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World J Gastroenterol 2007 October 7; 13(37): 4967-4973
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, *Series Editor*

Novel interactions of mitochondria and reactive oxygen/nitrogen species in alcohol mediated liver disease

Sudheer K Mantena, Adrienne L King, Kelly K Andringa, Aimee Landar, Victor Darley-Usmar, Shannon M Bailey

Sudheer K Mantena, Adrienne L King, Kelly K Andringa, Shannon M Bailey, Department of Environmental Health Sciences, University of Alabama at Birmingham, Birmingham, AL 35294, United States
Aimee Landar, Victor Darley-Usmar, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, United States
Supported by NIH/NIAAA grants AA13395 (VDU) and AA15172 (SMB)
Correspondence to: Shannon M Bailey, Department of Environmental Health Sciences, Center for Free Radical Biology, University of Alabama at Birmingham, Ryals Building, Room 623,1530 3rd Avenue South, Birmingham, AL 35294, United States. sbailey@uab.edu
Telephone: +1-205-9347070 Fax: +1-205-9756341
Received: June 30, 2007 Revised: July 18, 2007

Abstract

Mitochondrial dysfunction is known to be a contributing factor to a number of diseases including chronic alcohol induced liver injury. While there is a detailed understanding of the metabolic pathways and proteins of the liver mitochondrion, little is known regarding how changes in the mitochondrial proteome may contribute to the development of hepatic pathologies. Emerging evidence indicates that reactive oxygen and nitrogen species disrupt mitochondrial function through post-translational modifications to the mitochondrial proteome. Indeed, various new affinity labeling reagents are available to test the hypothesis that post-translational modification of proteins by reactive species contributes to mitochondrial dysfunction and alcoholic fatty liver disease. Specialized proteomic techniques are also now available, which allow for identification of defects in the assembly of multi-protein complexes in mitochondria and the resolution of the highly hydrophobic proteins of the inner membrane. In this review knowledge gained from the study of changes to the mitochondrial proteome in alcoholic hepatotoxicity will be described and placed into a mechanistic framework to increase understanding of the role of mitochondrial dysfunction in liver disease.

Key words: Mitochondria; Alcohol; Liver; Oxidative stress; Nitric oxide; Proteomics; Post-translational modifications

Mantena SK, King AL, Andringa KK, Landar A, Darley-Usmar V, Bailey SM. Novel interactions of mitochondria and reactive oxygen/nitrogen species in alcohol mediated liver disease. *World J Gastroenterol* 2007; 13(37): 4967-4973

http://www.wjgnet.com/1007-9327/13/4967.asp

INTRODUCTION

Long term heavy alcohol consumption is the most prevalent cause of liver-related morbidity and mortality in the United States. Excessive alcohol consumption is estimated to be the third leading cause of preventable death in the United States with up to 12 000 deaths each year attributed to alcoholic liver disease[1]. While it has long been held that the severity of alcoholic liver disease is dependent on the dose and duration of alcohol consumption, it has become increasingly clear that other factors may play a significant role in the development of liver disease[2]. Studies show that only 25% of heavy drinkers will develop alcoholic steatohepatitis and even less than 10% will progress to cirrhosis[3]. These observations have led to the general hypothesis that environmental, genetic, metabolic and/or viral factors, also referred to as "hits", may influence the progression from simple fatty liver (steatosis) to more serious liver diseases[2].

Evidence supports a "multi-hit" hypothesis in the pathophysiologic sequelae of alcoholic liver disease wherein the "first-hit" involves lipid accumulation in hepatocytes (steatosis), followed by "second-hits" that lead to more serious conditions such as alcoholic steatohepatitis, fibrosis, cirrhosis, and cancer. Examples of "second-hits" include metabolic stressors such as obesity, hypercholesterolemia, and hyperglycemia, which are components of the cardiometabolic syndrome, and environmental stressors, which may include dietary factors and pollutants such as tobacco smoke. Recent epidemiologic and clinical studies indicate that environmental tobacco smoke, a widespread toxicant, may accelerate fibrogenesis and increase the severity of a number of chronic liver diseases including hepatocellular carcinoma, hepatitis C-mediated liver injury, and primary biliary cirrhosis[4-8]. Moreover, alcohol, tobacco smoke, and obesity have been shown to be synergistic risk factors for hepatocellular carcinoma in patients[9]. Taken together,

these studies clearly highlight the need to identify the molecular targets and mechanisms through which these potential stressors or "hits" interact to accelerate and worsen alcoholic liver disease.

Studies suggest a potential mechanistic link among chronic alcohol consumption and many of the proposed secondary stressors, which include increased oxidative damage, hypoxic stress, and disrupted redox cellular signaling. Interestingly, the mitochondrion is a primary target for many of these metabolic derangements, which can also differentially exacerbate hepatic pro-inflammatory responses. Furthermore, the mitochondrial proteome is exquisitely sensitive to modifications by reactive oxygen and nitrogen species (ROS/RNS) and thus offers a unique opportunity to investigate the molecular mechanisms underlying hepatic pathobiology from chronic alcohol consumption. Accordingly, this review article will present an overview of the emerging new roles of mitochondria and reactive species in the development of alcoholic liver disease.

MITOCHONDRIAL DYSFUNCTION IN ALCOHOLIC LIVER DISEASE: BIOENERGETIC DEFECTS AND OXIDANT PRODUCTION

The effects of chronic alcohol consumption on liver have been extensively studied and have advanced our understanding of the molecular mechanisms responsible for alcohol hepatotoxicity. In general, chronic consumption of alcohol causes liver disease *via* oxidative stress, hypoxia, upregulation of pro-inflammatory cytokines, and bioenergetic defects involving the interactions of multiple liver cell types. Early in the disease process gut-derived endotoxin[10] activates Kupffer cells, which release a variety of potentially toxic substances including cytokines and ROS/RNS that negatively affect hepatic stellate cells and hepatocyte functions[11]. Consequently, a number of responses occur in hepatocytes including increased ROS/RNS production, mitochondrial damage, and altered nitric oxide (NO)-dependent control of respiration[12-14]. This profound disruption in mitochondrial metabolism contributes, in part, to liver disease by placing hepatocytes under bioenergetic stress. This is important because the inability to maintain hepatic ATP levels will predispose the liver to permanent damage due to a depression in the ATP requiring anabolic pathways responsible for replacing damaged and/or lost cellular macromolecules.

One critical change to hepatic mitochondria after chronic consumption of alcohol is a decrease in the rate of ATP synthesis. Conclusive evidence shows that chronic alcohol exposure depresses the activities of all the oxidative phosphorylation complexes by approximately 30%-50%, except Complex Ⅱ[15,16]. Inhibition of mitochondrial protein synthesis due to alcohol-mediated damage to the mitochondrial DNA[13,17] and ribosomes[18,19] is proposed to contribute to decreased functioning of the oxidative phosphorylation system and depressed rates of ATP synthesis[20,21]. Recently, studies by Cunningham and colleagues have shown that both hepatic energy charge and NADH-linked respiration are decreased in mitochondria isolated from liver of non-human primates allowed to consume alcohol for 18 mo[22]; thus recapitulating the findings observed in rodent studies. This alcohol-dependent loss of mitochondrial function is also predicted to cause further increases in ROS/RNS production and oxidative damage to the organelle and liver.

During electron transport in the respiratory chain, electrons can "leak" from the respiratory complexes and be passed one at a time to molecular oxygen (O_2) to form low amounts of superoxide anion ($O_2^{\bullet-}$), which is proposed to be increased in hepatocytes due to alcohol-dependent alterations in mitochondria. Mitochondria contribute to the production of ROS in hepatocytes when alcohol is consumed through the metabolism of alcohol *via* distinct oxidative mechanisms and chronic alcohol-related alterations to the oxidative phosphorylation system[23,24]. First, ethanol metabolism increases the availability of reducing equivalents (i.e. NADH) to the mitochondrion, which results in the redox active semiquinone intermediates within Complexes Ⅰ and Ⅲ to be in a more "reduced" state, thereby facilitating the reduction of O_2 to $O_2^{\bullet-}$[25,26] (Figure 1). Moreover, in the chronic alcohol consumer, it is postulated that mitochondrial production of ROS is elevated not only due to increased NADH delivery to the respiratory chain, but also as a consequence of molecular defects to the respiratory complexes caused by chronic alcohol consumption[13,27-29].

The mechanism of $O_2^{\bullet-}$ production by Complex Ⅲ (ubiquinone cytochrome *c* reductase) is well understood, as it is linked to the reactions of the ubiquinone or "Q"-cycle[30]. As the levels of cytochrome *b* are decreased by chronic alcohol consumption[27,29] this defect is postulated to decrease the rate of re-oxidation of the ubisemiquinone anion ($Q^{\bullet-}$). This resulting increase in the steady-state levels of $Q^{\bullet-}$ would subsequently lead to increased $O_2^{\bullet-}$ in mitochondria from alcohol exposed subjects (Figure 1A). In contrast, the mechanism responsible for increased $O_2^{\bullet-}$ production by Complex Ⅰ (NADH dehydrogenase) in response to chronic alcohol consumption remains undefined because the sites responsible for generating $O_2^{\bullet-}$ are not known. For example, the semiquinone in the flavin mononucleotide and the various iron-sulfur centers of Complex I have all been implicated as potential sites of $O_2^{\bullet-}$ production (Figure 1B). Recent studies have demonstrated that the majority of $O_2^{\bullet-}$ generated in mitochondria respiring on the Complex Ⅱ-linked substrate succinate occurs *via* reverse electron transport into Complex Ⅰ[31-33]. This mechanism involves reverse electron flow from succinate to NAD^+ providing reducing equivalents to the redox carriers of Complex Ⅰ, which then function as sites of $O_2^{\bullet-}$ production (Figure 1C). The contribution of reverse electron flow through Complex Ⅰ may be enhanced as a consequence of alcohol-dependent defects to the complex[27,28]. Similarly, mitochondrial levels of ROS may be increased by chronic alcohol consumption as a consequence of increased mitochondrial CYP450 2E1 levels[34] and as a by-product from matrix enzymes such as α-ketoglutarate dehydrogenase[35,36]. These higher rates of ROS production in the alcohol-damaged mitochondria

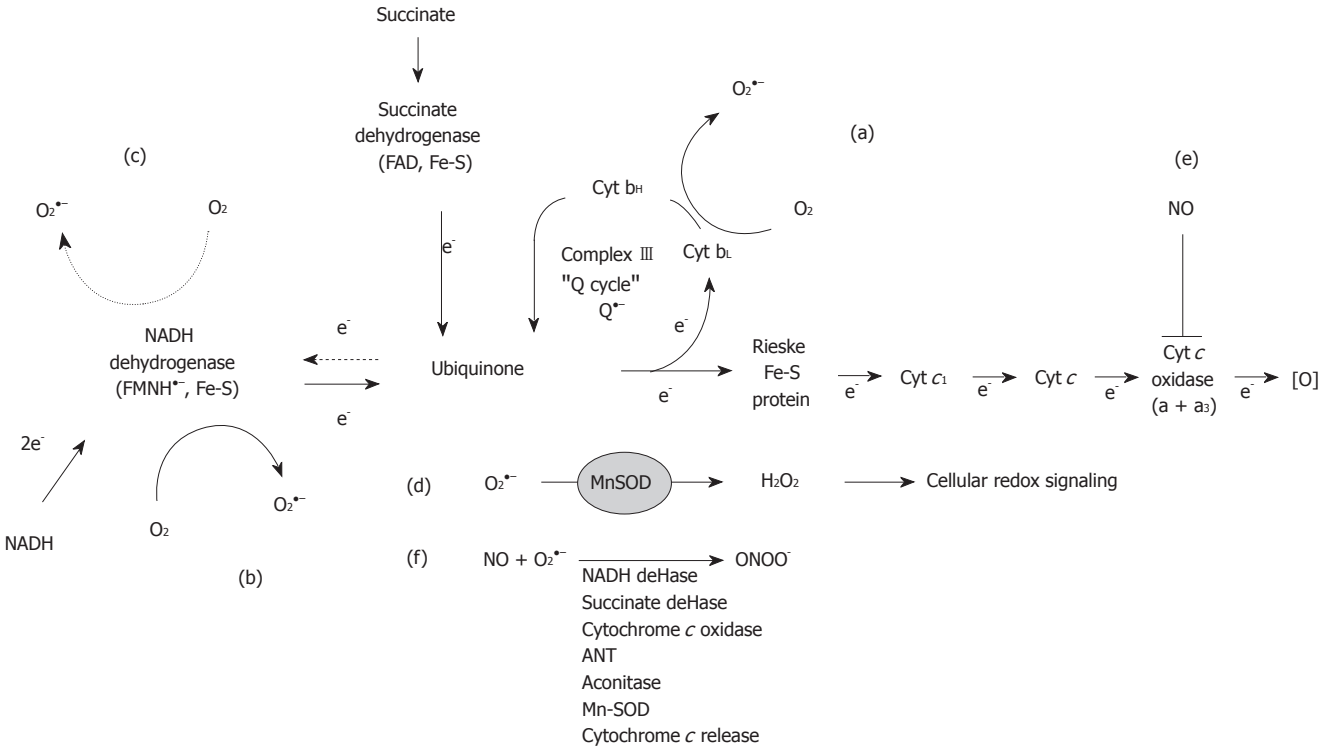

Figure 1 Superoxide production and reactive nitrogen species reactions in mitochondria. During electron transfer superoxide ($O_2^{\bullet-}$) is generated within Complexes Ⅰ (b, c) and Ⅲ (a) due to the presence of stable semiquionine anion species. Solid arrows illustrate forward electron flow, whereas dashed arrows indicate reverse electron flow through Complex Ⅱ to increase $O_2^{\bullet-}$ production in Complex Ⅰ (c). $O_2^{\bullet-}$ dismutation to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD) affects cellular redox signaling pathways (d). Increased iNOS expression leads to diffusion of nitric oxide (NO) into mitochondria where it inhibits cytochrome *c* oxidase (e), which increases $O_2^{\bullet-}$ generation.

are predicted to negatively affect mitochondrial function through oxidative damage to mitochondrial macromolecules and cellular function through modulation of cellular redox signaling pathways (Figure 1D).

NITRIC OXIDE, REACTIVE NITROGEN SPECIES, AND MITOCHONDRIA PHYSIOLOGY: IMPACT ON ALCOHOL HEPATOTOXICITY

In this section some of the key characteristics by which nitric oxide (NO) and RNS affect mitochondrial function in the context of alcoholic liver injury will be presented. As discussed above, several studies have demonstrated that chronic alcohol consumption increases hepatocyte ROS production presumably from the mitochondrial respiratory chain[12,25,37]. As these increased levels of ROS exceed those required for signal transduction and detoxification *via* antioxidant systems, mitochondrial DNA, protein, and lipid damage is predicted to be enhanced as a consequence of chronic alcohol exposure. In addition, NO production is increased in response to chronic alcohol *via* induction of inducible nitric oxide synthase (iNOS)[12,38]. This has important ramifications for toxicity because NO and its metabolite peroxynitrite ($ONOO^-$) have been implicated as key mediators of mitochondrial dysfunction[39,40]. Indeed, the detrimental effects of NO largely stem from excess NO diffusing into the mitochondrion and reacting with $O_2^{\bullet-}$ to produce $ONOO^-$, a reactive metabolite that can directly or indirectly participate in reactions leading to inactivation of mitochondrial proteins *via* post-translational modifications[41]. Moreover, it is known that NO can regulate mitochondrial function through reversible binding at the heme site in cytochrome *c* oxidase, which inhibits oxygen consumption[42,43] and through cross-talk mechanisms with soluble guanylate cyclase[44]. Potential impacts of NO and $ONOO^-$ on mitochondria are illustrated in Figure 1E and F.

Exposure of mitochondria to low concentrations of NO results in the reversible inhibition of cytochrome *c* oxidase activity due to the competition of NO with O_2 at the binuclear center of the enzyme[45-48]. Thus, NO has the effect to inhibit mitochondrial respiration. Studies from our laboratories have shown a novel role of NO in the pathogenesis of alcohol hepatotoxicity. We have demonstrated that the response of respiration to NO is altered by chronic alcohol consumption such that mitochondria from alcohol-fed animals are more sensitive to NO-dependent inhibition of respiration[14,38]. It is proposed that this loss of control of NO signaling per se, results in excessive inhibition of the respiratory chain leading to bioenergetic dysfunction (i.e. decreased ATP synthesis) and increased ROS production[49], which contribute to the development of alcoholic liver injury. The critical role of NO in alcohol hepatotoxicity is further supported because the increased sensitivity of mitochondrial respiration to inhibition by NO is absent in iNOS knockout mice fed alcohol chronically[38]. Therefore,

this finding supports the hypothesis that the early induction of iNOS modifies the control of NO-dependent respiration, which contributes to the development of alcohol-dependent steatosis and inflammation[38,50].

It has been shown that NO-mediated inhibition of respiration alters activation of hypoxia responsive targets in cells such that increased NO interferes with the upregulation of molecules required by cells to adapt to hypoxic stress[51,52]. This may also have the effect of changing oxygen gradients in tissues. Indeed, this is precisely the series of events that we propose occurs with NO in alcohol hepatotoxicity[49], which has been shown to be associated with tissue hypoxia[53,54]. Several studies have clearly demonstrated that acute alcohol exposure "steepens" the hepatic oxygen gradient as a consequence of ethanol oxidation[55,56], thus rendering the pericentral regions of the liver lobule hypoxic[53]. Thus, the effect of acute hypoxia in an individual actively drinking is proposed to be exaggerated in the chronic alcohol consumer as a consequence of the alcohol dependent increase in inhibition of mitochondrial respiration by NO[14]. In fact, studies by Arteel and colleagues demonstrate that chronic alcohol consumption increases liver hypoxia[54]. This concept is important to consider because it suggests that the interaction of hypoxia and disrupted NO signaling in mitochondria may accelerate the progression from steatosis to more severe liver pathologies. Moreover, it is these conditions that will lead to the irreversible post-translational modification and inactivation of mitochondrial proteins, which are proposed to contribute to alcohol-mediated mitochondrial dysfunction and hepatic pathobiology.

MODIFICATION TO THE LIVER MITOCHONDRIAL PROTEOME IN RESPONSE TO CHRONIC ALCOHOL CONSUMPTION

From the discussion above it is clear that increased NO and altered control of respiration by NO can lead to excessive ROS/RNS formation and the post-translational modification of mitochondrial proteins. Specifically, it has been shown that ROS, RNS, and reactive lipid species can modify critical amino acid residues thereby disrupting the catalytic function of proteins. As stated above, these toxic effects on proteins largely stem from the diffusion of NO into mitochondria and its reaction with $O_2^{\bullet-}$ to generate more reactive species, such as $ONOO^-$, other secondary RNS, and reactive lipid products. Because alterations in the redox state of protein thiols are important in regulating mitochondrial function such as respiration and oxidant production[57,58], the identification of proteins with oxidized and/or modified thiol groups is critical for elucidating the mitochondrial defects that contribute to alcoholic liver disease.

A number of reversible and irreversible modifications to cysteine residues are known to occur upon interaction of free sulfhydryl groups (-SH) with ROS, RNS, and reactive lipids. Reversible modifications to thiols include the formation of nitrosothiols (P-SNO), sulfenic acids (P-SOH) and mixed disulfides (P-SSG). Cysteine residues can also be irreversibly oxidized to higher oxidation states such as sulfinic ($P\text{-}SO_2H$) and sulfonic ($P\text{-}SO_3H$) acids by ROS and RNS. Each of these modifications has the potential to elicit a unique biological response that may disrupt normal mitochondrial functions. Studies have linked the oxidation and modification of protein thiols with induction of the mitochondrial permeability transition, alterations in energy metabolism, and oxidant production[59]. Our laboratories have shown an alcohol-dependent loss of function of the mitochondrial low K_m aldehyde dehydrogenase (ALDH) as a consequence of oxidative modification of thiols[60]. This is significant because inactivation of this important detoxification enzyme could potentially lead to increased levels of acetaldehyde and other reactive aldehyde species, which themselves have been shown to inactivate proteins through cysteine modifications[61,62]. Recently, Song and colleagues also demonstrated alcohol-dependent inactivation of ALDH and several β-oxidation enzymes *via* oxidation and nitrosation of thiols[63]. Taken together, these findings are consistent with the concept that modification of protein thiols may contribute to alcoholic steatosis and mitochondrial dysfunction through inactivation of proteins critical to the energy conservation pathways in liver.

While changes in the redox status of cysteine residues are known to affect mitochondrial activities, other types of post-translational modification should be considered as well. Tyrosine nitration ($P\text{-}NO_2$) is generally thought to induce permanent loss of protein function[64] however recent studies suggest that tyrosine nitration may be oxygen-regulated, target-selective, and reversible such that it may function as a "nitrative signaling" process controlling mitochondrial energy metabolism[65,66]. Studies have also established that 4-hydroxynonenal (4-HNE), a reactive lipid, may play an important role in alcohol hepatotoxicity *via* modification of key signaling proteins[67,68] and inhibition of cytochrome *c* oxidase activity[69,70]. 4-HNE has also been shown to inhibit several of the matrix dehydrogenase enzymes[71,72] and proteins of the β-oxidation system[73]. Moreover, electrophilic lipids like 4-HNE and the cyclopentenone 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 have been shown to co-localize to mitochondria and induce mitochondrial ROS production[74]. These results provide strong evidence suggesting that reactive lipids may disrupt mitochondrial function through selective targeting of mitochondrial enzymes.

In addition to post-translational modifications, it is also proposed that changes in the levels (i.e. abundances) of proteins that comprise the mitochondrial proteome may also negatively affect mitochondrial bioenergetics leading to liver injury. Early studies by Coleman and Cunningham established a key link between the chronic alcohol-related defects in Complexes Ⅰ, Ⅲ, Ⅳ, and Ⅴ and losses in the 13 mitochondrial encoded polypeptides and redox centers that comprise the oxidative phosphorylation system complexes[27,75,76]. Similarly, using a variety of proteomics approaches our laboratories have extended these findings to demonstrate a coordinated decrease in both mitochondrial and nuclear encoded subunits of the respiratory complexes, particularly those that comprise cytochrome *c* oxidase[12,13]. Moreover, proteomic analyses revealed that 40 additional mitochondrial proteins had altered levels in response

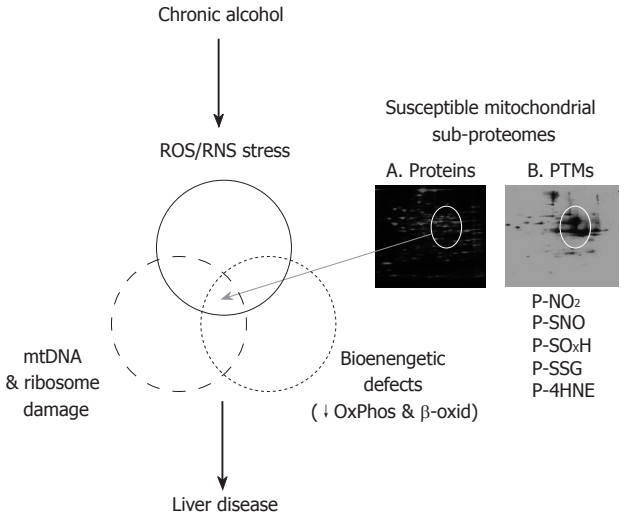

Figure 2 Overlapping alterations to the liver mitochondrial proteome contribute to the development of alcohol-induced liver disease. This figure illustrates the concept that there is a unique mitochondrial sub-proteome that when altered by chronic alcohol mediated mtDNA/ribosome damage, increased ROS/RNS production, and energy deficits contribute to the development of alcohol hepatotoxicity. These alterations involve changes in both protein levels (panel A) and post-translational modifications (PTMs, panel B) to this susceptible population of mitochondrial proteins.

to chronic alcohol consumption[13]. These studies are interesting in that, previously unidentified alterations in several key energy metabolism enzymes of β-oxidation, the TCA cycle, and amino acid metabolism, as well as several mitochondrial chaperones were found to be altered by chronic alcohol exposure. Importantly, these changes can also be linked to pathways with a clear impact on one of the primary pathologies of alcoholic liver disease, i.e. steatosis. These findings highlight the power of proteomics to detect previously unidentified alterations to "vulnerable" mitochondrial sub-proteomes following chronic alcohol consumption that contribute, in part, to the development of alcoholic liver disease (Figure 2). These alterations may include changes in protein levels as well as post-translational modifications to this susceptible sub-set of mitochondrial proteins.

In summary, mitochondria play a variety of roles in a number of essential cellular functions including energy production and homeostasis, redox cell signaling, and apoptosis. Disruption of mitochondrial function, manifested by the inability to maintain cellular ATP levels is critical to the development of chronic alcohol-induced liver injury. Moreover, as a primary source for the formation of ROS and RNS, the mitochondrion is recognized as a critical component involved in cellular stress responses. While the sequence of events leading to alcohol induced mitochondrial dysfunction and liver injury remains undefined, emerging evidence suggests that: (1) interaction of NO with the respiratory chain may predispose hepatocytes to hypoxic injury and (2) post-translational modifications of critical residues within mitochondrial proteins by reactive species may alter cellular functions including energy metabolism and redox signaling. Similarly, an alcohol-mediated increase in ROS/RNS can damage mitochondrial DNA and ribosomes resulting in decreased mitochondrial protein synthesis, which ultimately translates into a severe mitochondrial dysfunction. Investigations have provided novel mechanistic information regarding the impact of chronic alcohol consumption on the mitochondrial proteome; providing unique insight into the molecular mechanisms responsible for disease. This knowledge will clearly advance the design and testing of novel mitochondria-specific therapeutics in the treatment of alcoholic liver disease.

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