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Review

A Review of the Role of Reactive Oxygen and Nitrogen Species in Alcohol-induced Mitochondrial Dysfunction

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Our understanding of the mechanisms involved in the development of alcohol-induced liver disease has increased substantially in recent years. Specifically, reactive oxygen and nitrogen species have been identified as key components in initiating and possibly sustaining the pathogenic pathways responsible for the progression from alcohol-induced fatty liver to alcoholic hepatitis and cirrhosis. Ethanol has been demonstrated to increase the production of reactive oxygen and nitrogen species and decrease several antioxidant mechanisms in liver. However, the relative contribution of the proposed sites of ethanol-induced reactive species production within the liver is still not clear. It has been proposed that chronic ethanol-elicited alterations in mitochondria structure and function might result in increased production of reactive species at the level of the mitochondrion in liver from ethanol consumers. This in turn might result in oxidative modification and inactivation of mitochondrial macromolecules, thereby contributing further to mitochondrial dysfunction and a loss in hepatic energy conservation. Moreover, ethanol-related increases in reactive species may shift the balance between pro- and anti-apoptotic factors such that there is activation of the mitochondrial permeability transition, which would lead to increased cell death in the liver after chronic alcohol consumption. This article will examine the critical role of these reactive species in ethanol-induced liver injury with specific emphasis on how chronic ethanol-associated alterations to mitochondria influence the production of reactive oxygen and nitrogen species and how their production may disrupt hepatic energy conservation in the chronic alcohol abuser.

Keywords: Free radical; Mitochondria; Alcohol; Liver; Energy metabolism

MECHANISMS OF CHRONIC ETHANOL-RELATED LIVER INJURY

The development of alcohol-induced liver injury is thought to be influenced by numerous factors including direct physiochemical properties of ethanol, indirect effects on metabolism, oxidative stress, and immunologic and inflammatory events that are initiated by ethanol (Fig. 1). The direct effects of ethanol within the liver relate both to its physical and chemical properties. Ethanol can alter the physical properties of biological membranes and induce changes in structure.[1] Furthermore, the oxidative metabolism of ethanol generates numerous reactive species, which include acetaldehyde, reactive oxygen species (superoxide and hydrogen peroxide), reactive nitrogen species (nitric oxide and peroxynitrite), the 1-hydroxyethyl radical of ethanol, and various lipid peroxide species, which include 4-hydroxynonenal and malondialdehyde. These agents can oxidize or form adducts with macromolecules, thus compromising their respective functions within the cell. Thus, the ethanol-related generation of reactive species may result in the generation of important initiators that compromise the functional integrity of the hepatocyte, leading to cell injury or death which is central to the development of the later stages of alcoholic liver disease.

An equally important role of ethanol in the development of pathology is its participation in an

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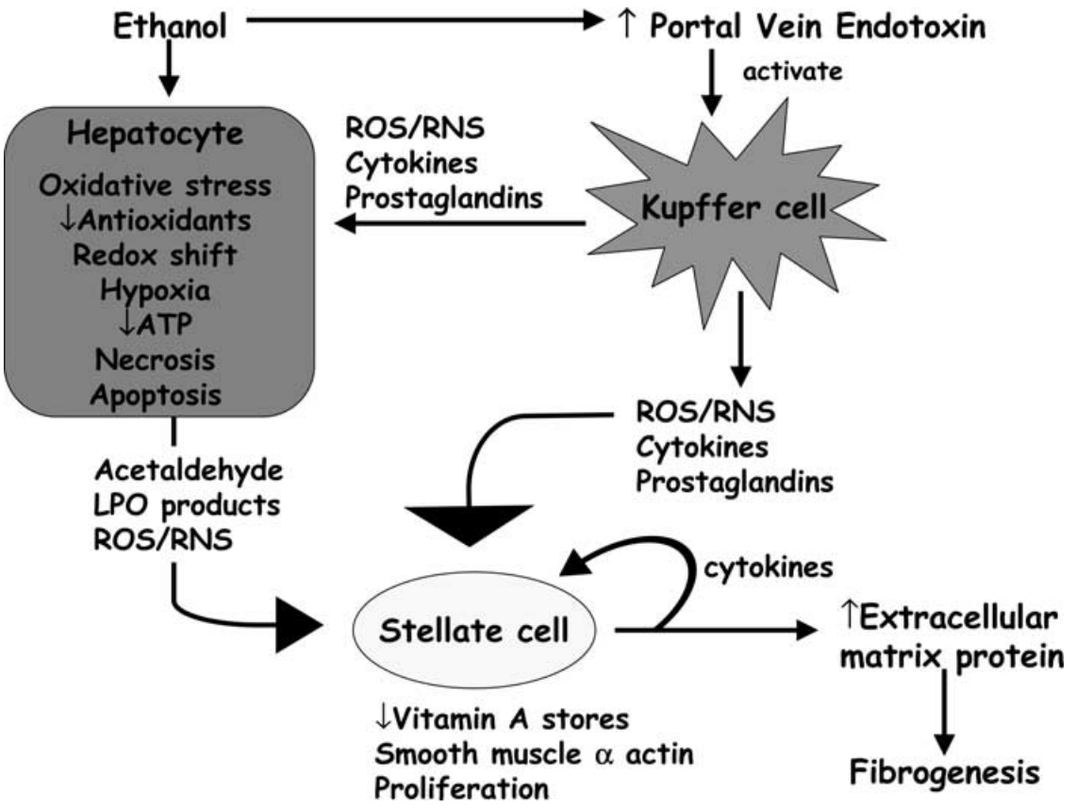

FIGURE 1 Effects of alcohol on liver cells. Chronic alcohol consumption triggers a series of events in hepatocytes, Kupffer cells, and hepatic stellate cells that are all believed to play important roles in the initiation and progression of alcohol-induced liver injury.

inflammatory response. There is strong evidence that in order to progress beyond the early fatty liver stage and develop hepatic inflammation and perivenous necrosis, increased exposure of the liver to gut-derived endotoxin must occur.[2] Elevated exposure to endotoxin is thought to be the result of an ethanol-related increase in the permeability of the gut to this pro-inflammatory agent.[3,4] Endotoxin then activates Kupffer cells, the resident macrophage of liver, which stimulates hepatocyte metabolic activity via elevated production of reactive species and pro-inflammatory cytokines (Fig. 1). This increased metabolic activity in hepatocytes,[5] which causes increased production of reactive species and results in the development of hypoxia in the perivenous region of the liver lobule, contributes to cell death, presumably by both necrotic and apoptotic mechanisms in hepatocytes.

An event apparently concurrent with chronic ethanol-induced inflammation is the transformation of the perisinusoidal hepatic stellate cell from its normal quiescent state to an "activated" state. Activation of stellate cells includes the loss of vitamin A stores, phenotypic change to a myofibroblast-like cell positive for smooth muscle α-actin, proliferation and increased production and secretion of extracellular matrix protein (Fig. 1).[6] The continued production of collagen by stellate cells, termed fibrogenesis, results ultimately in fibrosis, the deposition of collagen fibers within the liver lobule. The transition of stellate cells to activated myofibroblast-like cells and the production of collagen is largely controlled by cytokines produced within the stellate cell, other liver cells and infiltrating inflammatory cells.[7] Studies have demonstrated that reactive species and lipid peroxidation products appear to up-regulate the synthesis of fibrogenic cytokines and type 1 collagen.[8] Thus, ethanol-associated increases in reactive species and lipid peroxides in hepatocytes may play an important role in hepatic fibrogenesis, perhaps through their effects on Kupffer cells and stellate cells (Fig. 1).

IMPORTANCE OF REACTIVE OXYGEN AND NITROGEN SPECIES IN ETHANOL-INDUCED LIVER INJURY

Typically oxidative stress is defined as a situation where cells are exposed to conditions that increase the production of reactive species or decrease antioxidant defense mechanisms, or both. Over the past three decades considerable evidence has been accumulated which clearly demonstrates that

oxdative mechanisms contribute to the pathology observed in liver following chronic exposure to ethanol.[9–12] In the rat model of alcohol-induced liver toxicity in which ethanol is administered via intragastric infusion, the importance of oxidative stress was supported by the observation of a significant correlation between lipid peroxidation products and hepatic collagen accumulation[13] and perivenular or bridging fibrosis.[14] Moreover, these bioreactive aldehydes induced the expression of collagen in stellate cells in culture.[13] Studies by Greenwel and colleagues[15] reported a direct connection between reactive species production and fibrogenesis by demonstrating that acetaldehyde-induced upregulation of α1(I) procollagen gene expression in cultured stellate cells was mediated, in part, by increases in hydrogen peroxide. These studies have been extended and involvement of the PI3K and ERK1/2 signal transduction pathways have been implicated in acetaldehyde-induced upregulation of α2(I) collagen and fibronectin gene expression in human stellate cells.[16]

Oxidative mechanisms and free radical formation are likely involved in the progression of alcohol-induced liver injury, as demonstrated by increased levels of lipid radicals[17,18] and the 1-hydroxyethyl radical of ethanol[17,19] in the bile of rats administered ethanol by intragastric infusion. Although the mechanisms responsible for the production of these free radical species are not clear, it is possible that enhanced production of reactive species via the NADPH oxidase of Kupffer cells may be involved because the concentration of the 1-hydroxylethyl radical of ethanol and hepatic damage were decreased by the Kupffer cell inhibitor, gadolinium chloride.[19] However, the participation of oxidative mechanisms within hepatocytes cannot be discounted because ethanol-induced reactive species detected in the bile would be expected to originate from hepatocytes and not the sinusoidal Kupffer cells.[2]

Reactive nitrogen species have also been implicated in alcohol-induced liver injury. Nitric oxide is produced by hepatocytes and Kupffer cells from L-arginine via the enzyme, nitric oxide synthase. Changes in nitric oxide production induced by acute and chronic treatments have been conflicting with both increased[20,21] and decreased[22,23] nitric oxide production observed as a consequence of chronic ethanol exposure. Similar conflicting results have been reported for nitric oxide levels after acute ethanol exposure.[24] These ethanol-related alterations in liver nitric oxide levels have been proposed to be derived from the Kupffer cells primarily, with few changes attributed to hepatocyte nitric oxide production. However, very low concentrations of ethanol greatly potentiated TNFα/LPS-induced nitric oxide production in isolated rat hepatocytes.[25] Furthermore, gadolinium chloride, a Kupffer cell inhibitor, did not abolish the increase in nitric oxide measured in the effluent perfusate of livers infused with ethanol, suggesting ethanol stimulated hepatocyte nitric oxide production.[26] The diffusion-limited reaction of nitric oxide with superoxide yields peroxynitrite, a highly reactive oxidant, which has also been implicated in ethanol-induced injury in hypoxic liver.[27] In contrast, others have argued that ethanol-related increases in nitric oxide are protective against oxidative injury by scavenging superoxide or downregulating its production.[28–30] These conflicting results warrant further investigation into the potential role of reactive nitrogen species and the mechanisms responsible for their generation in the alcoholic liver. Taken together, the data presented in the above paragraphs undeniably supports the hypothesis that increased concentrations of reactive oxygen and nitrogen species might be critical for the initiation and progression of alcoholic liver disease.

CHRONIC ETHANOL-RELATED ALTERATIONS TO THE OXIDATIVE PHOSPHORYLATION SYSTEM

Some of the earliest changes induced by ethanol consumption occur at the level of the mitochondrion. The major change to hepatic mitochondria after chronic ethanol consumption is a decrease in the rate of ATP synthesis. Because the mitochondrial oxidative phosphorylation system is a multi-component structure, ethanol-related lesions to any or all of the respiratory complexes could give rise to an overall decrease in the rate of ATP synthesis. The oxidative phosphorylation system is composed of five functional complexes (Fig. 2). Complex I (NADH-ubiquinone reductase) and complex II (succinate dehydrogenase) transfer electrons from NADH and succinate, respectively, to ubiquinone. Complex III (ubiquinone-cytochrome c reductase) transfers an electron from reduced ubiquinone to cytochrome c and complex IV (cytochrome oxidase) facilitates electron transfer from cytochrome c to molecular oxygen. Complex V is the mitochondrial ATP synthase.

To date, there is conclusive evidence that chronic ethanol consumption decreases the activities of all of the oxidative phosphorylation components, except complex II (see reviews by Hoek[31] and Cunningham *et al.*[32]) Chronic ethanol consumption decreases the activity and heme content of cytochrome oxidase to 50–70% of control values.[33,34] Similarly, cytochrome b content of complex III[34,35] and select iron-sulfur centers of complex I[36] and ATP synthase activity[37,38] are also depressed in mitochondria isolated from animals fed ethanol chronically. Evidence has been provided that decreases in the concentrations and activities of these specific segments of the oxidative phosphorylation system

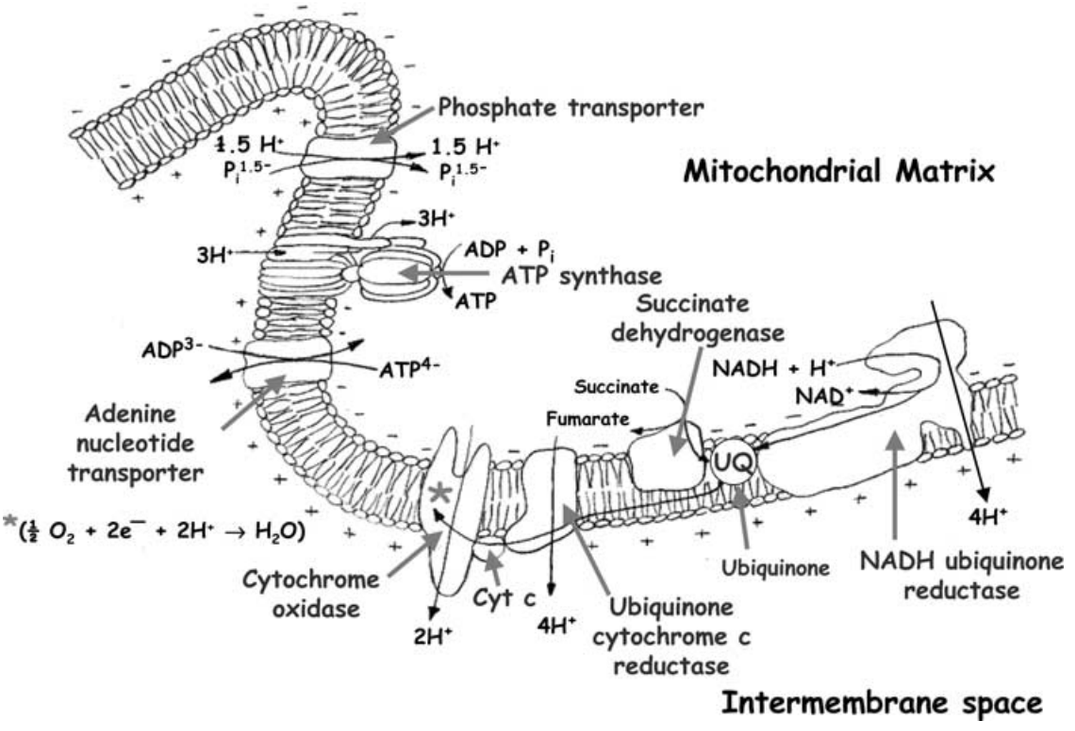

FIGURE 2 Components of the oxidative phosphorylation system as arranged in the mitochondrial inner membrane.

contribute to the chronic ethanol-related depression in the rate of ATP synthesis.[33]

The mammalian mitochondrion has a circular genome that encodes for 13 polypeptides of the oxidative phosphorylation system, which include seven subunits of the NADH ubiquinone reductase complex, three subunits of cytochrome oxidase, two subunits of the membrane-spanning (F_o) portion of the ATP synthase and cytochrome b. Interestingly, these polypeptides are all components of those segments of the oxidative phosphorylation system that exhibit decreased activity due to chronic ethanol consumption. These early observations suggested that chronic ethanol feeding impaired the biosynthesis of these mitochondrially encoded polypeptides. Studies by Coleman and Cunningham[35] subsequently demonstrated that the steady-state concentrations and rate of synthesis of all 13 mitochondrially encoded polypeptides were decreased in mitochondria from ethanol-fed animals. The ethanol-related lesion in the mitochondrial protein synthesis mechanism was due to decreased translation because mitochondrial DNA replication and transcription were unaffected in this model of chronic alcohol consumption.[39]

Studies by Cahill *et al.*[40] established that the chronic ethanol-related depression in mitochondrial protein synthesis was due to structural and functional alterations to the mitochondrial ribosome. This work has been extended by Patel and Cunningham[41] in a study which demonstrated that the sedimentation and diffusion coefficients of mitochondrial ribosomes isolated from animals chronically fed ethanol were significantly lower than those measured in controls. These observations indicated that an ethanol-related shape change occurred in the mitochondrial ribosome. Moreover, mitochondrial ribosomes from ethanol-fed animals had a greater tendency to dissociate into the two ribosomal subunits which resulted in decreased translational capacity as compared to controls.[41] These findings demonstrate that chronic ethanol-related losses in mitochondrial ribosome structure and function lead to impaired mitochondrial protein synthesis which might, in turn, contribute to the ethanol-related depression in ATP synthesis by mitochondria. These disturbances in structure and loss of function of the oxidative phosphorylation system are also likely to be associated with increased production of reactive species and oxidative injury to the mitochondrion in livers from chronic ethanol consumers. This suggestion will be presented in detail in the following sections.

MITOCHONDRIA AS A SOURCE OF REACTIVE SPECIES IN HEPATOCYTES EXPOSED TO ETHANOL

Despite an increasing body of evidence that demonstrates a role of reactive oxygen and nitrogen

Figure 3

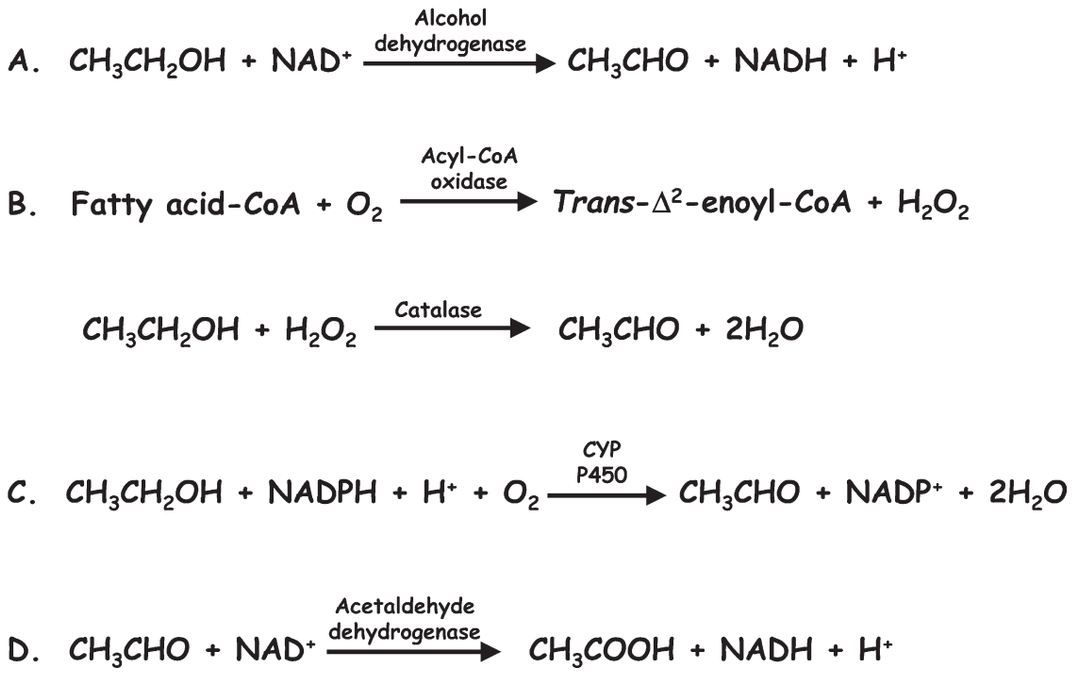

FIGURE 3 Reactions involved in the oxidative metabolism of ethanol and acetaldehyde.

species in ethanol-induced liver injury, the relative contribution of the mitochondrion, the cytochrome P450 system, and other proposed intracellular sites, like xanthine oxidase, nitric oxide synthase or peroxisomes, to the increase of reactive species within hepatocytes exposed to ethanol is still not established. Moreover, the contribution of non-parenchymal cells including the Kupffer cells and endothelial cells to the overall increase in liver oxidant production is also not known. We propose that hepatocyte mitochondria may play a significant role in the production of reactive species when ethanol is consumed. The ability of ethanol to stimulate the mitochondrial production of reactive species in hepatocytes is linked to the chronic ethanol-induced alterations to the oxidative phosphorylation system that were discussed above and the metabolism of ethanol via distinct oxidative processes. These concepts will be expanded on in subsequent sections.

The metabolism of ethanol via cytosolic alcohol dehydrogenase is the major pathway for the oxidation of ethanol in liver because the alcohol dehydrogenase isoenzyme in the adult human liver has a K_m value in the range of 0.5–1.0 mM (Fig. 3A). Therefore, under conditions of moderate ethanol consumption hepatic alcohol dehydrogenase will metabolize the majority of the ethanol. However, peroxisomal β-oxidation of fatty acids might also participate in ethanol metabolism as hydrogen peroxide generated during β-oxidation might serve as a co-substrate for the oxidation of ethanol via catalase (Fig. 3B).[42] It is possible that the catalase-mediated reaction might contribute more to ethanol oxidation due to increased peroxisomal oxidation of fatty acids in chronic alcohol consumers because there is typically an increase in liver fatty acids observed in these individuals.[43]

The microsomal system also contributes to ethanol metabolism via catalysis by several cytochrome P450 isoenzymes including 2E1, 1A2 and 3A4 (Fig. 3C).[44] Chronic ethanol consumption induces the 2E1 isoform, thus its contribution to ethanol metabolism may be increased in the chronic alcohol consumer. It has been demonstrated that the 2E1 isoform can generate hydrogen peroxide in the presence and absence of oxidizable cosubstrate[45] and a high correlation between the production of reactive oxygen species and the amount of 2E1 protein was found in liver microsomal samples isolated from rats fed ethanol chronically.[46] Moreover, the increased rate of hydroxyl radical production induced in microsomes isolated from chronic ethanol fed rats may cause oxidative damage to the hepatocyte as a result of chronic ethanol consumption.[47] Furthermore, studies by Nieto *et al.*[48,49] demonstrate that reactive species released from HepG2 cells over-expressing 2E1 stimulate proliferation and collagen expression in hepatic stellate cells in co-culture. These results indicate that reactive species derived from 2E1 in hepatocytes might help to trigger those early events that eventually lead to liver fibrosis in

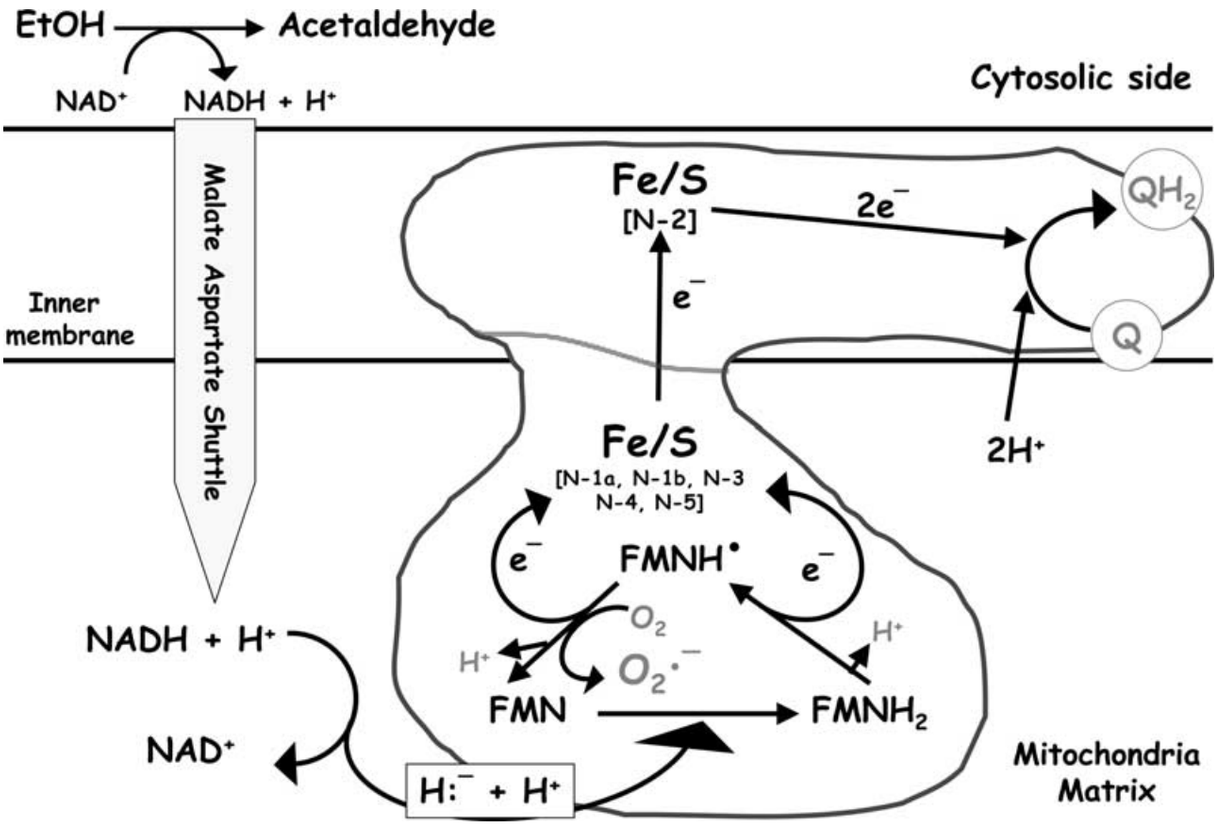

FIGURE 4 Ethanol (EtOH)-induced stimulation of superoxide anion ($O_2^{\bullet -}$) via the NADH ubiquinone reductase. The ability of the flavin mononucleotide to serve as both a one- and two-electron donor results from the presence of a stable semiquinone intermediate, $FMNH^{\bullet}$, in complex I and it is this form that can transfer its unpaired electron to molecular oxygen to generate the superoxide anion. Only six of the 8–9 iron-sulfur centers present within complex I are shown in this model. Release of protons (H^+) is to the aqueous environment.

the alcohol abuser. Thus, in any discussion of the role of the mitochondrion in the generation of reactive inflammatory mediators, it must be recognized that cytochrome P450 isozymes also contribute to oxidant levels in hepatocytes, particularly after chronic ethanol consumption.

Metabolism of ethanol by any of the mechanisms listed above gives rise to acetaldehyde, which is metabolized by hepatic aldehyde dehydrogenase in the mitochondria matrix (Fig. 3D). As a consequence of ethanol oxidation by alcohol dehydrogenase and the succeeding oxidation of acetaldehyde there is a significant increase in the hepatic NADH/NAD^+ ratio. This shift in the cellular redox state occurs both in the cytoplasm and the mitochondrion as determined by an increase in the lactate/pyruate and β-hydroxybutyrate/acetoacetate ratios, respectively.[50,51] When ethanol is oxidized the mitochondrial, low K_m aldehyde dehydrogenase generates most of the NADH within the mitochondrion whereas the reducing equivalents of cytosolic NADH generated during ethanol oxidation by alcohol dehydrogenase are transported into mitochondria primarily via the malate-aspartate shuttle system. Thus, ethanol oxidation increases the availability of oxidizable NADH to the mitochondrial electron transport chain.

It is well established that the mitochondrion can be a significant source of reactive oxygen species, specifically superoxide anion and hydrogen peroxide.[52] Studies indicate that the NADH-ubiquinone reductase (complex I) may generate superoxide during the re-oxidation of the flavin mononucleotide present within the enzyme complex (Fig. 4).[53] Electron transfer within the ubiquinone-cytochrome c reductase (complex III) involves a ubiquinone semiquinone intermediate[54] that has been shown to reduce molecular oxygen to superoxide (Fig. 5).[55] Thus, in the chronic ethanol consumer increased production and steady-state levels of reactive species in the mitochondrion would be expected, not only due to increased NADH generated during ethanol oxidation, but also as a consequence of those chronic ethanol-induced lesions to mitochondrial complexes I and III. Evidence for acute and chronic ethanol consumption to increase reactive oxygen species production within mitochondria will be presented in the following sections.

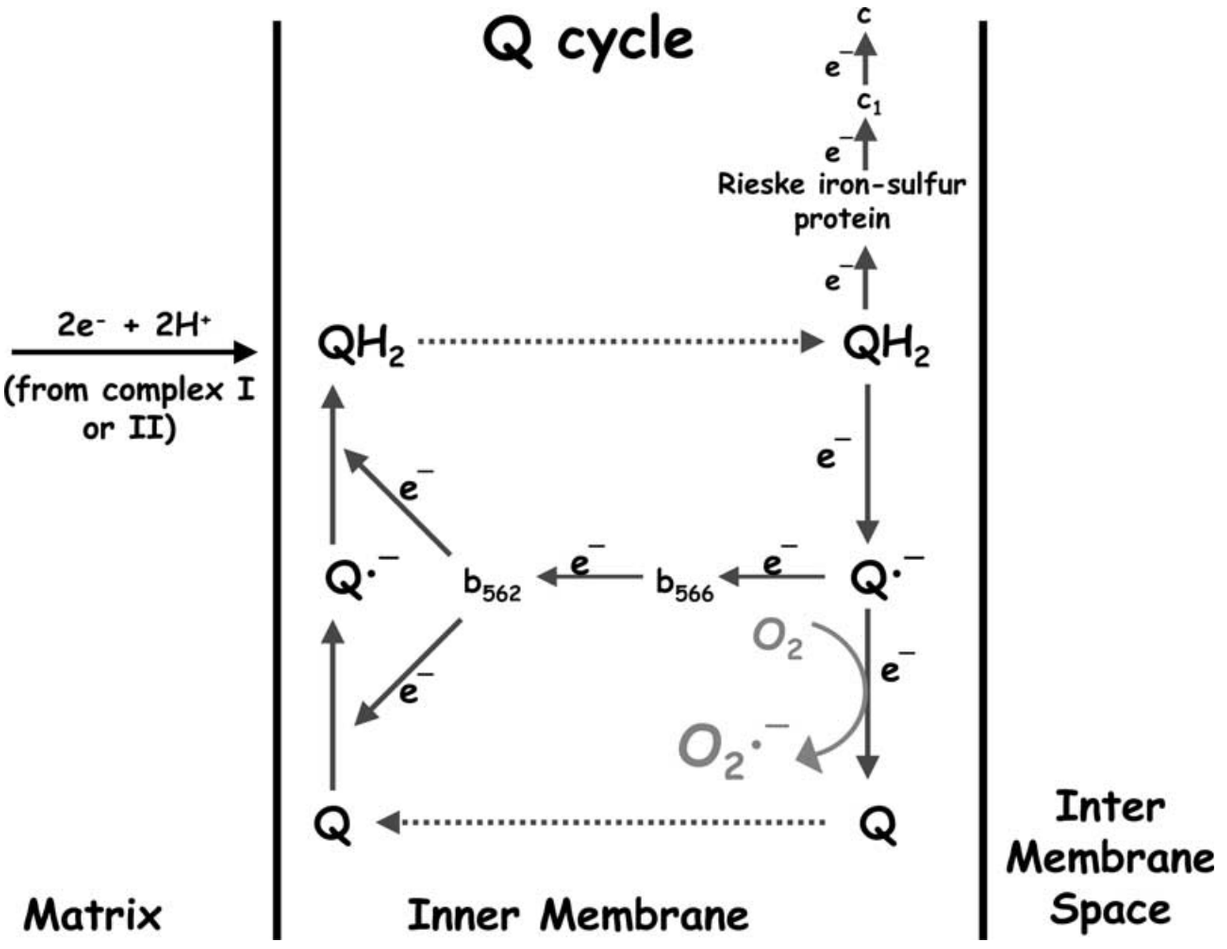

FIGURE 5 Generation of superoxide anion ($O_2^{\bullet -}$) during the Q cycle present within the ubiquinone-cytochrome c reductase. The core of the Q cycle is that ubiquinol (QH_2) undergoes a two-cycle reoxidation in which the ubquinone semiquinone anion ($Q^{\bullet -}$) is generated, which can transfer its unpaired electron to molecular oxygen and generate superoxide anion. For a more detailed description of the Q cycle please refer to Schultz and Chan.[80]

ACUTE ALCOHOL EXPOSURE AND INCREASED MITOCHONDRIAL REACTIVE OXYGEN SPECIES IN HEPATOCYTES

As discussed earlier, there is convincing evidence that acute exposure to ethanol stimulates the production of reactive species within liver. It is also well-established that under normal physiologic conditions the mitochondrial electron transport chain generates the majority of reactive oxygen species within the hepatocytes,[52] which strongly suggests that during ethanol oxidation superoxide anion production by mitochondria would be increased as a consequence of elevated intracellular concentrations of NADH. Recent studies by Bailey and colleagues strongly suggest that acute exposure to ethanol stimulates the production of reactive oxygen species at the level of the mitochondrion.[51,56]

In these studies[51,56] hepatocytes were isolated from the livers of ethanol-naïve rats and incubated with low, pharmacologically relevant concentrations of ethanol 1 mM (4.6 mg/dl) and 10 mM (46 mg/dl) and levels of reactive oxygen species and cell viability were measured one hour later. For these studies dichlorofluorescin diacetate was used to quantify reactive species because the green fluorescence intensity of the oxidized product, dichlorofluorescein, is positively correlated to the steady-state levels of reactive oxygen species in the hepatocyte. Alcohol-exposed cells were co-incubated with various inhibitors of mitochondrial electron transport and ethanol metabolism. This allowed for the assessment of the contribution of mitochondria and importance of ethanol metabolism in stimulating reactive oxygen species production within the hepatocyte. Acute ethanol treatment caused a dose-dependent increase in reactive oxygen species levels that was associated with mild decreases in hepatocyte viability, which were prevented by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Cyanamide, an aldehyde dehydrogenase inhibitor, also prevented

the ethanol-induced increase in reactive oxygen species levels in hepatocytes. These observations emphasize that reactive oxygen species production following exposure to acute doses of ethanol is dependent on the generation of NADH and its reoxidation by the mitochondrial electron transport chain.[51]

Experiments with the mitochondrial respiratory inhibitor, antimycin, were done to provide evidence for the involvement of complex III in generating reactive oxygen species from acute ethanol exposure. Antimycin increases the concentration of the ubiquinone semiquinone anion present within complex III, which can readily transfer its unpaired electron to molecular oxygen to produce superoxide anion. Therefore, in the presence of antimycin mitochondrial superoxide production would be elevated due to the oxidation of endogenous substrates and further stimulated by the added substrate, ethanol. Antimycin pretreatment markedly increased hepatocyte reactive species production, which was further enhanced by the added substrate ethanol and attenuated by 4-methylpyrazole.[51,56] These observations are consistent with the idea that the ubiquinone semiquinone associated with complex III participates in the ethanol-associated increase in reactive oxygen species (Fig. 5).

Another potential source of reactive oxygen species during ethanol oxidation is the flavin mononucleotide (FMN) coenzyme of complex I. Like ubiquinone, the FMN of complex I can also exist as a semiquinone anion and it is this form of the enzyme that can transfer an unpaired electron to molecular oxygen to generate superoxide (Fig. 4). Thus, inhibitors of electron transfer through complex I were used to demonstrate that during ethanol oxidation there is increased superoxide production from the FMN semiquinone radical.[56] Diphenyliodonium, an inhibitor that blocks electron flow through the FMN of complex I,[57,58] decreased basal levels of reactive oxygen species production in untreated cells and completely blocked the ethanol-related increase in reactive oxygen species. Rotenone blocks the transfer of an electron from the iron-sulfur center N-2 to ubiquinone,[59] which inhibits electron flow into complex III. However, reduction of molecular oxygen to superoxide can still occur at the FMN site in complex I in the presence of rotenone. Incubation with rotenone increased reactive oxygen species levels in both untreated and ethanol-treated hepatocytes.[56] These findings indicate that if the FMN of complex I can accept electrons, superoxide production can by augmented by acute exposure to ethanol (Fig. 4). The data obtained with these two inhibitors, working at different sites within complex I, suggests that the semiquinone form of FMN also contributes to acute ethanol-related increases in reactive oxygen species levels in hepatocytes.

MITOCHONDRIA AS A SOURCE OF REACTIVE SPECIES IN HEPATOCYTES ISOLATED FROM RATS FED ETHANOL CHRONICALLY

Evidence for increased mitochondrial generation of reactive oxygen species following chronic ethanol exposure comes primarily from indirect measures of lipid peroxidation products in isolated mitochondria from ethanol-fed rats.[14,60] Similarly, other studies have shown that mitochondria isolated from rats fed ethanol generated increased reactive species.[61,62] However, measures of reactive oxygen species production in intact cells under strictly controlled oxygen tensions are required to more accurately determine whether the mitochondrial contribution to reactive oxygen species formation is increased following chronic ethanol consumption. Studies by Bailey and Cunningham[51,63] and others [64] observed that chronic ethanol consumption significantly increased the steady-state levels of reactive oxygen species in hepatocytes isolated from the livers of rats fed the Lieber-DeCarli ethanol-containing diet.[65] Hepatocytes from ethanol-fed rats also have significantly lower levels of ATP after incubations,[63,66] which is due, in part, to defects to the oxidative phosphorylation system induced by the chronic ethanol exposure.[31,32] Therefore, it is not unexpected that hepatocytes from ethanol-fed animals were significantly less viable following incubations[51,63,66] as a consequence of the inability to maintain normal levels of ATP during exposure to increased reactive oxygen species.

It is likely that chronic ethanol-induced changes to the oxidative phosphorylation system are largely responsible for increased reactive oxygen species production in hepatocytes isolated from livers of ethanol-fed rats. It is well established that there are chronic ethanol-associated lesions in both complex I and III of the respiratory chain. Early studies by Thayer and colleagues[36] demonstrated that concentrations of the iron-sulfur centers in complex I are decreased following chronic ethanol consumption. This would have the effect of impeding the transfer of an electron from the FMN of complex I to ubiquinone, which might result in an accumulation of the FMN semiquinone and lead to enhanced production of superoxide at this site in the respiratory chain (Fig. 4). Furthermore, chronic ethanol consumption also decreases cytochrome b concentrations.[34,35] This defect would decrease the rate of re-oxidation of the ubiquinone semiquinone anion, which is generated during the Q-cycle of complex III. This will have the effect of increasing the steady-state levels of the ubiquinone semiquinone anion, which would lead to increased transfer of an electron to oxygen, thus enhancing the production of superoxide within the complex III site of mitochondria from ethanol-fed rats (Fig. 5). This hypothesis is

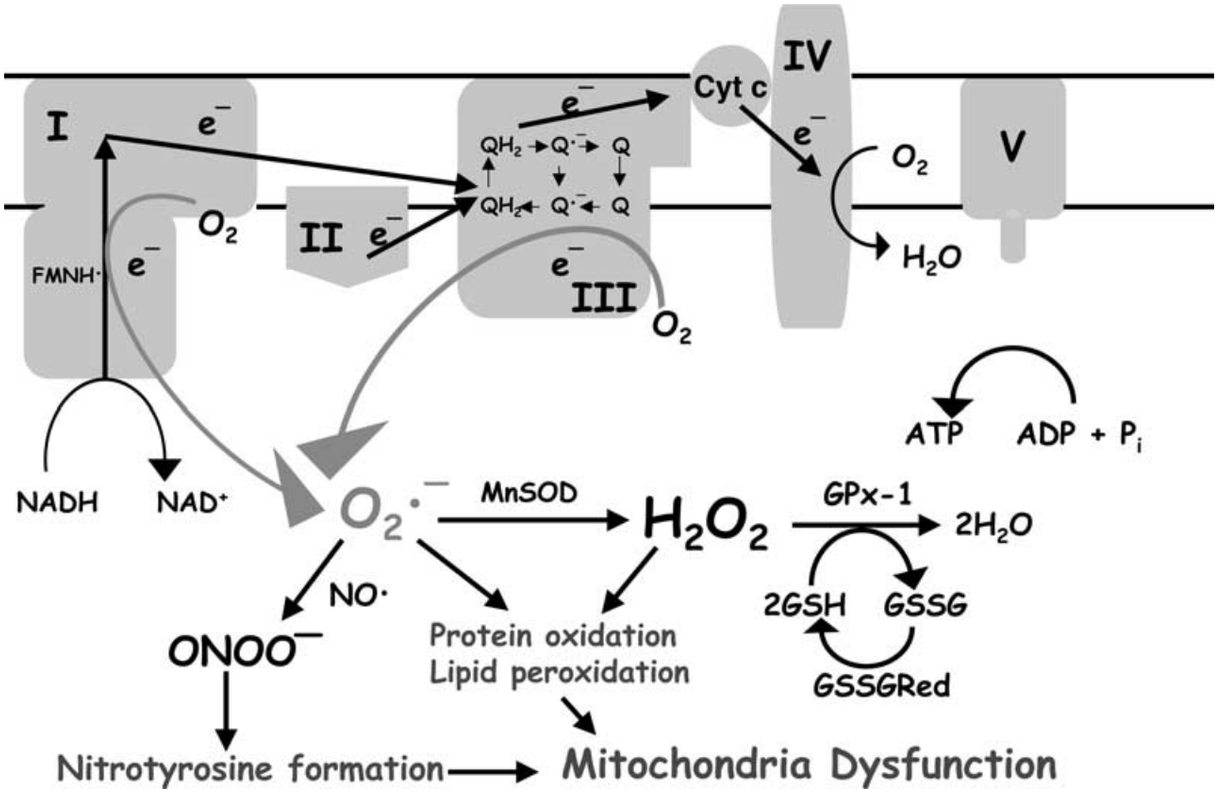

FIGURE 6 Mitochondrial as a source and target of chronic ethanol-elicited increases in reactive species in hepatocytes. Excess reducing equivalents from NADH generated during ethanol metabolism are transported into the mitochondria and as a consequence of this increased flux the electron carriers of the respiratory chain will be in a more reduced state, which will facilitate the transfer of an electron to molecular oxygen to produce superoxide ($O_2^{\bullet -}$) within complexes I and III. Elevated levels of reactive oxygen species will also be observed in the chronic ethanol consumer due to the ethanol-induced lesions to complexes I and III, which promote superoxide production. Furthermore, decreases in mitochondrial antioxidants, specifically glutathione peroxidase-1 (GPx-1) will lead to elevated levels of hydrogen peroxide (H_2O_2). Superoxide can react with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^-$), a potent oxidizing species. Proteins and lipids are highly susceptible to oxidation and as such might be critical targets of oxidative attack by ethanol-elicited increases in reactive oxygen and nitrogen species. Thus, oxidative modification/inactivation of mitochondrial electron transport chain proteins might disrupt mitochondrial function, which could seriously compromise energy metabolism and have detrimental effects on hepatocyte cell viability.

supported by experiments in which hepatocytes isolated from rats fed ethanol chronically generated 200% more reactive species when pretreated with the complex III inhibitor, antimycin, as compared to levels measured in hepatocytes from control-fed rats exposed to antimycin.[51] This observation is consistent with increased superoxide production associated with damage to the cytochrome bc_1 region of mitochondrial complex III.[31,32]

These chronic ethanol-elicited effects to mitochondria would facilitate the transfer of electrons to molecular oxygen and thus enhance the production of superoxide and hydrogen peroxide within mitochondria of the chronic alcohol consumer (Fig. 6). Similarly, it has been suggested that chronic ethanol-related increases in the pro-inflammatory cytokine tumor necrosis factor-α may also stimulate superoxide production at complex III via increases in lipid signaling mediators, like ceramide.[67] These observations suggest that the increase in reactive oxygen species observed in hepatocytes from ethanol-fed rats could be attributed, at least in part, to increased mitochondrial production as a consequence of the chronic ethanol-induced lesions to the mitochondrial oxidative phosphorylation system.

CHRONIC ETHANOL-RELATED INCREASES IN REACTIVE SPECIES DISRUPT HEPATIC ENERGY METABOLISM VIA OXIDATIVE MODIFICATION AND INACTIVATION OF MITOCHONDRIAL PROTEINS

Previous studies have demonstrated that chronic consumption of ethanol significantly decreases the viability of hepatocytes,[51,63,66] which might be related to ethanol-elicited increases in reactive species[51,63] and the inability to maintain sufficient concentrations of ATP[63,66] that are required to fuel the metabolic and repair mechanisms needed to protect the cell from chronic ethanol-related stresses. As mentioned earlier, lowered ATP production in

mitochondria isolated from chronic ethanol-fed animals has been attributed primarily to the ethanol-related reduction in the synthesis of mitochondrially-encoded polypeptides that make up certain segments of the respiratory chain.[35] However, it is possible that oxidative modification and inactivation of proteins of the electron transport chain by the ethanol-induced reactive species could also contribute to the ethanol-related decrease in ATP production and hepatic energy metabolism.

Evidence supports the idea that mitochondrial electron transport chain proteins are susceptible to attack and inactivation by reactive species. Several studies have demonstrated that NADH dehydrogenase, succinate dehydrogenase and ATP synthase activities are depressed following exposure to a variety of reactive species, including superoxide, hydrogen peroxide and peroxynitrite.[68,69] Because previous studies have indicated that the function of NADH dehydrogenase, cytochrome oxidase and ATP synthase[31,32] are depressed by chronic ethanol consumption it is logical to propose that ethanol-related increases in reactive species might be contributing to the inactivation of these proteins via post-translational oxidative modifications and thereby contributing to the chronic ethanol-related depression in the hepatic energy state (Fig. 6).

Emerging evidence suggests that ethanol exposure increases the levels of oxidized proteins in liver. Previous studies have demonstrated that acute[70,71] and chronic[72,73] exposure to ethanol increases the levels of protein carbonyl content in whole liver tissue. More recently, Lieber and colleagues[74] have demonstrated increased nitrotyrosine-protein adduct immunoreactivity in the cytosol of livers from rats fed ethanol chronically, which suggests chronic ethanol exposure increases peroxynitrite levels. Chronic ethanol consumption has also been shown to elicit a much greater increase in the content of protein carbonyl groups in mitochondrial proteins as compared to the increase induced in cytosolic carbonyl content.[75] Reasons for this increased susceptibility of mitochondrial proteins to oxidative modification include the possibility that the oxidative attack by ethanol-elicited reactive species might be highest in the mitochondrion where they are being generated. Liver mitochondria do not contain catalase and chronic ethanol consumption depresses glutathione peroxidase-1 activity,[75] thus mitochondrial proteins are lacking in two antioxidant protection mechanisms against increased levels of hydrogen peroxide. Furthermore, increased levels of oxidized proteins in mitochondria might also be a consequence of an ethanol-related downregulation of those proteolytic systems present within mitochondria that remove damaged and oxidized proteins.[76,77]

Whether these oxidative modifications to mitochondrial proteins contribute to the depressed energy state observed in liver following chronic ethanol consumption is not known. However, studies by Henderson and colleagues have demonstrated that liver cytochrome c oxidase was susceptible to oxidative modification and inactivation by the lipid peroxidation products 4-hydroxynonenal[78] and malondialdehyde[79] induced by short-term ethanol exposure. Taken together the above observations demonstrate that mitochondrial proteins might be more susceptible to oxidative modification as a consequence of increased exposure to reactive oxygen and nitrogen species during long-term exposure to ethanol. Thus, it is likely that chronic ethanol-elicited increases in reactive species might have the effect of inactivating mitochondrial proteins, which would diminish hepatic energy conservation and eventually contribute to the development of alcoholic liver disease in the chronic alcohol abuser.

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