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## Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease

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### Abstract

Nonalcoholic fatty liver disease (NAFLD) is today considered the most common form of chronic liver disease, affecting a high proportion of the population worldwide. NAFLD encompasses a large spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis. Obesity, hyperglycemia, type 2 diabetes and hypertriglyceridemia are the most important risk factors. The pathogenesis of NAFLD and its progression to fibrosis and chronic liver disease is still unknown. Accumulating evidence indicates that mitochondrial dysfunction plays a key role in the physiopathology of NAFLD, although the mechanisms underlying this dysfunction are still unclear. Oxidative stress is considered an important factor in producing lethal hepatocyte injury associated with NAFLD. Mitochondrial respiratory chain is the main subcellular source of reactive oxygen species (ROS), which may damage mitochondrial proteins, lipids and mitochondrial DNA. Cardiolipin, a phospholipid located at the level of the inner mitochondrial membrane, plays an important role in several

reactions and processes involved in mitochondrial bioenergetics as well as in mitochondrial dependent steps of apoptosis. This phospholipid is particularly susceptible to ROS attack. Cardiolipin peroxidation has been associated with mitochondrial dysfunction in multiple tissues in several physiopathological conditions, including NAFLD. In this review, we focus on the potential roles played by oxidative stress and cardiolipin alterations in mitochondrial dysfunction associated with NAFLD.

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**Key words:** Oxidative stress; Cardiolipin; Mitochondrial bioenergetics; Antioxidants; Nonalcoholic fatty liver disease

**Core tip:** Nonalcoholic fatty liver disease (NAFLD) is considered the most common form of chronic liver disease. Mitochondrial dysfunction and oxidative stress play a key role in the physiopathology of NAFLD. Mitochondrial respiratory chain is the main source of reactive oxygen species, which may damage mitochondrial proteins, lipids and mitochondrial DNA. Cardiolipin, a phospholipid of the inner mitochondrial membrane, plays an important role in mitochondrial bioenergetics and in apoptosis. Cardiolipin abnormalities have been associated with mitochondrial dysfunction in several physiopathological conditions, including NAFLD. In this review, we focus on the potential roles played by oxidative stress and cardiolipin alterations in mitochondrial dysfunction in NAFLD.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease, affecting a high proportion of the population worldwide<sup>[1,2]</sup>. NAFLD refers to the wide spectrum of liver damage including several pathological conditions such as nonalcoholic steatohepatitis (NASH), advanced fibrosis and cirrhosis<sup>[3-5]</sup>. A number of predisposing factors have been related to NAFLD, such as obesity, diabetes, dyslipidemia, drugs and parenteral nutrition<sup>[6,7]</sup>. However, the pathogenesis of NAFLD and its progression to fibrosis and chronic liver disease is still unknown. The leading hypothesis for nonalcoholic-induced liver disease is the two-hits model<sup>[8]</sup>. The first hit is an initial metabolic alteration, like insulin resistance, hyperglycemia and hyperlipidemia and the accumulation of triglyceride in hepatocytes, leading to steatosis. The second hit triggers the progression to more severe liver pathologies like steatohepatitis, fibrosis and cirrhosis of liver. Examples of the second hit could be genetic and environmental factors like diet, smoke and pollutants leading to mitochondrial dysfunction. All these factors are recognized to induce oxidative stress, mainly *via* mitochondrial alterations. Mitochondrial dysfunction impairs fat homeostasis in liver and also leads to an overproduction of reactive oxygen species (ROS). These reactive oxygen species are considered to play an important role in inducing lethal hepatocyte injury associated with NAFLD<sup>[9-13]</sup>. Oxidative stress may cause damage at cellular level, such as membrane lipid peroxidation, cell degeneration and necrosis, cell death by apoptosis<sup>[14-16]</sup>, proinflammatory cytokine expression, liver stellate cell activation and fibrogenesis<sup>[11,17-19]</sup>.

It is well known that the mitochondrial respiratory chain is an important source of ROS at subcellular level and hence a potential contributing factor to NAFLD. Results obtained in our laboratory demonstrated enhanced mitochondrial ROS formation associated with an impairment of the mitochondrial respiratory chain in rats fed a choline-deficient diet (CDD)<sup>[20-22]</sup>. Similar results have been reported in patients with nonalcoholic steatosis<sup>[23]</sup>. In addition, it has been shown that patients with steatohepatitis present with ultrastructural mitochondrial alterations<sup>[24,25]</sup> and impairment of hepatic ATP synthesis<sup>[26]</sup>.

Cardiolipin is a dimeric phospholipid found almost exclusively in the inner mitochondrial membrane (IMM) where it plays a pivotal role in several reactions and processes involved in mitochondrial bioenergetics, primarily the process of the oxidative phosphorylation<sup>[27-31]</sup>. In addition, cardiolipin is believed to participate in several steps of the intrinsic (mitochondrial) pathway of the apoptotic process<sup>[32-35]</sup>, in mitochondrial morphology and dynamics, including fusion and fission processes, as well as in protein translocation, insertion and assembly into mitochondria<sup>[36,37]</sup>. Cardiolipin alterations are considered an important contributing factor in mitochondrial dysfunction in multiple tissues in several physiopathological situations<sup>[37-39]</sup>. Cardiolipin oxidation has been shown to be involved in mitochondrial dysfunction in insulin re-

sistance<sup>[40]</sup>, obesity<sup>[41]</sup> and NAFLD<sup>[22]</sup>. In this review, we focus on the potential roles played by oxidative stress and cardiolipin alterations in mitochondrial dysfunction associated with nonalcoholic fatty liver disease.

## MITOCHONDRIAL ABNORMALITIES IN NAFLD

The mechanisms responsible for NAFLD are still not fully elucidated. Reduced capacity to oxidize fatty acids, increased delivery and transport of free fatty acids (FFAs) into the liver and enhanced hepatic fatty acid synthesis are considered important factors in the pathogenesis of fatty liver. Structural and functional mitochondrial alterations have been shown to contribute to the pathogenesis of NAFLD. Structural alterations include morphological changes, ultrastructural lesions and depletion of mitochondrial DNA (mtDNA), while functional alterations include the activity of respiratory chain complexes and the mitochondrial  $\beta$ -oxidation<sup>[19]</sup>. Abnormal morphological changes in liver mitochondria have been observed in patients and animal models of NASH<sup>[24,42]</sup>. Results obtained by electronic microscopy revealed that mitochondria in a mouse model with fatty oxidation defect and hepatic steatosis are large and swollen, limited in number, and that the mitochondrial matrix has paracrystalline inclusions and hypodensity<sup>[13]</sup>. These ultrastructural mitochondrial perturbations in patients with NAFLD may be indicative of altered mitochondrial functions, primarily the process of oxidative phosphorylation<sup>[23,43]</sup>. NAFLD, often found in patients with insulin resistance, obesity and type 2 diabetes, is associated with decreased oxygen consumption and ATP generation, reduced total mtDNA and mtDNA transcription factor A, and reduced content of respiratory proteins in the fat, muscle and liver<sup>[44]</sup>. MtDNA depletion in hepatocytes impairs mitochondrial function and causes hepatic steatosis and other liver injury<sup>[45]</sup>. Patients with NASH have decreased expression of mtDNA encoded polypeptides<sup>[46]</sup> and impaired activity of respiratory chain complexes<sup>[23]</sup>.

Another important factor underlying the mitochondrial dysfunction found in NAFLD patients and animal models is tumor necrosis factor (TNF- $\alpha$ ). The involvement of this cytokine in the pathogenesis of NASH is well documented<sup>[4]</sup>. High blood TNF- $\alpha$  levels have been found in patients with nonalcoholic fatty liver disease<sup>[47]</sup>. The levels of TNF- $\alpha$  in liver tissue of ob/ob mice were much higher than in normal mice<sup>[48]</sup>. The likely sources of hepatic TNF- $\alpha$  are hepatocytes and Kupffer cells<sup>[49]</sup>. TNF- $\alpha$  induces mitochondrial perturbations such as swelling with a lighter matrix and abnormal morphological alterations in the membrane. In addition, TNF- $\alpha$ -induced mitochondrial swelling causes a bursting of the membrane leading to an interference between respiratory chain complexes I and III<sup>[50]</sup>. Treatment of ob/ob mice with anti-TNF- $\alpha$  has a beneficial effect on mitochondrial respiratory chain complexes (I, III, IV and V) activity,  $\alpha$ -oxidation activity and liver histology<sup>[48]</sup>. TNF- $\alpha$  may

also alter electron transport chain (ETC) by inducing hypoxia-inducible factor -1 $\alpha$  and mtDNA damage<sup>[18,48,51]</sup>.

## MITOCHONDRIA AS SOURCE OF ROS PRODUCTION

Oxidative stress is a condition due to an altered balance between the production of ROS and/or reactive nitrogen species (RNS) and the antioxidant defences capacity. The resulting imbalance appears to be involved in various physiopathological situations in which an oxidative insult causes tissue damage and cell death. The effects of ROS and RNS are not always injurious but, under physiological conditions, they represent essential signaling roles and physiological regulatory mechanisms in several vital cellular processes<sup>[52,53]</sup>.

The redox state of the ETC components is considered the main factor involved in mitochondrial ROS generation. Electron transfer through the mitochondrial respiratory chain generates an electrochemical gradient which is utilized to synthesize ATP. In certain metabolic situations, such as high-fat or high-glucose states, inhibited ETC complexes activity or exposure to xenobiotics, the mitochondrial electrochemical gradient is high. Under these conditions the life of superoxide-generating electron transport intermediates, such as the ubisemiquinone radical, is prolonged, facilitating the transfer of electrons one at a time to oxygen, thus increasing the superoxide anion (O<sub>2</sub><sup>•-</sup>) generation.

Mitochondria are the primary source of cellular reactive oxygen species. It has been reported that approximately 0.2%-2% of the oxygen consumed by the cell is converted by mitochondria to ROS, mainly through the production of superoxide anion (O<sub>2</sub><sup>•-</sup>)<sup>[54]</sup>. Mitochondria consume around 90% of a cell's oxygen to produce ATP through the process of oxidative phosphorylation. This process, however, comes with additional cost, the production of potentially harmful ROS. The mitochondrial electron transport chain is considered the main source of ROS. The primary oxygen radical species generated by mitochondria is superoxide anion which is then converted to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by spontaneous dismutation or by superoxide dismutase (SOD), present both within the mitochondria and in the cytosol. Hydrogen peroxide, in turn, is converted into water by glutathione peroxidase or catalase, otherwise, in the presence of divalent cations such as iron, H<sub>2</sub>O<sub>2</sub> can undergo Fenton's reaction to produce hydroxyl radical <sup>•</sup>OH.

Mechanisms of ROS production by mitochondrial respiratory chain complexes and the sites of their production have been reviewed extensively<sup>[55]</sup>. The two major sites of O<sub>2</sub><sup>•-</sup> production are complex I (NADH-coenzyme Q reductase) and complex III (ubiquinone-cytochrome c oxidoreductase). Mitochondria can produce superoxide anion, predominantly from complex I, likely at the site of FMN<sup>[55]</sup> and from complex III, at the site of the unstable ubisemiquinone molecules<sup>[56,57]</sup> or the cytochrome b<sup>[58]</sup>. ROS are also generated, to a

lesser extent, outside of the mitochondrion by nonenzymatic and/or enzymatic reactions. Reactions involved in extra-mitochondrial ROS production include NADPH oxidase, xanthine oxidase, D-amino oxidase, the P-450 cytochromes and proline and lysine hydroxylase, uncoupled NO synthase. ROS may also be generated by FFA oxidation in peroxisomes and microsomes and from Kupffer cell activation. In the obese and in patients with NASH, the pro-oxidant cytochrome P450 2E1 (CYP2E1) isoform activity is increased<sup>[59]</sup>, likely induced by FFA or ketones. This microsomal enzyme, in addition to its involvement in the degradation of xenobiotics, promotes FFA  $\beta$ -oxidation, during which ROS are generated<sup>[60]</sup>. Kupffer cells may also generate ROS *via* the NADPH-oxidase system<sup>[61]</sup>.

Mitochondrial ROS generation might be regulated by nitric oxide (NO)<sup>[62]</sup>. This compound can be converted to other RNS such as nitroxyl anion (NO<sup>-</sup>) or the toxic peroxynitrite (ONOO<sup>-</sup>)<sup>[62]</sup>. It has been reported that mitochondria can produce NO from mitochondrial nitric oxide synthase (iNOS)<sup>[62,63]</sup>; however, this has been questioned<sup>[64]</sup>. NO may affect mitochondrial respiratory chain function by reacting with cytochrome c oxidase, thus interrupting electron transfer to oxygen<sup>[65]</sup>. In addition, peroxynitrite, a product resulting from NO reaction with O<sub>2</sub><sup>•-</sup>, inhibits the activity of various proteins, including electron transport chain complexes<sup>[66]</sup>. Mechanism through which peroxynitrite exert this effect are varied and include its oxidative potential<sup>[67]</sup> and its ability to damage DNA<sup>[68]</sup>. Oxidizing reactivity of ONOO<sup>-</sup> is comparable to that of <sup>•</sup>OH<sup>[69]</sup>. Endogenous NO can regulate mitochondrial production of ROS by two mechanisms: at low levels NO can increase O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> generation by modulating the rate of oxygen consumption at the level of cytochrome c oxidase<sup>[70]</sup>, whereas at high levels, NO inhibits H<sub>2</sub>O<sub>2</sub> production by reacting with O<sub>2</sub><sup>•-</sup>, resulting in ONOO<sup>-</sup> formation<sup>[71]</sup>. NO and other RNS are increased in response to chronic alcohol consumption through induction of iNOS<sup>[72]</sup>. Studies also report increased iNOS expression in the liver of ob/ob mice<sup>[48]</sup>. iNOS induction is linked to alterations in the NO-dependent control of mitochondrial respiration (at the level of cytochrome c oxidase), which potentially contributes to the development of alcoholic steatohepatitis. The importance of understanding the role of NO in the modulation of the mitochondrial electron transport chain activity in hepatic physiology and pathophysiology is supported by various studies, indicating that localized NO production can occur within the organelle through a specific mitochondrial NOS isoform or *via* metabolism of nitrite to NO<sup>[73,74]</sup>.

Within the mitochondrial matrix, manganese-SOD converts O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide can be further metabolized by glutathione peroxidase (Gpx I) and peroxiredoxin (Prx III) or can diffuse from the mitochondria into the cytosol. O<sub>2</sub><sup>•-</sup> is unable to cross the mitochondrial membrane except in its protonated form, which is a small fraction of this radical at physiological pH<sup>[75]</sup>. The O<sub>2</sub><sup>•-</sup>



present in the intermembrane space might be scavenged by the oxidized form of cytochrome c or can diffuse into the cytosol through the voltage dependent anion channel (VDAC)<sup>[76]</sup>.

Important mitochondrial antioxidant defence systems are represented by glutathione (GSH) and multiple GSH-linked antioxidant enzymes. Among these are Gpx 1, mainly located in the cytosol, and Gpx 4, also known as phospholipid hydroperoxide glutathione peroxidase, predominantly linked to the mitochondrial membrane. These enzymes catalyze the reduction of H<sub>2</sub>O<sub>2</sub> and lipid hydroperoxides.

Depletion of mitochondrial GSH has been implicated in the development of alcoholic liver disease in that GSH participates in pathways responsible for ROS detoxification<sup>[77]</sup>. It has been also proposed that depletion of mitochondrial GSH sensitizes hepatocytes from alcohol-fed animals to TNF $\alpha$ -induced cell death<sup>[78]</sup>. Conflicting effects of NAFLD on mitochondrial GSH levels have also been reported. For example, decreased<sup>[48]</sup> and increased<sup>[79]</sup> levels of mitochondria GSH have been reported in a murine model of NASH such as the ob/ob mice. It is possible that the age of animals and their diet could explain, at least in part, the differences observed in mitochondrial GSH<sup>[80,81]</sup>. Depletion of mitochondrial GSH levels could also be due to its reduced uptake by mitochondria as a result of enhanced levels of cholesterol within the inner mitochondrial membrane<sup>[82]</sup> and the decrease in synthesis of S-adenosylmethionine, the major methyl donor in liver and precursor to GSH<sup>[83]</sup>. Recently, lower hepatic expression of the Mu-class glutathione S-transferase was reported in obese patients with steatosis compared to obese individuals without NAFLD<sup>[84]</sup>. Thus, low levels of GSH and decreased expression and/or activity of some glutathione-related enzymes may have additional deleterious effects on fatty liver. These findings demonstrate that although GSH is a potential player in the pathogenesis of fatty liver disease, additional studies are necessary to delineate the precise role of mitochondrial GSH alterations in the pathogenesis of alcohol and non-alcohol mediated mitochondrial dysfunction and liver injury.

## MITOCHONDRIAL OXIDATIVE DAMAGE IN NAFLD

Mitochondrial ROS generation is considered an important contributing factor to various liver diseases *via* the accumulation of mtDNA mutations that could lead to lethal cell injury<sup>[85]</sup> through the impairment of several bioenergetic reactions involved in the oxidative phosphorylation. In addition, due to its close proximity to the ETC, the lack of protective histones and incomplete repair mechanisms, mtDNA is highly prone to oxidative damage, leading to DNA breaks and the occurrence of somatic of mtDNA mutations<sup>[55]</sup>. Mitochondrial DNA encodes 13 proteins involved in ETC functioning, as well as the two mitochondrial ribosomal RNAs and all the mi-

tochondrial transfer RNAs required for the synthesis of mtDNA-encoded polypeptides within mitochondria.

During NASH, different types of mtDNA damage have been detected, including deletions, point mutations and increased 8-hydroxydeoxyguanosine level<sup>[24,86]</sup>. Reduced levels of mtDNA have also been reported in patients with NASH<sup>[87]</sup> that seem to be responsible for the impairment of mitochondrial function and development and progression of liver steatosis and other liver injuries. Reduced expression of mtDNA-encoded polypeptides has been reported in patients with NASH, further supporting a role for mtDNA depletion in mitochondrial dysfunction<sup>[88]</sup>. Interestingly, although loss of mtDNA has been demonstrated in the livers of patients with NASH, mtDNA level was shown to be markedly enhanced in patients with fatty liver but without inflammation or fibrosis<sup>[89]</sup>. Increased levels of mutations in the mitochondrially encoded subunit I and subunit II genes of complex I and complex IV, respectively, have been detected in the livers of NASH patients compared to patients with simple fatty livers<sup>[90]</sup>. The accumulation of mtDNA mutations may lead to mitochondrial respiratory chain dysfunction, which in turn results in an increase in mitochondrial ROS production and subsequent accumulation of more mtDNA mutations. This triggers a vicious cycle of oxidative damage in which mitochondrial dysfunction produces larger amounts of reactive oxygen species which in turn can induce further oxidative damage to mitochondrial structure and function, as well as to other cellular components. Damage to mtDNA can be propagated as mitochondria and cell divide<sup>[91]</sup>. Therefore, oxidative damage to mtDNA could have severe implications for mitochondrial dysfunction in fatty liver, allowing the physiological consequences of the damage to be amplified.

In addition to mtDNA and proteins, phospholipid constituents of the mitochondrial membranes are particularly prone to ROS-induced oxidative attack, especially the long chain polyunsaturated fatty acids. Phospholipids are the most abundant lipid components of the mitochondrial membranes where they play multiple roles. Phospholipids modulate the membrane permeability barrier, the structural and functional properties of membrane-associated enzymatic activities and provide a matrix for the assembly and function of a variety of catalytic processes. Polyunsaturated fatty acids (PUFA) are essential components of mitochondrial phospholipids. The presence of high concentration of PUFA in their structure render mitochondrial phospholipids a prime target for reactions with oxidizing agents and enables them to participate in long free radical chain reactions, producing a variety of by-products of lipid peroxidation process, such as hydroperoxides and endoperoxides. These compounds may undergo fragmentation to produce several reactive intermediates, among them, malondialdehyde and the most reactive 4-hydroxy-trans-2-nonenal. These two by-products of lipid peroxidation have been shown to interact with and inactivate ETC components, includ-

ing cytochrome c oxidase, by forming adducts with this enzyme<sup>[92]</sup>, contributing to triggering mitochondrial dysfunction in NASH<sup>[93,94]</sup>.

Phospholipid peroxidation is considered an important contributing factor in mitochondrial dysfunction in several physiopathological conditions as well as during the aging process<sup>[95]</sup>. In fact, phospholipid peroxidation alters the structural organization of the mitochondrial bilayer, perturbing membrane fluidity and permeability. These alterations, in turn, affect mitochondrial ETC activity, the oxidative phosphorylation process, inner membrane barrier properties, maintenance of mitochondrial membrane potential and mitochondrial Ca<sup>2+</sup> buffer capacity.

Cardiolipin is a phospholipid component of the inner mitochondrial membrane, rich in unsaturated fatty acids, particularly linoleic acid in heart and liver tissues or arachidonic and docosahexaenoic acid in brain tissue mitochondria<sup>[96]</sup>. Due to its high content of unsaturated fatty acids, cardiolipin is highly sensitive to ROS-induced oxidative damage. In fact, the presence of a methylene bridge between two double bonds of CL fatty acids makes these compounds highly prone to oxidative damage. In addition, CL molecules seem to be a primary target for ROS attack due to their location in the inner mitochondrial membrane in the proximity of the sites of ROS generation, notably the ETC complexes. Normal CL can be measured directly by a normal-phase HPLC technique with UV detection at 206 nm, while oxidized cardiolipin form can be detected with the same HPLC technique with detection at 235 nm (indicative of conjugated dienes)<sup>[96]</sup>. Results obtained in our laboratory have demonstrated an oxidation/depletion of cardiolipin in hepatic mitochondria isolated from rat fed with CDD, an experimental animal model of non-alcoholic fatty liver<sup>[22]</sup>.

## ROLE OF CARDIOLIPIN IN MITOCHONDRIAL BIOENERGETICS

Cardiolipin plays pleiotropic functions in mitochondria. This phospholipid is particularly abundant in biological membranes involved in the generation of an electrochemical gradient that is used to produce ATP, such the bacterial plasma membrane and the inner mitochondrial membrane<sup>[28]</sup>. The association between CL and these energy-transducing membranes suggests a critical role for CL in mitochondrial bioenergetics. Cardiolipin has been shown to interact with and to be required for optimal functioning of several inner mitochondrial membrane proteins and enzymes<sup>[27-31,97]</sup>, including among others, the ETC complexes involved in the oxidative phosphorylation process and ADP/ATP translocator. Crystallographic studies have demonstrated the presence of few tightly bound CL molecules in each of the crystal structures of complex III, complex IV and ADP/ATP carrier<sup>[98]</sup>. These results suggest that CL is an integral component of these proteins, the presence of which is critical to folding, structure and function.

Mitochondrial respiratory chain complexes are organized into higher oligomeric structures, generically called supercomplexes or respirosomes<sup>[99]</sup>. Cardiolipin, due to its dimeric structure, seems to affect the assembly, stabilization and functional activity of such respiratory supercomplexes. Indeed, in mitochondria lacking CL, a respiratory supercomplex formed by complex III and IV is destabilized<sup>[100]</sup>. Similarly, dimeric organization of ADP/ATP carrier and other ADP/ATP carrier-containing supercomplexes are destabilized in CL-deficient mitochondria<sup>[101]</sup>. Altogether, these findings indicate the potential role played by CL in the supramolecular organization of proteins involved in mitochondrial bioenergetics. Thus, abnormalities in CL structure, content and fatty acids composition may have a deleterious effect in mitochondrial function in several physiopathological conditions<sup>[37,38,96,102]</sup>, including fatty liver diseases<sup>[22]</sup>.

## CARDIOLIPIN OXIDATION AND RESPIRATORY CHAIN DYSFUNCTION IN NAFLD

As discussed earlier in this review, CL molecules seem to be required for functional activity of several mitochondrial inner membrane proteins, including respiratory chain complexes involved in the oxidative phosphorylation process. Cardiolipin oxidation may significantly affect the respiratory chain complexes activity and mitochondrial function. Results from our laboratory showed that exposure of beef heart submitochondrial particles to mitochondrial-mediated ROS generation or ROS generating systems resulted in an oxidation and depletion of mitochondrial CL and in a significant decrease of the complexes I, III and IV activity<sup>[103-105]</sup>. Moreover, exogenously added CL-liposomes were able to significantly prevent the ROS-mediated decrease of the activity of these respiratory chain complexes<sup>[103-105]</sup>. Thus, the oxidation and depletion of mitochondrial CL seem to be responsible for the ROS-induced alterations of the respiratory chain complexes I, III and IV activities.

Choline deficient diet (CDD) is a well-known experimental model to induce fatty liver in rats<sup>[106]</sup>. The depletion of choline interferes with the synthesis and/or secretion of very low-density lipoproteins and thus inhibits the transport of triglycerides outside of the hepatocytes, resulting in fatty liver in rats. Steatosis induced by the CDD is characterized by pathological and biochemical similarities with fatty liver in humans. Reactive oxygen species have been shown to have an important role in hepatic tissue injury in fatty liver<sup>[18,19]</sup>. Alterations in mitochondrial respiratory chain activity seem to be involved in the oxygen radicals production and thus they are considered a potential contributing factor in the pathogenesis of NAFLD<sup>[12,20,23]</sup>. Due to the fact that ROS are a highly reactive and short lived species, it is likely that mitochondrial-mediated ROS production leads to primary reactions and damage to cellular com-

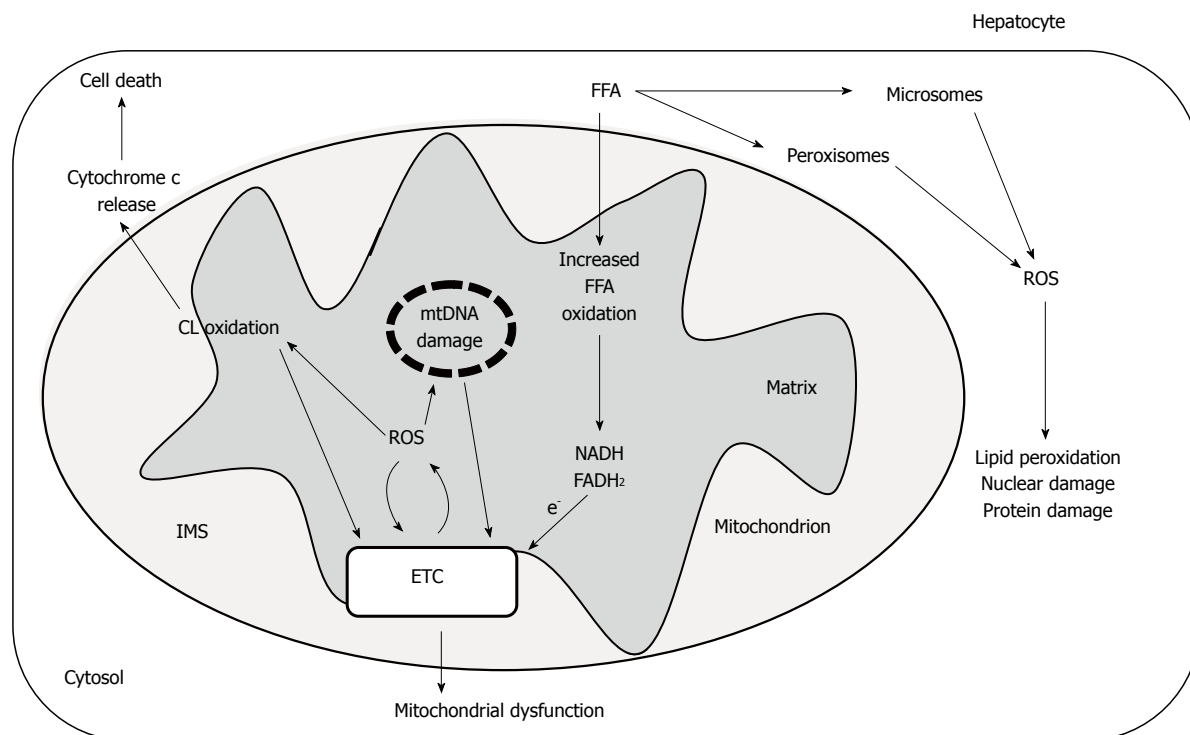


Figure 1 Possible role of reactive oxygen species and cardiolipin oxidation in hepatic mitochondrial dysfunction in nonalcoholic steatohepatitis.

ponents located near the site of their production. Therefore, mitochondria could be considered both as a major source of ROS production and as a major target of their oxidative attack. The effect of ROS should be greatest at the level of mitochondrial membrane constituents, especially the protein complexes involved in the electron transport chain and phospholipid constituents rich in unsaturated fatty acids, particularly cardiolipin. Results obtained in our and other laboratories have shown that the activity of complex I is significantly diminished in liver mitochondria isolated from CDD rats compared to control animals<sup>[20,22]</sup>. Complex I has been shown to also be affected in the liver of patients with NASH<sup>[23]</sup>. The molecular mechanism underlying this alteration in complex I activity was also explored. *In vitro* experiments demonstrated that some CL molecules are linked to complex I and are required for functional activity of this enzyme complex<sup>[97,107]</sup>. As mentioned above, the content of CL decreased in liver mitochondria from CDD animals, while the level of oxidized cardiolipin increased<sup>[22]</sup>. Furthermore, the lowered activity of complex I in liver mitochondria from CDD animals could be restored to the level of control liver by the addition of exogenous CL. Other major phospholipid components of the mitochondrial membranes, such as phosphatidylcholine and phosphatidylethanolamine and peroxidized CL, were unable to replace CL in this effect. Moreover, oxidized cardiolipin markedly inhibited the activity of complex I in beef heart submitochondrial particles (personal observations). These results suggest that the observed dysfunction of complex I in hepatic mitochondria from CDD animals could be attributed, at

least in part, to ROS-induced cardiolipin alterations.

The activity of complex I is considered a rate-limiting step for the mitochondrial ETC and thus is an important factor for the regulation of the oxidative phosphorylation process. In addition, complex I is an important locus of mitochondrial ROS production and therefore a likely source of reactive oxygen species in fatty liver diseases. The impairment of complex I, observed in liver mitochondria from CDD animals and attributable to ROS induced cardiolipin damage, may generate more superoxide radicals, triggering a vicious cycle of ROS-induced mitochondrial membrane damage that leads to mitochondrial malfunction and subsequently to hepatocyte injury (Figure 1). Long-chain free fatty acids which accumulate in nonalcoholic fatty liver are considered the initial source of this ROS damaging cycle<sup>[18]</sup>. These compounds have been shown to promote ROS production in mitochondria, although the mechanism(s) underlying this effect is still unclear<sup>[108,109]</sup>.

It is now accepted that individual components of the ETC may exist as large macromolecular assemblies, or so called supercomplexes<sup>[99]</sup>. Several biochemical and biophysical lines of evidence support the existence of such supercomplexes. Such supercomplex organization of ETC would likely increase the efficiency of electron/proton translocation and hence that of ATP synthesis<sup>[101]</sup>. Another important consequence of the supercomplex organization of ETC is that of minimizing the production of ROS<sup>[110]</sup>. Experimental evidence indicates that cardiolipin molecules participate in the structural organization and stabilization of these supercomplexes<sup>[100]</sup>. As mentioned above, the content of CL declines, while that of its

oxidized form increases in liver mitochondria from CDD animals. On this basis, it could be predicted that these CL alterations might be involved in the destabilization of the respiratory chain supercomplexes in fatty liver. Thus, the CL-dependent alterations of the ETC organization in supercomplexes could be another factor contributing to ROS generation and to mitochondrial bioenergetic decay in NAFLD. Cardiolipin-dependent destabilization of mitochondrial respiratory chain supercomplexes has been already reported in some pathophysiological situations, such as Barth syndrome<sup>[111]</sup> and diabetes<sup>[112]</sup>.

## APOPTOSIS IN NAFLD

Cell death in the liver and peripheral tissues has emerged as an important mechanism involved in the development and progression of NAFLD. An increase in hepatocyte cell death by apoptosis is typically present in patients with NAFLD and in experimental models of steatohepatitis<sup>[15,16]</sup>. Apoptosis may be executed by two fundamental pathways<sup>[113]</sup>: the extrinsic pathway is mediated by death receptors on the cellular surface and the intrinsic pathway is organelle-based. The extrinsic pathway is initiated by death receptors, including Fas, TNF-receptors and TNF-related apoptosis-inducing ligand receptors. These receptors trigger intracellular cascades that activate death-inducing proteolytic enzymes, especially caspases. Apoptosis can be initiated by mitochondria in the intrinsic pathway. In liver cells, mitochondrial dysfunction plays a critical role by amplifying the apoptotic signal and integrating both pathways into a final common pathway. Mitochondrial dysfunction results in the release of several proapoptotic proteins into the cytosol, including cytochrome c. This hemoprotein then forms an activation complex with apoptotic protease activating factor 1 and caspase 9, known as the apoptosome. This complex activates the downstream effectors caspases 3, 6 and 7, which execute the final apoptotic changes<sup>[114]</sup>. The mitochondrial events are regulated by the Bcl-2 family proteins, which comprises the antiapoptotic proteins Bcl-2 and Bcl-xL, the proapoptotic proteins, such as Bax and Bak, and the BH3-only proteins, such as Bad, Bim and Bid, which provide crosstalk between intrinsic and extrinsic pathways<sup>[115]</sup>. Indeed, activated caspase 8 cleaves the cytosolic protein Bid to a truncated active fragment t-Bid, which translocates to mitochondria and induces cytochrome c release<sup>[116]</sup>. Cardiolipin has been shown to be involved in several steps of the intrinsic pathway of the apoptotic process<sup>[32,33]</sup>, including mitochondrial permeability transition pore (MPTP) and cytochrome c release from mitochondria<sup>[117]</sup>.

## CARDIOLIPIN OXIDATION, MPTP AND CYTOCHROME C RELEASE FROM MITOCHONDRIA

MPTP is defined as the sudden increase of mitochondri-

al inner membrane permeability to low molecular weight solutes (1.5 kDa) in response to many stimuli, including high levels of  $\text{Ca}^{2+}$  and oxidant stress. MPTP activation by a combination of elevated intramitochondrial  $\text{Ca}^{2+}$  and oxidative stress promotes the collapse of transmembrane ion gradients, resulting in membrane depolarization, uncoupling of oxidative phosphorylation and ATP depletion<sup>[118,119]</sup>. Under conditions of low ATP levels, the cells are unable to maintain structural and functional integrity, including ion homeostasis. This results in outer membrane permeabilization and cytochrome c release into the cytoplasm to initiate pro-apoptotic signals. The duration of MPTP opening directly affects maintenance of ATP stores and cellular integrity. If MPTP opening is transient, the cell can recover; while in conditions of prolonged MPTP opening and ATP depletion, irreversible damage and cell death occur, predominantly through necrosis. A number of molecules were accepted as key structural components of the MPTP, including cyclophilin-D, a peptidyl-prolyl cis-trans isomerase, in the matrix, ADP/ATP and phosphate carriers in the inner membrane and VDAC (also known as porin) in the outer membrane<sup>[120]</sup>. Very recently, it was reported that reconstituted dimers of the  $\text{F}_0\text{F}_1$  ATP synthase, incorporated into lipid bilayers, form  $\text{Ca}^{2+}$ -activated channels with properties identical to those of the mitochondrial megachannel, the electrophysiological equivalent of the MPTP, indicating dimers of the  $\text{F}_0\text{F}_1$  ATP synthase to be a new putative component of the MPTP<sup>[121]</sup>.

$\text{Ca}^{2+}$  ions are considered the main inducers of the MPTP opening. Our studies have shown that exogenous added oxidized CL sensitized mitochondria to  $\text{Ca}^{2+}$ -induced MPTP opening<sup>[117]</sup>. Similarly, oxidation of endogenous CL by tert-Butyl hydroperoxide resulted in MPTP opening<sup>[122]</sup>.  $\text{Ca}^{2+}$  and oxidized CL seem to play a coordinated role in MPTP opening by interacting with components of the MPTP, probably ADP/ATP carrier. It was recently reported that hepatic mitochondria from CDD rats were more vulnerable to  $\text{Ca}^{2+}$ -induced MPTP activation in comparison to the respective control animals<sup>[123]</sup>. Pretreatment with cyclosporine A completely prevented  $\text{Ca}^{2+}$ -dependent mitochondrial swelling. It is conceivable that oxidized CL, the level of which increases in mitochondria from CDD animals<sup>[22]</sup>, could be a contributing factor to the enhanced susceptibility of these mitochondria to  $\text{Ca}^{2+}$ -dependent MPTP opening. Interestingly, the induction of MPTP opening by oxidized CL and  $\text{Ca}^{2+}$  is associated with the release of cytochrome c from mitochondria<sup>[117]</sup>.

Release of cytochrome c, as well as other proteins, from the mitochondria to the cytosol appears to play a central role in the induction of the apoptotic cascade that ultimately leads to the programmed cell death<sup>[117]</sup>. Cardiolipin has been implicated in the process of apoptosis in animal cells through its interaction with cytochrome c<sup>[32,33,117]</sup>. This hemoprotein is normally bound to the outer leaflet of the inner mitochondrial membrane, primarily to CL molecules<sup>[124]</sup>. The strength of the bind-



ing of cytochrome c to mitochondria is dependent on the fatty acyl chain composition of CL. Oxidation of CL promotes the detachment of cytochrome c from mitochondrial membrane<sup>[32,35]</sup>. Experimental evidence indicates that cytochrome c release from mitochondria during apoptosis occurs *via* a two-step process. This mechanism first involves the detachment of cytochrome c from the outer leaflet of the inner mitochondrial membrane, followed by permeabilization of the outer mitochondrial membrane and the release of cytochrome c into the cytosol space<sup>[33]</sup>. Oxidized cardiolipin, in the presence of Ca<sup>2+</sup>, induces the mitochondrial permeability transition pore opening<sup>[117]</sup>. In addition, CL oxidation seems to promote the permeabilization of the outer mitochondrial membrane, probably interacting with Bcl2 family proteins, such as Bax and Bid<sup>[125]</sup>. Thus, oxidized CL may have a critical role in several steps of the apoptotic process such as: the translocation of t-Bid to mitochondria with subsequent oligomerization of Bax and Bak leading to membrane permeabilization; cytochrome c dissociation from the inner mitochondrial membrane; and activation of MPTP, followed by the release of this hemoprotein from the mitochondrial intermembrane space to the cytosol (Figure 1). The fact that CL oxidation/depletion has been detected in liver mitochondria from CDD animals<sup>[22]</sup> suggests a potential role of these CL alterations in the apoptotic process associated with fatty liver. Indeed, apoptosis is an important mechanism of cell death in patients with NASH<sup>[126]</sup>. Thus, modulation of CL vulnerability to peroxidation may be a potential target for sensitizing cells to apoptosis in fatty liver. Another consequence of cytochrome c release from mitochondria is that it interferes with electron transport through ETC, resulting in ROS formation and triggering a new vicious cycle, which will worsen the disturbance.

## MITOCHONDRIAL-TARGETED ANTIOXIDANTS IN NAFLD

Several therapeutic agents have been tested for their ability to alleviate fatty liver diseases; however, no proven drug therapies have emerged as being highly effective. As mitochondrial oxidative stress is considered an important factor in the etiology of fatty liver, the majority of drug discovery efforts to date have centered on compounds that prevent mitochondrial ROS overproduction. Both traditional and new antioxidant compounds have been shown to reduce the development and/or reverse the pathological effects of fatty liver disease, presumably through their capability to decrease oxidative stress in tissues. Antioxidants which have been shown to be particularly effective in treating fatty liver disease include N-acetyl-L-cysteine, zinc, ursodeoxycholic acid,  $\alpha$ -tocopherol (vitamin E), lipoic acid, carnitine, S-adenosyl-L-methionine and polyphenolic compounds, such as silybin and resveratrol, just to name a few. Further information on antioxidant therapy for treatment of fatty liver diseases can be found in the following excellent

review articles<sup>[127-132]</sup>.

Despite a large number of experimental studies encouraging the antioxidant therapy in the treatment of NAFLD, there are few clinical trials that support the efficacy of these compounds. Some antioxidants have been shown to have a positive effect and others as ineffective, probably due to the fact that these compounds do not reach the site of free radical generation, especially when mitochondria are the primary source of ROS. It should be considered that in certain physiological conditions the effect of ROS are not injurious but may have important signaling roles in physiological regulatory mechanisms. For example, mitochondrial-mediated ROS production has been shown to play an important signaling role in the glucose-stimulated insulin secretory pathway in B-cells and is also involved in insulin signaling and sensitivity<sup>[133]</sup>. Recently, new antioxidant compounds have been synthesized by attaching them to a lipophilic triphenylphosphonium moiety in order to target these agents to the mitochondrion<sup>[134]</sup>. These new modified antioxidant agents have been shown to accumulate several hundredfold in the mitochondrion in response to the mitochondrial membrane potential. Indeed, mitochondrial-targeted ubiquinone, modified by conjugation to lipophilic cations, was shown to reduce cardiac ischemia/reperfusion injury<sup>[135]</sup>.

As discussed above, CL alterations, especially CL oxidation, may be involved in mitochondrial dysfunction, as well as in mitochondrial-mediated steps of the apoptotic process in NAFLD, such as MPTP opening and cytochrome c release from mitochondria. Therefore, compounds that target CL on the IMM preventing its oxidation would be particularly useful in improving mitochondrial function and reducing ROS generation. New mitochondrial-targeted antioxidant agents are cationic derivatives of plastoquinones which are selectively accumulated within mitochondria<sup>[136]</sup>. The mechanism by which these antioxidant compounds exert their antioxidant activity includes, in particular, prevention of mitochondrial cardiolipin oxidation by ROS attack. Although these mitochondria-targeted antioxidant agents can decrease mitochondrial ROS production, recent studies indicate they impair several reactions involved in mitochondrial bioenergetics<sup>[137]</sup>. Therefore, more studies are needed to demonstrate the safety and efficacy of these antioxidant agents in diseases for which mitochondrial dysfunction and oxidative stress are proposed to be important factors in their etiology, including NAFLD.

Melatonin, a hormonal product of the pineal gland, has been shown to protect against physiopathological states characterized by ROS overproduction<sup>[138]</sup>. The mechanism(s) by which this compound exerts these protective effects are not well established. Experimental evidence indicates that some of the protective effects of melatonin may be produced through its action at mitochondrial level by preventing ROS-induced cardiolipin oxidation<sup>[139,140]</sup>. In this respect, our studies have demonstrated that melatonin is able to prevent cardiolipin per-



oxidation in mitochondria isolated from heart and brain tissues. This effect may underlie the protection afforded by melatonin against mitochondrial dysfunction in heart ischemia/reperfusion<sup>[141]</sup> and brain aging<sup>[142]</sup>.

Another new family of agents that target CL in the inner mitochondrial membrane are the SS (Szeto-Schiller) peptide antioxidants<sup>[143]</sup>. These compounds have been shown to optimize cristae architecture, improve mitochondrial bioenergetics and reduce ROS production. In addition, these antioxidant peptides have been evaluated in numerous preclinical disease models characterized by bioenergetic failure. Additional studies are needed to better investigate the potential efficacy of these new CL-targeted antioxidants in diseases for which oxidant stress, CL abnormalities and mitochondrial dysfunction play a role in pathology, including fatty liver diseases.

## CONCLUSION

Strong evidence indicates that the pathogenesis of non-alcoholic fatty liver disease is linked to mitochondrial structural and functional alterations. ROS overproduction and altered oxidative phosphorylation are important factors involved in mitochondrial dysfunction. Oxidative damage to mitochondria alters mitochondrial respiratory chain complexes and mitochondrial DNA to partially block the flow of electrons. The subsequent increase in mitochondrial ROS formation triggers a vicious cycle of damage amplification. CL, the signature phospholipid of the mitochondria, is particularly prone to ROS-induced oxidative damage. This phospholipid plays a pivotal role in modulating the activity of a variety of mitochondrial bioenergetic reactions and processes, especially oxidative phosphorylation and coupled respiration. Cardiolipin is also an important player in different stages of the mitochondrial apoptotic process. A number of studies have demonstrated the involvement of CL alterations in mitochondrial dysfunction in multiple tissues in a variety of physiopathological states. In this review, we highlighted the potential roles played by CL alterations in mitochondrial bioenergetics decay and in the mitochondrial apoptotic process associated with nonalcoholic fatty liver disease. Thus, CL oxidation seems to affect the activity of several mitochondrial bioenergetic reactions in fatty liver, including electron transport chain and mitochondrial permeability transition, as well as the release of cytochrome c from mitochondria. The development of new and effective antioxidant strategies aimed at reducing the production of oxidants and hence CL oxidation in mitochondria offers great promise for the prevention and treatment of liver disease.

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