 731.6–279.2, 293.2; EAA-L₄CL, m/z 685.6–279.2; EAA-L₅OCL, m/z 686.6–279.2. Data acquisition and analysis were performed using X’calibur software, version 2.0.

The area under the curve (AUC) for M₄CL, CL, KODE-CL, HODE-CL, EAA-CL and MLCL was determined and the AUC of each CL in each sample was normalized to M₄CL AUC. The corrected values were used to calculate the ratio of corresponding CL over the total CL and was expressed as percent of total CL. Data are presented as the mean ± SEM.

**t-Bid induces CL oxidation in isolated mitochondria**

Mouse liver mitochondria were isolated as previously described [15,18]. Fifteen microgram of mitochondria were incubated in 100 μl experimental buffer (250 mM sucrose, 10 mM Hepes, pH 7.4, 1 mM ATP, 5 mM succinate, 0.08 mM ADP, 1 mM dithiothreitol, and 2 mM K₂HPO₄, pH 7.4). After addition of 10 ng t-Bid, the samples were incubated for 30 min at room temperature. Mitochondria were pelleted by centrifugation at 8000 rpm for 10 min at 4 °C. The oxidation products of CL in the pellets were extracted as described above.

**ApAP inhibits CL oxidation and in isolated mitochondria**

Fifteen microgram of energized mitochondria were pre-incubated with 0–400 μM ApAP for 10 min. CL oxidation induced by t-Bid was processed as described above.

**2.7. Statistical analysis**

Results are expressed as means ± SD. Statistical analysis was performed using t tests. A probability value of p < 0.05 was considered statistically significant.

**Results**

**Formation of Lipid electrophile EAA-CL from cardiolipin oxidation in vitro during t-Bid induced cyt c release from mitochondria**

Mitochondria play a central role in cell survival and apoptosis. The apoptotic pathways are highly orchestrated by pro- and anti-apoptotic Bcl-2 superfamily protein members. BID is an abundant pro-apoptotic protein of the Bcl-2 family that is crucial for death receptor-mediated apoptosis in many cell types. It has been well established that t-Bid, a truncated form of BID, facilitates the disruption of the mitochondrial trans-membrane potential and the release of apoptogenic proteins including cyt c. During this process, t-Bid also causes the remodeling of mitochondrial cristae and the release of apoptogenic proteins including cyt c. To test the biological relevance of lipid electrophile generation from CL oxidation during apoptosis, we treated mice liver mitochondria with t-Bid to induce cyt c release and identified the production of EAA-CL using mass spectrometry (shown in Fig. 2). We performed LC–MS analysis using the collision-induced dissociation (CID) of m/z 685 (doubly charged, Fig. 2A), the putative structure of EAA-CL, in the negative ion mode. A fragment with m/z 279 in the CID spectrum (Fig. 2B) was consistent with the presence of linoleate side chains while m/z 171 and 311 represented the presence of side chains of truncated aldehyde at C9 and epoxyalcohol-aldehyde LA moiety respectively. Other fragments such as m/z 1091.5 and 307 were consistent with the proposed structure. Thus our MS data provided unambiguous evidence for the formation of this novel lipid electrophile from CL oxidation. In addition, other oxidation products such as HODE-CL were also identified by CID experiment (data not shown). We further performed quantitative analysis of the major oxidation products using multiple reaction monitoring (MRM) by monitoring the transition of each parent ion to their respective characteristic fragmentation (Fig. 2C). The MRM results clearly demonstrated the formation of multiple oxidation products including EAA-CL and HODE-CL. Their elution order on a reverse phase LC column was consistent with the polarity of each compound. Furthermore, our previous work demonstrated that ApAP served as an inhibitor of the peroxidase activity of cyt c/CL complex. We then tested the ability of ApAP to inhibit the
Formation of lipid electrophiles from CL oxidation in liver tissue of LDLR KO mice

Oxidative stress is a hallmark of atherogenesis and free radical lipid peroxidation has been associated with every stage of this disease [20]. LDLR knockout (KO) is a well-established mouse model used to study atherosclerosis. 4-HNE was identified in mouse atherosclerotic lesions and liver, and levels of 4-HNE were associated with cell death in these tissues [21]. We employed this mouse model to test if EAA-CL and other cardiolipin oxidation products were formed in the liver during the formation of atherosclerotic plagues in the aorta. Western diet feeding for 16 weeks resulted in significant atherosclerotic lesion formation in the aortas as revealed by Red Oil O staining (Fig. 3A). We analyzed the liver tissues in these animals and identified multiple oxidation products from CL oxidation including 4-HNE, EAA-CL, KODE-CL, and HODE-CL (Fig. 3B). Furthermore, levels of EAA-CL from liver tissue of Western diet group were significantly elevated compared to that from the control group. These data represent the first in vivo evidence for the formation of these novel lipid electrophiles, EAA-CL, from mitochondrial cardiolipin during atherosclerosis.

Besides the major cardiolipin species L4CL, mouse liver tissue contains other minor cardiolipins such as L3OCL. The structure of L3OCL has one of the LA side chains replaced by an oleic acid. According to our chemical mechanism of 4-HNE formation from CL, cross-chain peroxyl radical reaction occurs between two adjacent side chains; thus the presence of an unreactive oleic acid side chain in L3OCL may disrupt this reaction and lead to less lipid electrophile production through this mechanism. As shown in Fig. 4, our data showed that the formation of similar electrophiles EAA-CL from L3OCL was indeed significantly suppressed compared to those from L4CL, highlighting the importance of this cross-chain reaction during the formation of reactive electrophiles. Interestingly, formation of other major oxidation products, such as HODE-CL, from L3OCL was decreased compared to L4CL but did not reach statistical significance.

Discussion

Free radical-induced lipid peroxidation has been linked to multiple human diseases including atherosclerosis [8]. Among the complicated oxidation products, lipid electrophiles, including 4-HNE, generated from lipid oxidation have attracted increased attention due to their potential roles in altering protein structures and functions through covalent modification of critical nucleophilic amino acid residues [10,21,22]. Overwhelming evidence indicates that mitochondria play an essential role in ROS generation, lipid peroxidation, and the pleiotropic effects of 4-HNE in various biological processes. In contrast to the well-studied biology of 4-HNE, the chemical mechanisms for 4-HNE formation and cellular locations remain poorly defined [23,24]. In a previous report we proposed a novel chemical mechanism for the formation of 4-HNE and other reactive lipid aldehydes from mitochondrial cardiolipin oxidation [17]. In our current study, we provide evidence that this mechanism operates both in vitro in t-Bid induced cyt c release and in vivo in mice liver of atherosclerosis after Western diet feeding. Furthermore, formation of these bioactive lipid mediators can be dose-dependently inhibited by a widely used clinical reagent ApAP acting as a peroxidase inhibitor, corroborating with previous studies where ApAP was a potent inhibitor for peroxidase activity of cylooxygenase (COX)[25]. Hemoproteins including hemoglobin and myoglobin [26], and peroxidase of cyt c/CL complex [15]. Our findings have significant implications in linking mitochondrial lipid peroxidation and lipid electrophile production to important biological processes, such as...
apoptosis, and human diseases that are associated with oxidative stress.

The novel chemical mechanisms for the formation of 4-HNE from CL oxidation are based on the fact that the extent of oxidation for L4CL is far greater than other phospholipids. The susceptibility of L4CL to free radical oxidation has been attributed to the presence of four LA side chains in the same molecules and the rate-limiting step reaction of hydrogen atom abstraction by a peroxyl radical may occur through intra-molecular reactions. Furthermore, subsequent peroxyl radical addition reactions that form cross-chain products may be responsible for the formation of 4-HNE. A seminal work reported by Kagan et al. demonstrated that oxidation of CL by the peroxidase function of cyt c/CL complex was required to trigger intrinsic apoptotic pathways [27]. Recent studies from the same group also reported that CL oxidation was linked to traumatic brain injury [28] and mitophagy [5]. However, the role of bioactive lipid electrophiles from CL oxidation remains to be studied. Our studies suggest that 4-HNE formation from this process may also play a significant role in these events because 4-HNE has been shown to induce apoptosis and autophagy [10,29,30]. Moreover, it is well established that 4-HNE plays an important role in cardiovascular diseases. On one hand, 4-HNE directly suppresses contractile function, enhances ROS generation, and modulates multiple signal transduction pathways that are known to contribute to atherosclerosis, myocardial ischemia-reperfusion injury, heart failure, and cardiomyopathy [21]. On the other hand, levels of circulating anti-cardiolipin antibody are positively correlated with endothelial dysfunction and atherosclerosis. It is interesting to note that the anti-cardiolipin antibody actually recognized oxidized CL [31]. Formation of these epitopes is most likely through covalent modifications of immune responsive proteins by lipid electrophiles generated from CL oxidation [32]. Moreover, L4CL is the major CL species in the mitochondria of most mammalian tissues and it constitutes more than 70% of mitochondrial CLs in the heart [33]. Thus formation of 4-HNE via this novel mechanism is likely important in the context of cardiovascular diseases. Our current study clearly demonstrates that formation of 4-HNE from CL operates in vivo and has significant pathophysiological relevance with the detection of the characteristic product EAA-CL and other CL oxidation products (Fig. 3). This mechanism is further substantiated by the observation that the presence of only one un-oxidized fatty acid (such as oleic acid) in some minor cardiolipin species significantly decreases the formation of this characteristic bioactive lipid and other oxidation products such as HODE-CL (Fig. 4).

Modulating the formation of 4-HNE and other lipid electrophiles from CL oxidation in mitochondria may represent a viable approach to attenuate the cytotoxicity of these bioactive lipid electrophiles. Several strategies have been explored to inhibit the oxidation of CL in mitochondria [34]. The peroxidase activity of cyt c/CL complex is activated by hydrogen peroxide or lipid hydroperoxides; thus scavenging H2O2 or reducing lipid hydroperoxides may lead to the inhibition of apoptosis [35]. In fact, liver apoptosis and cyt c release were suppressed in transgenic mice overexpressing phospholipid hydroperoxide glutathione peroxidase (PHGPX), the enzyme that reduces lipid hydroperoxides. Levels of cardiolipin oxidation products in these transgenic animals were also suppressed compared to the wild type animals [36]. Furthermore, reduction of mitochondrial hydrogen peroxide by overexpressing mitochondria-specific peroxiredoxin 3 (Prdx3/Prx3) led to reduced levels of F2-IsopS, a widely accepted standard for oxidative stress in vivo [37,38], and 4-HNE [39]. Inhibition of the peroxidase activity of cyt c/CL complex represents a novel approach for controlling CL oxidation and its related consequences. ApAP is one of the most widely used analgesics. Previous studies show that it inhibited peroxidase activity of COX by reducing the protoporphyrin radical cation in the peroxidase active site of the enzyme, thereby blocking formation of the catalytic tyrosyl radical in the cyclooxygenase site [25,40]. Later studies demonstrated that ApAP could protect the kidney from oxidative damage following rhadomyolysis by inhibiting the peroxide-driven lipid peroxidation catalyzed by myoglobin and hemoglobin [41]. We reported recently that ApAP inhibited cyt c reox cycling-induced lipid peroxidation [15]. In current studies, in addition to suppression of the CL oxidation products such as HODE-CL, lipid electrophiles including EAA-CL are also significantly inhibited during t-Bid induced cyt c release (Fig. 2). It is still unclear, however, how the phospholipid containing lipid electrophiles, such as EAA-CL, are metabolized or repaired. The metabolic pathways for these novel phospholipid electrophiles and their biological activities are currently under investigation.

In summary, we provide evidence that, in addition to 4-HNE, a novel class of CL containing lipid electrophiles, EAA-CL, are generated from mitochondrial CL oxidation in vitro and in vivo in the context of apoptosis and atherosclerosis. The widely used clinical reagent ApAP can inhibit CL oxidation and generation of these lipid electrophiles. This study suggests a potential link between mitochondrial CL oxidation, lipid electrophile generation, mitochondrial dysfunction, and apoptosis during the progression of atherosclerosis.

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