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## Review Article

## Role of lipid peroxidation derived 4-hydroxynonenal (4-HNE) in cancer: Focusing on mitochondria

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## ABSTRACT

Oxidative stress-induced lipid peroxidation has been associated with human physiology and diseases including cancer. Overwhelming data suggest that reactive lipid mediators generated from this process, such as 4-hydroxynonenal (4-HNE), are biomarkers for oxidative stress and important players for mediating a number of signaling pathways. The biological effects of 4-HNE are primarily due to covalent modification of important biomolecules including proteins, DNA, and phospholipids containing amino group. In this review, we summarize recent progress on the role of 4-HNE in pathogenesis of cancer and focus on the involvement of mitochondria: generation of 4-HNE from oxidation of mitochondria-specific phospholipid cardiolipin; covalent modification of mitochondrial proteins, lipids, and DNA; potential therapeutic strategies for targeting mitochondrial ROS generation, lipid peroxidation, and 4-HNE.

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## Contents

Introduction	194
Formation and catabolism of 4-HNE in mitochondria	195
4-HNE mitochondrial protein adducts in cancer	196
4-HNE mitochondrial DNA adducts in cancer	197
4-HNE mitochondrial lipid adducts in cancer	197
Therapeutic strategies for manipulation of ROS and lipid peroxidation in cancer: implication for mitochondrial 4-HNE	197
Conclusions	198
Acknowledgements	198
References	198

**Abbreviations:** 4-HNE, 4-hydroxy-2-alkenals; 4-ONE, 4-oxo-2-nonenal; 8-oxo-dG, 8-oxoguanine; AA, arachidonic acid; ADH, alcohol dehydrogenases; AKRs, aldo-keto reductases; ALDH, aldehyde dehydrogenases; ALDH2, aldehyde dehydrogenase 2 family(mitochondrial); As<sub>2</sub>O<sub>3</sub>, arsenic trioxide; ATP5B, ATP synthase subunit β; CL, cardiolipin; DHA, docosahexaenoic acid; DHN, 1,4-dihydroxy-2-nonenone; DOX, doxorubicin; EAA-CL, epoxyalcohol-aldehyde-cardiolipin; ETC, electron transport chain; FapyA, 4,6-diamino-5-formamidopyrimidine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; GSH, glutathione; GS-HNE, glutathionyl-HNE; GSTs, glutathione-S-transferases; HADHA, trifunctional enzyme subunit α; HNA, 4-hydroxy-2-nonenic acid; HODE, hydroxyoctadecadienoic acid; HpODE, hydroperoxyoctadecadienoic acid; IMM, inner membrane of mitochondria; KODE, keto-octadecadienoic acid; L<sub>4</sub>CL, tetralinoleoyl cardiolipin; LA, linoleic acid; LPO, lipid peroxidation; mtDNA, mitochondrial DNA; NDUFS2, NADH dehydrogenase [ubiquinone] iron-sulfur protein 2; OMM, outer membrane of mitochondria; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SDHA, succinate dehydrogenase [ubiquinone] flavoprotein subunit; UADT, upper aero digestive track; UCPs, mitochondrial uncoupling proteins

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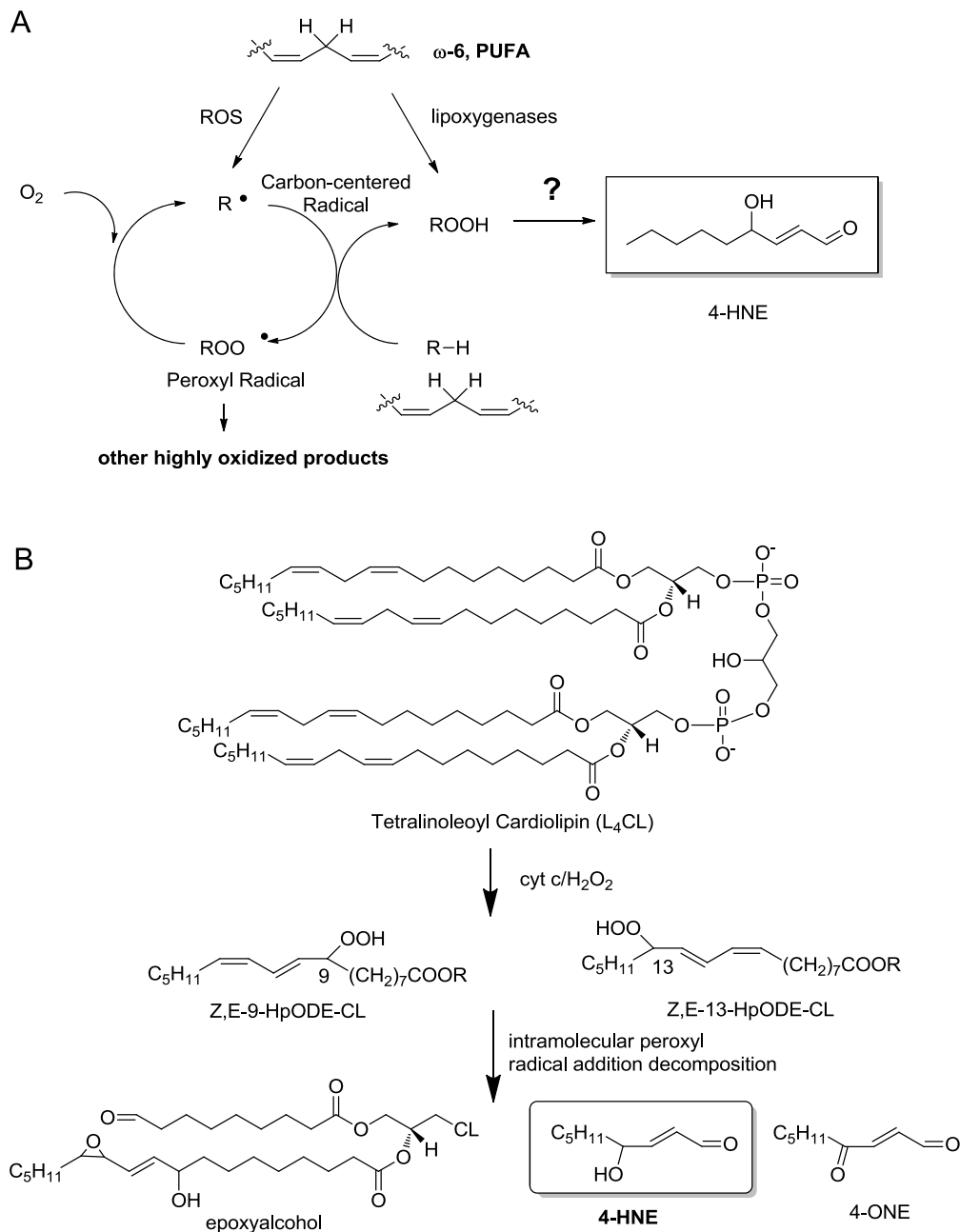
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## Introduction

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radicals, singlet oxygen, and lipid peroxyl radicals, are ubiquitous and considered as byproducts of aerobic life [1]. Most of these chemically reactive molecules are short-lived and react with surrounding molecules at the site of formation while some of the more stable molecules diffuse and cause damages far away from their sites of generation. Overproduction of these ROS, termed oxidative stress, may provoke oxidation of polyunsaturated fatty acids (PUFAs) in cellular membranes through free radical chain reactions and form lipid hydroperoxides as primary products [2]; some of these primary oxidation products may decompose and lead to the formation of

reactive lipid electrophiles. Among these lipid peroxidation (LPO) products, 4-hydroxy-2-nonenals (4-HNE) represents one of the most bioactive and well-studied lipid alkenals [3]. 4-HNE can modulate a number of signaling processes mainly through forming covalent adducts with nucleophilic functional groups in proteins, nucleic acids, and membrane lipids. These properties have been extensively summarized in some excellent reviews [4–10].

Mitochondria are vital for cellular bioenergetics and regarded as the major cellular site for ROS production [11]. Our previous works demonstrated that mitochondria are also an important site for 4-HNE formation [12,13]. Furthermore, it has been well documented that oxidative stress and ROS generation are intimately associated with cancer [14,15]. Accumulating data suggest that mitochondrial macromolecular adducts from 4-HNE are involved



**Fig. 1.** Chemical mechanisms for 4-HNE formation from lipid peroxidation. (A) General scheme for the formation of 4-HNE from decomposition of lipid hydroperoxides that can be generated from free radical oxidation of  $\omega$ -6 PUFA or enzymatic oxidation by lipoxygenases. (B) Lipid electrophiles generated from oxidation of mitochondrial cardiolipin: oxidation of L<sub>4</sub>CL by the peroxidase activity of cyt c and CL complex in the presence of H<sub>2</sub>O<sub>2</sub> results in the formation of hydroperoxyoctadecadienoic acid (HpODE), 9-HpODE-CL and 13-HpODE-CL. 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), 4-HNE, and 4-oxo-2-nonenal (4-ONE).

in the initiation and progression of cancer [16,17]. In this review, we summarize the recent progress on understanding the role of 4-HNE in cancer and focus on the involvement of mitochondria: formation of 4-HNE from oxidation of cardiolipin, covalent modification of mitochondrial biomolecules including proteins, DNA and lipids, and therapeutic targeting the mitochondrial pathways induced by 4-HNE in the context of cancer pathogenesis.

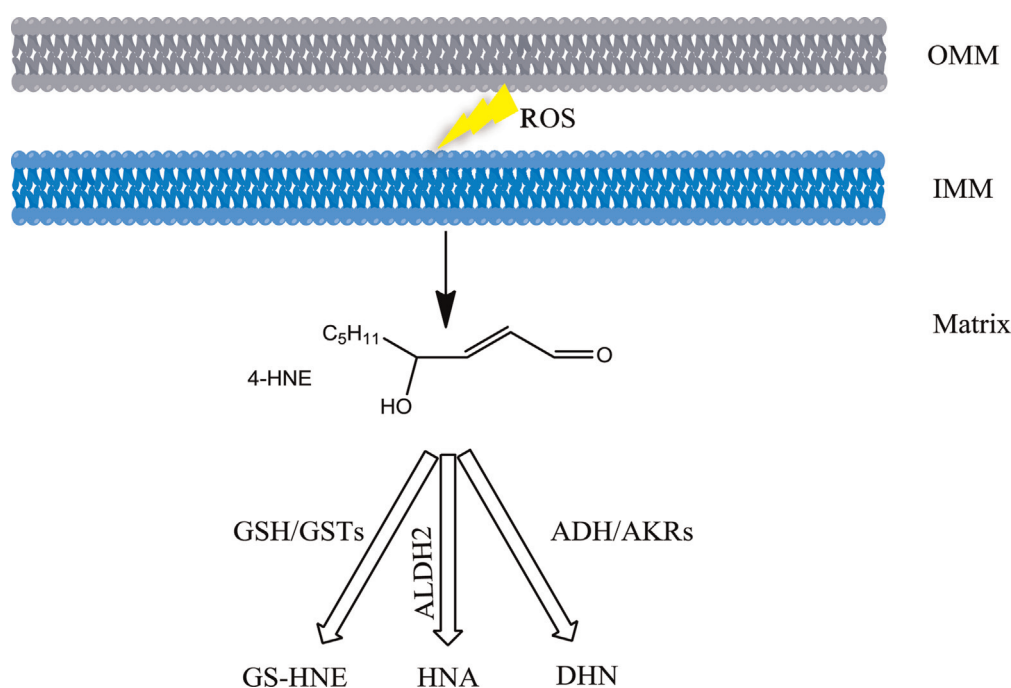
### Formation and catabolism of 4-HNE in mitochondria

Mitochondrion is the “power house” for most eukaryotic cells, including cancer cells, for its role in ATP production through oxidative phosphorylation. During this process, significant amount of superoxide are produced at complexes I and III of the electron transport chain (ETC). Most of the superoxide is converted to hydrogen peroxide by superoxide dismutases (SOD). One of the main targets of ROS is the membrane lipid bilayers, especially the PUFA chains in lipids. Peroxidation of PUFAs generates an array of primary lipid oxidation products and lipid electrophiles, among which 4-HNE is one of the well-studied active lipid electrophiles [5]. In contrast to its well established biological consequences, chemical mechanisms that led to 4-HNE formation remain to be clearly elucidated [18,19]. It is generally accepted that 4-HNE is derived from decomposition of hydroperoxide of  $\omega$ -6 PUFAs at the sn-2 position of glycerophospholipids in cellular membranes (Fig. 1A). Thus phospholipids containing linoleic acid (LA, 18:2,  $\omega$ -6) and arachidonic acid (AA, 20:4,  $\omega$ -6) on cytoplasmic membranes are considered the major source for 4-HNE production. Our recent work, however, demonstrated that oxidation of mitochondrial phospholipid cardiolipin also led to formation of significant amount of 4-HNE and other oxidation products *via* a novel chemical mechanism that involved cross-chain peroxy radical addition and decomposition [20–22] (Fig. 1B).

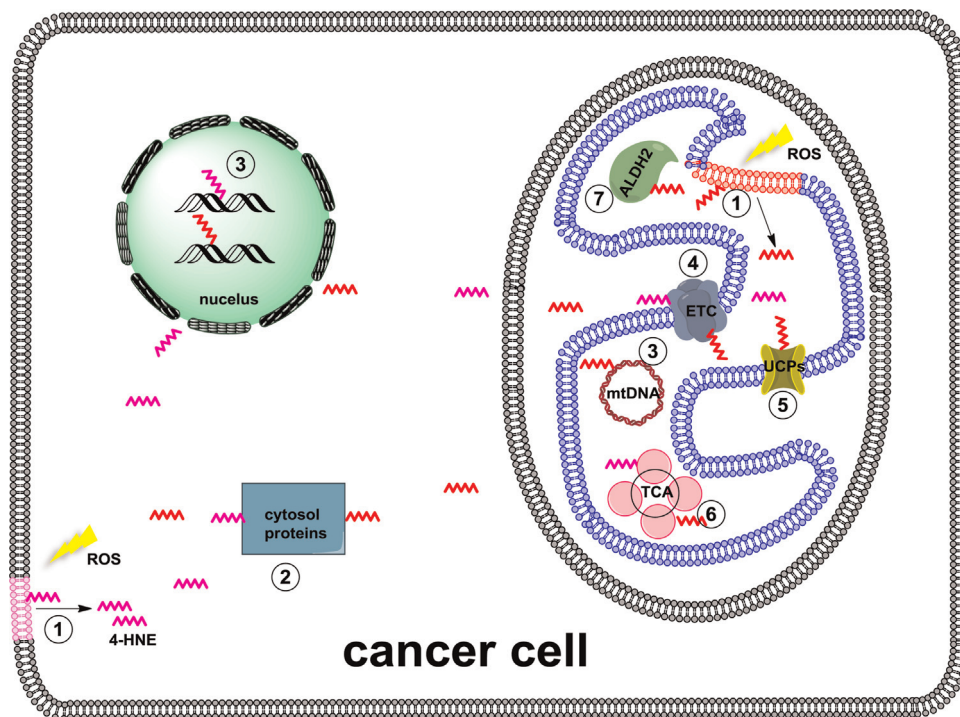
Cardiolipin is a unique class of mitochondrial specific phospholipids that reside almost exclusively in the inner membrane of mitochondria (IMM) and are critical for maintaining the structural

integrity of mitochondrial membranes and the function of multiple protein complexes in the ETC [23]. In most mammalian tissues, tetralinoleoylcardiolipin ( $L_4$ CL) is the major form of cardiolipins, which contains four linoleic acid chains in the same molecule. Incorporation of four LA side chains in  $L_4$ CL and its association with mitochondria render  $L_4$ CL to be readily oxidized by ROS and then generate electrophiles through this novel “arm to arm” reaction [20]. We provided further evidence that formation of 4-HNE and other lipid electrophiles from CL played an important role in intrinsic apoptosis in the context of atherosclerosis [13]. This process appears to be involved in cancer. Human kidney cancer tissue showed greater staining for 4-HNE protein adducts both in cytoplasm and mitochondria compared to adjacent tissue [24]. Furthermore, about 30% of the 4-HNE adducted proteins locate in mitochondria and a majority of them are the members of ETC [25,26].

There are three major detoxification pathways to convert 4-HNE to less reactive chemical species and control their steady-state intracellular concentrations (Fig. 2). Firstly, the main catabolic reactions are the formation of adducts with glutathione (GSH), which occurs spontaneously or can be catalyzed by glutathione-S-transferases (GSTs). Secondly, 4-HNE can be reduced to 1,4-dihydroxy-2-nonenone (DHN) by aldo-keto reductases (AKRs) or alcohol dehydrogenases (ADH). Thirdly, 4-HNE can be oxidized to 4-hydroxy-2-nonenic acid (HNA) by aldehyde dehydrogenase (ALDH). Throughout more than 1.5 billion years of obligate endosymbiotic co-evolution, mitochondria have developed a complex system to sense and react to oxidative stress similar to their host. GSH is one of the most important hydrophilic antioxidant in mitochondria for maintaining the redox balance. Similarly, a significant amount of AKRs and ADH is expressed in mitochondria. ALDH2, one member of ALDH family, is exclusively located in mitochondria. It is noteworthy that the superoxide dismutases (SOD) are the well-known antioxidant enzymes that convert superoxide into hydrogen peroxide. Among them, MnSOD resides in mitochondria whereas Cu/ZnSOD is located in cytosol.



**Fig. 2.** Catabolism of 4-HNE in mitochondria. ROS induced lipid peroxidation in IMM and OMM (outer membrane of mitochondria) leads to 4-HNE formation. In matrix, 4-HNE conjugation with GSH produces glutathionyl-HNE (GS-HNE); this process occurs spontaneously or can be catalyzed by GSTs. 4-HNE is reduced to 1,4-dihydroxy-2-nonenone (DHN) catalyzed ADH or AKRs. ALDH2 catalyzes the oxidation of 4-HNE to form 4-hydroxy-2-nonenic acid (HNA).



**Fig. 3.** A schematic view of 4-HNE macromolecule adducts in cancer cell. 4-HNE macromolecule adducts are involved in cancer initiation, progression, metabolic reprogramming, and cell death. 4-HNE (depicted as a zigzag line) is produced through ROS-induced lipid peroxidation of mitochondrial and plasma membranes. Biological consequences of 4-HNE adduction: 1, reducing membrane integrity; 2, affecting protein function in cytosol; 3, causing nuclear and mitochondrial DNA damage; 4, inhibiting ETC activity; 5, activating UCPs activity; 6, reducing TCA activity; 7, inhibiting ALDH2 activity.

#### 4-HNE mitochondrial protein adducts in cancer

As mentioned previously, proteins, DNA and membrane lipids are the primary targets for 4-HNE attack. It was estimated that 1–8% of the 4-HNE formed in cells will modify proteins [27], among which, about 30% 4-HNE target proteins locate in mitochondria [25,26]. Elevated status of oxidative stress has been associated with a majority of cancer types and thus 4-HNE is believed to be a major player that contributes to the mutagenic and carcinogenic effects of lipid peroxidation. Formation of 4-HNE protein adducts in renal and colon cancer tissues has been related to the growth and progression of kidney and colon cancer [4]. Increased levels of 4-HNE have been shown to be associated with liver cancer initiation in animal models and humans [28]. A recent study demonstrated that RLIP76 regulated pancreatic tumorigenesis through maintaining cellular levels of 4-HNE, GS-HNE and GS-DHN [29]. Furthermore, modulation of oxidative stress can be utilized for therapeutic purpose in cancer prevention and treatment [30]. Doxorubicin (DOX) is one of the most effective anticancer drugs and oxidative stress is a primary mechanism of DOX-induced toxicity to cancer cells. However, the cardiac side effects limit its clinical use. Using a redox proteomics method, Zhao et al. [25] identified several HNE-modified mitochondrial proteins in cardiac mitochondria from DOX-treated mice. Most of the proteins are related to mitochondrial energy metabolism, such as ATP synthase subunit  $\beta$  (ATP5B), succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA) and NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2) in ETC, and trifunctional enzyme subunit  $\alpha$  (HADHA) in citric acid cycle. The enzymatic activities of these proteins were significantly reduced in DOX-treated mice. Treatment of an SOD mimetic averted the production of DOX-induced HNE-protein adducts and mitochondrial dysfunction. These results imply that robust 4-HNE formation can induce cancer cell death through affecting mitochondrial function. Nevertheless, mild mitochondrial function impairment is a major

contributing factor in cancer. Studies from Anotnio and Paolo showed that partial inhibition of ETC complexes improved tumor cell migration *in vitro* and *in vivo*, and the metastasis can be prevented with a mitochondria-targeted superoxide scavenger mito-TEMPO [31,32]. It should be noted that cardiolipin interacts with a number of IMM proteins including the ETC complexes and this interaction is required for optimal activity of complex I (NADH ubiquinone oxidoreductase), complex III (ubiquinone cytochrome c oxidoreductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase). Furthermore, any alteration in the cardiolipin structure, content and composition may lead to mitochondrial dysfunction [33–35]. Constitutive 4-HNE levels might contribute to adaptive response in protecting cancer cells against oxidative damage. Superoxide production is sensitive to the proton motive force and it can be significantly reduced by mild uncoupling of oxidative phosphorylation through action of mitochondrial uncoupling proteins (UCPs). UCP2, the widely expressed UCP, has been shown to be up-regulated in a number of aggressive human cancers and plays an important role in cancer initiation, progression, and metabolic reprogramming as an antioxidant defense [36]. It has been strongly supported by the fact that UCPs can be activated by ROS [37,38] and several reports showed that ROS activated UCPs through a free radical chain reaction and 4-HNE formation; 4-HNE induced uncoupling was inhibited by inhibitors of UCPs [39,40]. These studies suggest that 4-HNE may act as a feed-back mechanism through activating UCPs to mitigate excessive ROS production and oxidative damage in cancer.

ALDH2, located in the mitochondrial matrix, is better known for its critical role in ethanol metabolism. ALDH2 is also capable of metabolizing endogenous aldehydic products derived from lipid peroxidation, such as 4-HNE, thus protecting mitochondria from these toxic agents [41]. Chronic consumption of ethanol has been causally linked to the development of upper aero digestive track (UADT) cancers, liver cancer, colorectal cancer, and breast cancer.

Studies from Asian countries confirmed a significant association between ALDH2 enzyme deficiency and UADT cancer risk [42–44]. Excessive ethanol consumption enhances lipid peroxidation by overwhelming antioxidant defense system in tissues and the aldehydic products of lipid peroxidation can covalently bind to proteins in tissues [45,46]. Doorn et al. showed that 4-HNE was both a substrate and an inhibitor of ALDH2; inhibition of ALDH2 by 4-HNE is reversible at low concentration and become irreversible when the concentration of 4-HNE reaches 10  $\mu$ M [47]. This inhibition appears to be a result of 4-HNE-cysteine adduct at the ALDH2 active site. Thus 4-HNE-induced ALDH2 inactivation may have an important role in the progression of some cancers.

#### 4-HNE mitochondrial DNA adducts in cancer

Mitochondria are unique organelles because they contain their own DNA which is responsible for encoding 2 ribosomal RNAs, 22 transfer RNAs and 13 protein subunits of the ETC. Mitochondrial DNA (mtDNA) is organized into protein–DNA complexes called nucleoids within the mitochondrial matrix. This kind of structure can provide some protection to the genome while mtDNA remains in close proximity to the ETC which is considered the main source of ROS within the cell. Therefore, it is predictable that mtDNA is highly susceptible to oxidative damage by ROS. Indeed, more oxidative damage to mtDNA has been widely observed than those of nuclear DNA. However, whether these damages are directly from mitochondrial ROS remain to be clearly defined. Exposure to ROS can result in a number of oxidative modifications to DNA including the lesions 8-oxoguanine (8-oxo-dG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), 4,6-diamino-5-formamidopyrimidine (FapyA), and thymine glycol. These mtDNA modifications can cause point mutations, deletions, and strand breaks; thus the unrepaired oxidative damage may lead to the production of dysfunctional proteins. 8-Oxo-dG represents the most commonly studied oxidative lesions and increased levels of 8-oxo-dG are found in mtDNA from patients with arsenical skin cancers [48], glioma [49], urinary bladder and renal cancer [50].

4-HNE, the product of lipid peroxidation, is mutagenic and genotoxic in viruses, bacteria and mammalian cells. It reacts with all four DNA bases but with different efficiency: G > C > A > T [44]. 4-HNE-dG represents the best biomarker of the genotoxic effects of 4-HNE and these adducts are primarily found in nuclear DNA. A classic example of etiological relevance of 4-HNE-dG in human cancers is 4-HNE-dG induced p53 mutation. P53 is a well-known tumor suppressor and a mutational hotspot in human cancers, especially in hepatocellular carcinoma. 4-HNE-dG adducts were preferentially formed at the third base of codon 249 in the p53 gene, causing gene mutation and affecting diverse biological processes including cell cycle arrest, apoptosis, DNA repair, and differentiation [51]. However, modification of mtDNA by 4-HNE and other lipid electrophile has yet to be discovered.

#### 4-HNE mitochondrial lipid adducts in cancer

Mitochondria are dynamic organelles surrounded by two membranes with a well-defined lipid composition; most of these lipids are synthesized in the endoplasmic reticulum. However, *de novo* lipid synthesis and remodeling of mitochondrial lipids are important for maintaining the structural integrity and function of mitochondria. The most abundant phospholipids in mitochondrial membrane are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), representing about 40–55% and 30–45% of total mitochondrial phospholipids, respectively. CL accounts for around 15% of mitochondrial phospholipids. Other lipids, such as

phosphatidylserine (PS), sphingolipids and sterols, are present at modest or even trace amounts [52]. Phospholipids containing amino group, such as PE and PS, can undergo both a Michael addition and a Schiff base formation with 4-HNE [53]. Since the low amount of PS in mitochondria, PE become the primary targets of 4-HNE adduction. PE Michael adducts were found in human urine and blood in oxidative stress related situations such as aging and diabetes [54], while the biological relevance is much less documented compared to protein and DNA adducts. Guichardant et al. [55] demonstrated that 4-HNE adduction made PE a poor substrate for secreted phospholipase A<sub>2</sub> and was not cleaved by phospholipase D. Plasmalogen, an important subclass of PE, is covalently modified by 4-HNE and this modification may alter its antioxidant potential. However, all these lipid modification by 4-HNE have not been shown to be derived from mitochondrial lipids.

#### Therapeutic strategies for manipulation of ROS and lipid peroxidation in cancer: implication for mitochondrial 4-HNE

Evidence provided thus far clearly demonstrated that 4-HNE played an important role in cancer through mitochondria. 4-HNE at low concentrations can protect cancer cells against further damage (e.g., UCPs reduce superoxide production) whereas cells undergo apoptosis or even necrosis at high concentrations when adduct load on mitochondrial macromolecules overwhelms the protective mechanisms. Thus, strategies focusing on manipulating mitochondrial ROS generation, lipid peroxidation, and 4-HNE formation may provide therapeutic value to prevent or treat cancer.

While moderate ETC inhibition and subsequent increase in mitochondrial ROS production promote tumor cell migration and metastasis, specific scavenging of mitochondrial superoxide can be a therapeutic option to prevent spontaneous tumor metastasis. Studies by Paolo et al. showed that treatment with mitochondrial specific superoxide scavenger mitoTEMPO, an SOD<sub>2</sub> mimetic, prevented the metastatic phenotype in human and mouse cancer cells [32]. MitoTEMPO impairs the formation of ROS including H<sub>2</sub>O<sub>2</sub> and lipid peroxides [56]. Similar to mitoTEMPO, mitoQ, a mitochondria-targeted form of coenzyme Q<sub>10</sub>, is currently being tested in clinical trials [57]. These findings may offer a rationale to test mitochondria-targeted superoxide scavengers in metastasis prevention trials.

Robust ROS formation is a common mechanism for some anticancer drugs to kill cancer cells, such as DOX and arsenic trioxide (As<sub>2</sub>O<sub>3</sub>). However, only limited reports have taken into account of the roles played by the lipid peroxidation products induced by ROS. Reports showed that some PUFAs, such docosahexaenoic acid (DHA), can sensitize various tumor cells such as breast cancer, ovarian cancer, and cervical cancer, to ROS-inducing anticancer agents. The cytotoxic effect of combined treatments was due to induction of apoptosis, preceded by increased production of intracellular aldehydes of lipid peroxidation. These effects can be abolished by treatment of antioxidant vitamin E [58]. A recent study demonstrated that ketogenic diet (high in LAs, and low in carbohydrates and protein) enhanced radio-chemo-therapy responses in lung cancer xenografts by a mechanism that may involve increased oxidative stress [59]. Ketogenic diets may enrich mitochondrial CL with LA and at the same time force cells to rely on mitochondrial oxidative metabolism for energy production. According to our studies, LA-containing CL is an abundant source of 4-HNE under oxidative stress condition and predisposes cancer cells to undergo apoptosis. Indeed tumors from mice treated with ketogenic diet and radiation showed significantly increased levels of 4-HNE-modified proteins. Therefore, these results suggest that ketogenic diet (high in PUFAs, i.e., DHA, LA, and arachidonic acid)

could serve as an effective adjuvant for improving responses to radio-chemo-therapies in the treatment of cancers by a mechanism linking mitochondrial 4-HNE formation, oxidative stress, and lipid peroxidation.

## Conclusions

Lipid peroxidation-derived 4-HNE is a prototypical reactive lipid electrophile that readily forms covalent adducts with nucleophilic functional groups in macromolecule such as proteins, DNA, and lipids (Fig. 3). A body of work have shown that generation of 4-HNE macromolecule adducts plays important pathological roles in cancer through interactions with mitochondria. First of all, mitochondria are one of the most important cellular sites of 4-HNE production, presumably from oxidation of abundant PUFA-containing lipids, such as L<sub>4</sub>CL. Emerging evidence suggest that this process play a critical role in apoptosis. Secondly, in response to the toxicity of 4-HNE, mitochondria have developed a number of defense mechanisms to convert 4-HNE to less reactive chemical species and minimize its toxic effects. Thirdly, 4-HNE macromolecule adducts in mitochondria are involved in the cancer initiation and progression by modulating mitochondrial function and metabolic reprogramming. 4-HNE protein adducts have been widely studied but the mtDNA modification by lipid electrophiles has yet to emerge. The biological consequence of PE modification remains to be defined, especially in the context of cancer. Last but not the least, manipulation of mitochondrial ROS generation, lipid peroxidation, and production of lipid electrophiles may be a viable approach for cancer prevention and treatment.

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