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Mitochondrial cholesterol accumulation in alcoholic liver disease: Role of ASMase and endoplasmic reticulum stress



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ABSTRACT

Alcoholic liver disease (ALD) is a major cause of chronic liver disease and a growing health concern *in the world*. While the pathogenesis of ALD is poorly characterized key players identified in experimental models and patients, such as perturbations in mitochondrial structure and function, selective loss of antioxidant defense and susceptibility to inflammatory cytokines, contribute to ALD progression. Both oxidative stress and mitochondrial dysfunction compromise essential cellular functions and energy generation and hence are important pathogenic mechanisms of ALD. An important process mediating the mitochondrial disruption induced by alcohol intake is the trafficking of cholesterol to mitochondria, mediated by acid sphingomyelinase-induced endoplasmic reticulum stress, which contributes to increased cholesterol synthesis and *StARD1* upregulation. Mitochondrial cholesterol accumulation not only sensitizes to oxidative stress but it can contribute to the metabolic reprogramming in ALD, manifested by activation of the hypoxia inducible transcription factor 1 and stimulation of glycolysis and lactate secretion. Thus, a better understanding of the mechanisms underlying alcohol-mediated mitochondrial impairment and oxidative stress may lead to the identification of novel treatments for ALD. The present review briefly summarizes current knowledge on the cellular and molecular mechanisms contributing to alcohol-induced mitochondrial dysfunction and cholesterol accumulation and provides insights for potential therapeutic targets in ALD.

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Introduction

Alcoholic liver disease (ALD) encompasses a spectrum of liver alterations ranging from simple steatosis to more advanced stages such as alcoholic steatohepatitis (ASH), cirrhosis and hepatocellular carcinoma (HCC) [1]. Alcoholic hepatitis (AH), first described in 1961, is a separate clinical entity characterized by acute

inflammation, severe jaundice, anorexia, abdominal pain, nausea and vomiting, and exhibits a high-mortality rate [2]. The mechanisms underlying the transition from steatosis to ASH remain incompletely understood and include interactions between alcohol with genetic, environmental and nutritional factors [3–5]. Moreover, the dose, duration and type of alcohol consumption as well as drinking patterns and the presence of associated risk factors, including obesity, iron overload and concomitant infection with viral hepatitis can contribute to the progression of ALD. Chronic ASH is associated with a number of histologic changes, including the presence of Mallory's hyaline, megamitochondria, nuclear vacuolization, lobular inflammation, or perivenular and perisinusoidal fibrosis [3]. ALD, one of the oldest forms of liver injury known to humankind, is still a leading cause of liver-related morbidity and mortality and a major health issue in the world [6,7]. Up to 50% of cases of end-stage liver disease have alcohol as a major etiologic factor [6], which reflects the lack of effective therapies for the treatment of ALD due to our incomplete understanding of the basic mechanisms underlying alcohol-mediated cell damage [1,3,8]. Nevertheless, there have been important

Abbreviations: ALD, alcoholic liver disease; AH, alcoholic hepatitis; ASH, alcoholic steatohepatitis; ASMase, acid sphingomyelinase; CYP2E1, cytochrome P450 2E1; ER, endoplasmic reticulum; FFA, free fatty acids; GSH, glutathione; mGSH, mitochondrial GSH; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OXPHOS, oxidative phosphorylation; MRC, mitochondrial respiratory chain; ROS, reactive oxygen species; SH, steatohepatitis; SOD, superoxide dismutase; TG, triglycerides; TNF, tumor necrosis factor.

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recent progress about the mechanisms contributing to the progression of the disease from its initial stage of steatosis [9]. Cellular redox imbalance, exemplified by abnormal NAD^+/NADH ratio, endoplasmic reticulum (ER) stress and oxidative stress, impaired autophagy, and mitochondrial dysfunction are considered important contributory factors for ALD. The superimposition of these mechanisms with disruption of methionine metabolism and altered phosphatidylcholine homeostasis can accelerate liver deterioration and ALD progression [10]. Given the role of mitochondria in energy production, metabolism, cell death and generation of reactive oxygen/nitrogen species (ROS/RNS), alcohol-induced mitochondrial dysfunction is recognized as a key mechanism of ALD and is a hallmark of the disease. Here, we will briefly cover some of the most relevant aspects of mitochondrial dysfunction caused by alcohol consumption, *particularly the accumulation of cholesterol in mitochondria*, and its impact in oxidative stress and the underlying mechanisms, which may provide novel therapeutic opportunities for the treatment of ALD.

Mitochondrial alterations in ALD

Mitochondria are intracellular organelles with a double membrane that function as the cell's powerplants by generating the energy required for vital metabolic reactions and cellular homeostasis. Indispensable for this crucial role of mitochondria is the presence of the mitochondrial respiratory chain (MRC) and the oxidative phosphorylation (OXPHOS) that transduce electron transport into energy generation in the form of ATP upon ADP phosphorylation. The liver is one of the richest organs in terms of number and density of mitochondria; each hepatocyte can contain about 800 mitochondria [11]. Mitochondria provide the energy required for multiple cell functions by transforming nutrient influx into ATP. This process is associated with the generation of ROS/RNS due to the leakage of electrons from the MRC to molecular oxygen [12,13]. *While in vitro evidence indicated that between 0.12–2% of the oxygen consumed in OXPHOS is used to generate superoxide anion, this estimation cannot be extrapolated in vivo* [14]. In particular, hepatic mitochondria are recognized as a major source of ROS whose overproduction can contribute to ALD pathogenesis [15]. Under physiological conditions, most of the ROS generated by the MRC are detoxified by mitochondrial antioxidant defenses, primarily SOD2/MnSOD, which converts superoxide anion to hydrogen peroxide; hydrogen peroxide is subsequently detoxified by peroxiredoxin-III (Prx-III)/thioredoxin-2 (Trx-2) and the GSH redox cycle. Undetoxified ROS, particularly hydrogen peroxide, can diffuse out of mitochondria where they function as signaling molecules [15–18]. Accumulation of superoxide anion favors its interaction with nitric oxide to form the potent oxidant *peroxynitrite* that can target mitochondrial components; hydrogen peroxide accumulation contributes to oxidant dependent cell death upon formation of potent free radicals in the presence of metals in the Fenton reaction [15]. Mitochondria are also involved in key cellular functions such as Ca^{2+} homeostasis, heme biosynthesis, steroid hormone biosynthesis, nutrient metabolism, removal of ammonia, and activation of signaling pathways and cell death [15,19,20]. In this regard, emerging evidence indicates that mitochondria are intimately involved in cell signaling pathways, serving as platforms and effectors in multiple processes such as innate immunity, autophagy and cell death [21]. Emerging data also support a crucial, role for mitochondrial dynamics and physical interaction between mitochondria and the ER in signaling pathways and in the maintenance of ER and mitochondria homeostasis [21].

Mitochondrial dysfunction is frequently described as an early event in several chronic pathological conditions [15,18,22]. In the

liver, chronic diseases including ASH and nonalcoholic steatohepatitis (NASH), viral hepatitis, drug-induced hepatotoxicity and HCC, are characterized by mitochondrial dysfunction [15,18]. However, a growing body of evidence has also shown that *injured* cells with damaged mitochondria can develop cytoprotective mechanisms that ensure cellular energy homeostasis and limit cell death, including mitophagy, a specialized form of autophagy that removes damaged or dysfunctional mitochondria [23]. In addition, mitochondria are unique because they are the only cellular organelle that contains its own DNA encoding 13 polypeptides of the OXPHOS and MRC. Alterations in mitochondrial morphology and function are a hallmark of ALD. Early observations in alcoholic patients described the presence of megamitochondria that was associated with a milder form of ALD, lower incidence of cirrhosis and fewer complications with a good long-term hepatocellular survival [24,25]. Moreover, a common deletion of mitochondrial DNA is frequently observed in alcoholic patients with microvesicular steatosis [26]. Studies in experimental models indicated a wide range of mitochondrial functional alterations [19]. Early studies in the 70s described mitochondrial damage as an early and characteristic feature of ALD in experimental models in rat liver that were confirmed in subsequent investigations [27]. For instance, rats fed alcohol orally (Lieber–DeCarli liquid diet) exhibit lower mitochondrial respiration (state III) rates and lower respiratory control ratio (state III/state IV) in isolated liver mitochondria [28–30]. Also, chronic alcohol intake has been shown to impair hepatic mitochondrial OXPHOS (e.g. subunits of complexes I–V) by suppressing the synthesis of protein subunits encoded by mitochondrial DNA and decreasing mitochondrial protein synthesis by interfering with mitochondrial ribosomes [31]. The lower capacity of oxidative phosphorylation results in the accumulation of reduced respiratory carriers in complexes I and III, which stimulates superoxide anion generation [19]. A proteomic approach in rats fed alcohol reported the differential expression of hepatic mitochondrial proteins, 13 of which increased while 30 exhibited decreased by alcohol [32]. In contrast to alcohol feeding in rats, a recent study in mice fed alcohol via intragastric or oral administration highlights the dynamic changes in mitochondrial function and morphology caused by alcohol [33]. These findings include: 1) increased state III respiration in isolated liver mitochondria, associated with increased levels of complexes I, IV, and V incorporated into the MRC; 2) increased mitochondrial NAD^+ and NADH levels (2-fold), with no change in the redox status; 3) alteration in mitochondrial morphology, with increased numbers of elongated mitochondria; and 4) increased hepatic PGC-1 α expression, suggesting enhanced mitochondrial biogenesis. Interestingly, these mitochondrial perturbations in mice correlated with increased liver injury induced by alcohol feeding, which is more pronounced in the intragastric than in the oral model. Moreover, as mitochondria are the major consumers of molecular oxygen, the stimulation of mitochondrial respiration in mice fed alcohol may contribute to the characteristic hypermetabolic state and subsequent hypoxia and liver injury, which are preferentially seen in pericentral hepatocytes. The mechanisms underlying the contrast between these findings in mice, characterized by increased mitochondrial respiration, and rats with decreased mitochondrial respiration, remain to be fully understood. However, as alcohol feeding induces lower injury in rats than mice, the decreased mitochondrial respiration capacity following alcohol feeding may be considered a protective mechanism against ALD. These findings suggest that the increased mitochondrial respiration induced by alcohol in mice may reflect an adaptation for a more efficient alcohol metabolism, which requires steady supply of NAD^+ equivalents. Whether the increased mitochondrial respiration is causally linked to the stimulation of alcohol metabolism via alcohol dehydrogenase (ADH) and

acetaldehyde dehydrogenase (ALDH) through NAD⁺ regeneration from NADH remains to be established. Thus, the impact and mechanisms of the stimulation of mitochondrial respiration in alcohol-fed mice deserves further investigation.

ROS generation in ALD: source, defense and consequences

The oxidative metabolism of ethanol in the liver is an important source of mitochondrial-derived ROS, which induce oxidative stress and cell injury in ALD [7]. Alcohol is primarily metabolized by the concerted action of ADH in the cytosol and the low K_m ALDH in the mitochondria. Both enzymes are kinetically limited by the NAD⁺ availability, which is reduced to NADH upon oxidation of alcohol to acetaldehyde and acetate. The regeneration of NADH to NAD⁺ which occurs in the MRC ensures not only continued alcohol oxidation but constitutes an important source of ROS generation [34]. In addition to ADH/ALDH alcohol is also metabolized by CYP2E1, the predominant cytochrome P450 isoform that oxidizes ethanol to acetaldehyde. CYP2E1 is loosely coupled and prone to radical formation, including hydroxyethyl radicals [34]. Although the K_m of ADH for alcohol is lower than the K_m of CYP2E1 for alcohol, the latter pathway is inducible by alcohol and hence stands as a major contributor for the oxidative metabolism of alcohol and hence for ROS formation. This pathway is active mostly in the endoplasmic reticulum (ER), although a mitochondrial CYP2E1 isoform has been described [35,36]. CYP2E1 metabolizes a number of small molecules of toxicological interest including ethanol, acetaminophen, halothane, carbon tetrachloride and carcinogens such as nitrosamines [37]. It is well established that CYP2E1 causes oxidative stress through the production of ROS/RNS in vivo [38] and in vitro [39–41]. Interestingly, ethanol exposure increases the expression of CYP2E1 both in the ER and mitochondria [35]. There is evidence that mitochondrial CYP2E1 can induce deleterious effects. For instance, expression of a 40-kDa mitochondrial CYP2E1 favored oxidative stress and cytotoxicity in GSH-depleted HepG2 cells [42]. *Moreover, by introducing specific mutations in the N-terminal 40-amino acid sequence of the rat CYP2E1 that preferentially target CYP2E1 to ER or mitochondria, Bansal et al. reported a higher rate of GSH depletion in cells expressing mutant CYP2E1 targeted to mitochondria compared with cells expressing mutant CYP2E1 targeted to ER [43].* Finally, full-length CYP2E1 found in liver mitochondria from streptozotocin diabetic rats generated significant amount of ROS within these organelles [44]. Thus, alcohol oxidation by the ADH/ALDH system and the alcohol-inducible mitochondrial CYP2E1 are important sources of mitochondrial ROS generation in ALD.

As indicated above, the transfer of electrons from MRC to oxygen generates superoxide anion. The predominant defense against the accumulation of mitochondrial superoxide anion is MnSOD. MnSOD is essential for life, as illustrated by the neonatal lethality of mice deficient in MnSOD [45]. However, the role of alcohol on MnSOD regulation is controversial [46–48]. Homozygous mutations in the mitochondrial targeting sequence of MnSOD, which are associated with increased mitochondrial localization and activity, is a risk factor for progressive disease in patients with ALD [49]. *These findings may seem intriguing as elimination of superoxide anion by MnSOD might be expected to protect against ALD. The scavenging of superoxide by MnSOD generates hydrogen peroxide, and MnSOD activation in ALD may exceed the capacity to eliminate hydrogen peroxide, which is detoxified by the Prx-III/Trx-2 and the GSH redox cycle. A vital component of this redox cycle is mitochondrial GSH (mGSH), which ensures the elimination of hydrogen peroxide by GSH peroxidases (Gpx) [15,17]. Both antioxidant systems are of relevance in hydrogen peroxide elimination and protection against oxidative stress. For*

instance, deletion of the mitochondrial targeted Gpx4 is embryonic lethal [50], and kinetic competition analysis reported that an important amount of hydrogen peroxide generated in mitochondria targets PrxIII [51]. While the relative importance of Prx-III/Trx-2 vs mGSH/Gpx in protection and susceptibility to oxidant cell death has not been specifically addressed, recent findings have shown that selective mGSH depletion sensitized hepatocytes against hydrogen peroxide accumulation induced by superoxide scavenging with MnTBAP despite Prx-III/Trx-2 defense [52]. Moreover, depletion of mGSH results in Trx2 oxidation and impaired Prx-III reactivation and mGSH depletion in hypercholesterolemic pigs decreases the levels of the mitochondria-specific antioxidant enzymes such as MnSOD, Trx2, and PrxIII [53,54]. Importantly, it has been reported that alcohol consumption depletes mGSH selectively [55–59], and this effect is rapidly reversed upon alcohol withdrawal [60,61]. Furthermore, recent findings in rats fed with an ethanol-polyunsaturated fatty acid diet confirmed the depletion of mGSH that preceded ASH development, effects that were prevented by betaine treatment [62].

The depletion of mGSH by alcohol may be of relevance to ALD progression. Not only mGSH depletion contributes to oxidative stress, but mGSH determines the critical balance between mitochondrial antioxidant defenses necessary to ensure protection against oxidative stress [52]. MnSOD overexpression aggravates the deleterious effects of prolonged alcohol feeding on mitochondrial DNA in mice [63]. Alcohol-induced mGSH depletion sensitizes hepatocytes to TNF-induced cell death [64]. The mechanism of mGSH depletion-induced sensitization to TNF is independent of NF- κ B inactivation and involves mitochondrial ROS overproduction and cardiolipin peroxidation that determined mitochondrial outer membrane restructuring and mitochondrial outer membrane permeabilization [65]. Inflammation is an important feature of progressive ALD, particularly ASH and AH. Recent findings have reported increased production of IL-1 β and inflammasome assembly in mice fed alcohol [66], and mitochondrial ROS induce the redistribution of NLRP3 and ASC to mitochondria/ER contact sites and stimulate caspase 1-mediated IL-1 β processing [67]. Therefore, it is conceivable that mGSH depletion caused by alcohol feeding may contribute to the inflammasome activation and hence generation of IL-1 β characteristic of ALD. Consistent with these findings, we have observed that cholesterol feeding to mice synergized with alcohol to cause ASH, inflammasome activation and IL-1 β production (Conde de la Rosa, manuscript in preparation). Besides ROS, recent findings have demonstrated a ROS independent inflammasome activation involving the redistribution of cardiolipin from the inner to the outer mitochondrial membrane [68]. Whether mGSH depletion caused by alcohol contributes to cardiolipin-dependent inflammasome activation and IL-1 β deserves further investigation.

Mitochondrial cholesterol and ALD: regulation of mitochondrial GSH and metabolic reprogramming

The role of cholesterol in liver diseases has attracted increasing attention in recent years and it is currently considered a key player in ASH and NASH [69]. In particular, the trafficking of cholesterol to mitochondrial membranes sensitizes hepatocytes to TNF-mediated steatohepatitis and cell death by promoting mGSH depletion [70]. Indeed, cholesterol enrichment in isolated mitochondria impairs the mitochondrial transport of GSH resulting in mGSH depletion (Fig. 1). The effect of cholesterol in the mitochondrial transport of GSH is dependent on the changes of mitochondrial membrane dynamics; however, this effect is selective for the mGSH transport as other mitochondrial carriers are insensitive to cholesterol loading [71]. The cholesterol/phospholipid molar ratio

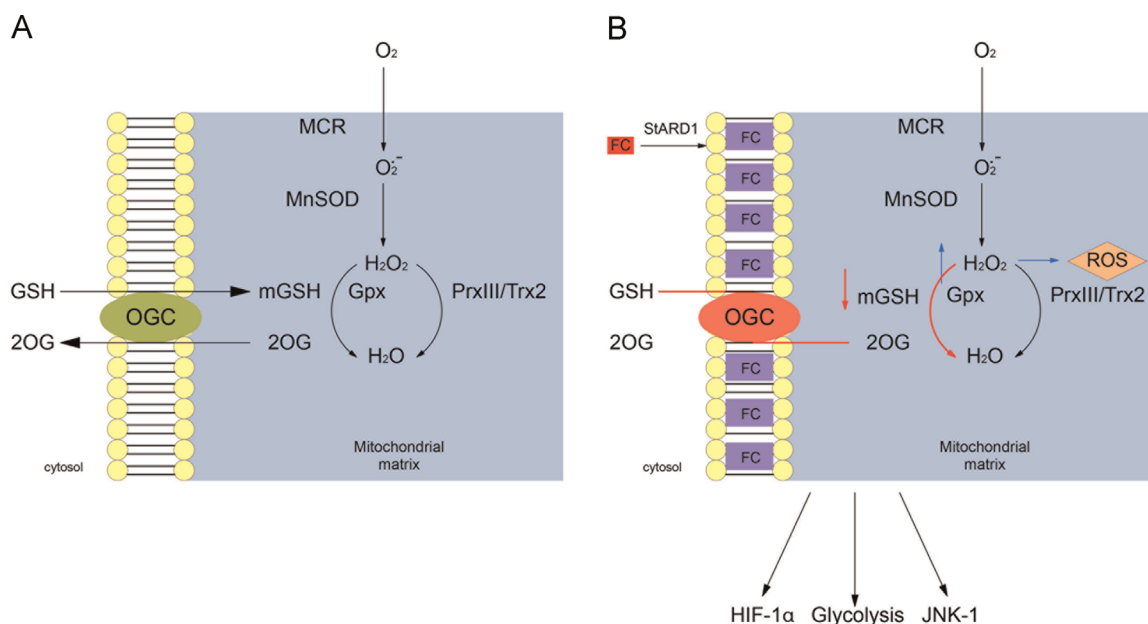


Fig. 1. Regulation of mitochondrial GSH transport by mitochondrial cholesterol. (A) The transport of GSH to mitochondrial matrix via the mGSH carrier OGC is dependent on membrane dynamics. Under physiological conditions the OGC carrier transports GSH into mitochondrial matrix by exchanging mitochondrial 2-oxoglutarate (2OG) by cytosolic GSH. The mitochondrial GSH/Gpx and the PrxIII/Trx are key mitochondrial antioxidant defenses to control the generation of hydrogen peroxide, which is largely formed from the dismutation of superoxide anion by MnSOD. Oxygen consumption in the mitochondrial respiratory chain (MCR) generates superoxide anion. (B) In alcohol fed models cholesterol accumulates in the mitochondrial inner membrane, which impairs OGC carrier by the loss of membrane fluidity resulting in the depletion of mitochondrial GSH. Mitochondrial GSH depletion compromises the elimination of hydrogen peroxide via the GSH redox cycle and Gpx and contributes to ROS overgeneration. Besides impairing the OGC carrier, mitochondrial cholesterol accumulation by alcohol can trigger lipotoxicity by activating JNK1 and contribute to the metabolic alterations in ALD reflected by activation of HIF1 α and glycolysis.

is a critical determinant of the fluidity of membrane bilayers and the increase in membrane cholesterol disrupts lipid organization and decreases the transition of liquid-ordered to liquid-disordered phases of membranes [72]. This change in membrane packing impacts in the activity of specific membrane proteins, such as the mGSH carrier (see below). However, the mitochondrial transport of S-adenosyl-L-methionine or the uptake of adenine nucleotide via the adenine nucleotide translocator are insensitive to the decrease in membrane fluidity induced by cholesterol loading [71,73]. Consequently, alcohol feeding caused mitochondrial cholesterol accumulation and subsequent mGSH depletion by stimulating the expression of the mitochondrial cholesterol carrier StARD1 [74], whose regulation is discussed in the next section. The accumulation of cholesterol to mitochondria has been recently reported in rats fed ethanol along with polyunsaturated fatty acids [62]. The impact of mitochondrial cholesterol accumulation in the depletion of mGSH is not an exclusive feature of ASH as it has been also reported in other contexts, including NASH and anthrax-induced cell death [75,76].

At the molecular level, the effect of cholesterol in the regulation of mGSH is mediated by the susceptibility of the 2-oxoglutarate carrier (OGC) to perturbations in membrane dynamics. Functional expression analyses in *Xenopus laevis* oocytes microinjected with OGC cRNA showed enhanced transport of GSH in isolated mitochondria [77]. Moreover, cholesterol enrichment impairs the transport kinetics of 2-oxoglutarate via the OGC by decreasing mitochondrial membrane fluidity. Restoration of membrane dynamics by the fatty acid analog A₂C improves the activity of OGC and mGSH transport despite cholesterol enrichment. In liver mitochondria and HepG2 cells the transport of GSH exhibits two kinetic components with high and low affinity [78]. Mitochondrial cholesterol accumulation in HepG2 cells induced by acetaldehyde treatment increased the Michaelis constant for the high- and low-affinity components, with a greater impact on the former [79]. However, kinetic analyses of 2-oxoglutarate transport in rat liver

mitochondria exhibited a single Michaelis–Menten component with kinetic parameters in the range of those reported previously for kidney mitochondria and a K_m close to the high K_m of mitochondrial GSH transport [77,80]. Overall, these findings suggest that the OGC accounts for the low-affinity high capacity of GSH transport in liver mitochondria.

Besides depleting mGSH levels, mitochondrial cholesterol has emerged as a key player in lipotoxicity. Recent studies in primary mouse hepatocytes loaded with free cholesterol upon incubation with LDL reported that mitochondrial cholesterol deposition caused hepatocyte apoptosis and necrosis by activating JNK1 [81]. These effects were accompanied by mitochondrial membrane pore transition, cytochrome c release, oxidative stress and ATP depletion. Deletion of JNK1 and TLR4 protected against mitochondrial cholesterol lipotoxicity. Moreover, HMGB1 was released into culture medium of wild type hepatocytes but not JNK1^{-/-} or TLR4^{-/-} hepatocytes loaded with free cholesterol, while anti-HMGB1 anti-serum prevented JNK activation and free cholesterol lipotoxicity in wild type hepatocytes. Consistent with these findings, HMGB1 overexpression has been reported in patients with ALD and HMGB1 plays a critical role in alcohol-induced liver injury [82]. Whether mitochondrial cholesterol contributes to alcohol-induced JNK activation and HMGB1 upregulation remains to be determined. Moreover, mitochondrial cholesterol accumulation in mouse hepatocytes has been recently shown to stabilize the hypoxia inducible transcription factor 1 α (HIF-1 α) in normoxia by a ROS dependent mechanism [83]. The impact of mitochondrial cholesterol in the prevention of HIF-1 α degradation was abolished by the depletion of mitochondrial DNA. Although mGSH levels were not examined in the preceding report, previous studies demonstrated that mGSH levels determine the survival of hepatocytes during hypoxia and that mGSH depletion stimulated hypoxia-induced ROS generation [84,85]. Given the role of hypoxia in alcohol induced steatosis and ALD [86], it is conceivable that mitochondrial cholesterol-mediated ROS generation via mGSH

depletion may contribute to alcohol-induced HIF-1 α activation. Finally, stimulated mitochondrial cholesterol trafficking by silencing the endolysosomal proteins Niemann Pick type C proteins 1/2 (NPC1/2) stimulates lactate secretion and decreases glutamine-dependent mitochondrial respiration and ATP transport across mitochondrial membranes [87]. In line with these findings, alcohol feeding in mice stimulates glycolysis and lactate secretion, suggesting that alcohol induces a metabolic shift from mitochondrial respiration to aerobic glycolysis [88]. Moreover, it is conceivable that mitochondrial cholesterol accumulation by alcohol can contribute to the mitochondrial dysfunction in ALD. For instance, mitochondrial cholesterol loading due to NPC1 mutations impairs the mitochondrial ATP synthase activity, which is restored upon cholesterol extraction by cyclodextrin. These findings suggest that alcohol-induced mitochondrial cholesterol accumulation may contribute to mitochondrial dysfunction and the metabolic reprogramming caused by alcohol consumption, characterized by HIF-1 α stabilization and stimulated glycolysis (Fig. 1).

Mechanisms of mitochondrial cholesterol regulation in ALD: role of ER stress

Given the emerging role of mitochondrial cholesterol in the regulation of mitochondrial function, membrane properties and transport functions, uncovering the mechanisms underlying the trafficking of cholesterol to mitochondria may be of potential relevance in ALD. The restricted mitochondrial cholesterol pool plays important physiological roles, such as in steroidogenesis in specialized tissues or bile acids synthesis in the liver. StARD1 is the founding member of a family of lipid transporting proteins that contain StAR-related lipid transfer (START) domains. StARD1 is an outer mitochondrial membrane protein which was first described and best characterized in steroidogenic cells where it plays an essential role in cholesterol transfer to the mitochondrial inner membrane for metabolism by CYP11A1 to generate pregnenolone, the precursor of steroids [89,90]. Despite similar features with StARD1, other START members cannot replace StARD1 deficiency as global StARD1 knockout mice die within 10 days due to adrenocortical lipoid hyperplasia [91]. StARD1 silencing decreases mitochondrial cholesterol levels in HepG2 cells and stimulates mitochondrial outer membrane permeabilization [92]. Moreover,

alcohol feeding upregulates liver StARD1 mRNA levels with similar results observed in patients with AH [74]. Interestingly, alcohol feeding, however, does not influence the expression of StARD3 (also known as MLN64), suggesting this particular member of the StART family plays a minor role in alcohol-induced mitochondrial cholesterol accumulation.

ER stress is a key event in ALD, contributing to steatosis and liver injury [93]. The ER plays an essential role in the integration of multiple metabolic signals and the maintenance of cell homeostasis, particularly protein synthesis and folding. Under stress conditions induced by protein misfolding, the ER triggers an adaptive response called uncoupled protein response (UPR). To resolve ER stress, UPR promotes a decrease in protein synthesis, and an increase in protein degradation and chaperone production for protein folding. Alcohol-induced perturbation in methionine metabolism is believed to contribute to ER stress, as betaine supplementation prevented ER stress, steatosis and liver injury in mice fed alcohol [94]. Moreover, tauroursodeoxycholic acid (TUDCA), a chemical chaperone that prevents ER stress [95], restores the mGSH pool in alcohol fed rats [59] and ameliorates alcohol-induced ER stress [74]. Emerging evidence has demonstrated that StARD1 is a previously unrecognized target of the UPR and ER stress signaling. Indeed, tunicamycin, an ER stress trigger, induces the expression of StARD1 in isolated hepatocytes and this effect is prevented by TUDCA treatment [74]. Moreover, mice fed a high cholesterol diet (HC) exhibited increased expression of StARD1. However, HC feeding downregulates the expression of SREBP-2-regulated target genes, including hydroxymethylglutaryl Co-A reductase, demonstrating that StARD1 is and an ER stress but not SREBP-2 regulated gene. In contrast to StARD1, the role of ER stress in the regulation of StART family members has been limited to StARD5 [96,97], with conflicting results reported for StARD4 [96,98]. As the UPR comprises three transducers, namely inositol requiring (IRE) 1 α , PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6 α , which are controlled by the master regulator glucose-regulated protein 78 (GRP78 also known as BiP), further work is needed to examine the relative contribution of IRE-1 α , PERK and ATF6 α arms in the regulation of StARD1 (Fig. 2). Besides ER stress, StARD1 activation is regulated at the transcriptional and post-translational levels. In murine steroidogenic cells StARD1 activity and subsequent steroidogenesis increases upon StARD1 phosphorylation at serine residues [99,100]. Whether or

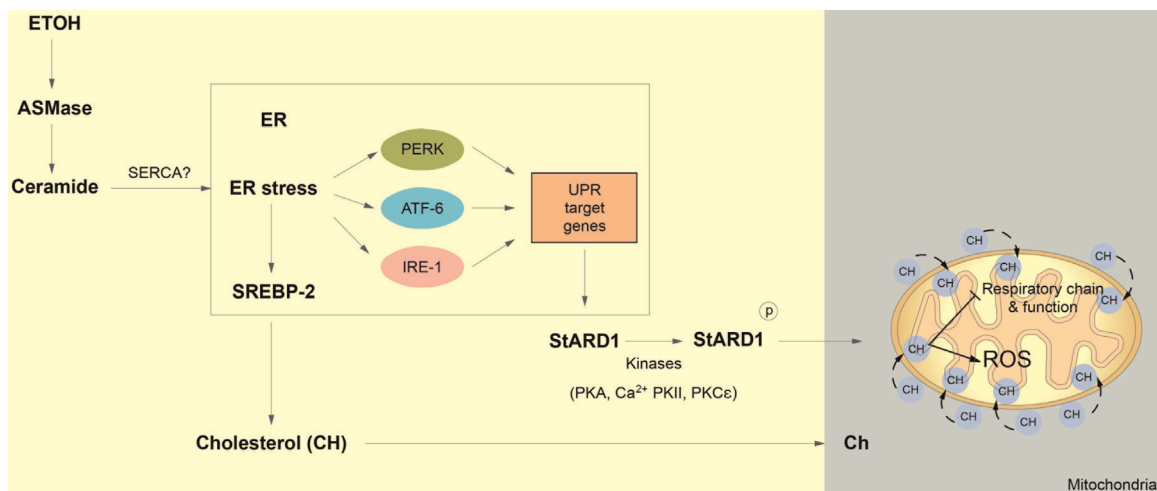


Fig. 2. Role of ER stress in the regulation of StARD1. A fundamental mechanism in the upregulation of StARD1 is the onset of ER stress by alcohol. Alcohol-mediated activation of ASMase generates ceramide, which in turn causes ER stress resulting in the activation of transcription factor SREBP-2 that regulate de novo cholesterol synthesis in the ER and the three arms of the unfolded protein response that induce the transcriptional regulation of StARD1. Whether ASMase-induced ceramide generation induces ER stress by regulating ER Ca²⁺ homeostasis via SERCA remains to be determined. Besides the regulation of StARD1 at the transcriptional level, phosphorylation events regulate StARD1 activity and hence may contribute to the regulation of mitochondrial cholesterol. Further work is required to determine the relative contribution of the 3 arms of the UPR in the transcriptional regulation of StARD1 and the putative StARD1 kinases that phosphorylate StARD1.

not StARD1 phosphorylation by alcohol regulates mitochondrial cholesterol homeostasis remains to be explored. If so, then the identification of putative kinases that phosphorylate and activate StARD1 may be of potential relevance in ALD. Overall, these findings indicate that the upregulation of StARD1 by alcohol-induced ER stress determines the accumulation of cholesterol in the mitochondrial inner membrane.

Acid sphingomyelinase regulates ER stress in ALD

Acid sphingomyelinase (ASMase) is a specific mechanism that generates ceramide, the prototype sphingolipid that in addition to its critical structural function in membrane bilayers, it regulates apoptosis, cellular senescence, stress response, inflammation and metabolism [101–103]. Sphingomyelin hydrolysis by ASMase generates a specific pool of ceramide that mediates TNF-induced hepatocellular injury and liver failure [104,105]. ASMase also regulates Fas-mediated liver failure [106] and is a critical apoptotic enzyme mediating radiation and chemotherapy induced cell death [102]. Besides its proapoptotic function, ASMase regulates liver fibrosis by controlling hepatic stellate cell transdifferentiation into a myofibroblast phenotype and by regulating cathepsin activation [107,108]. Recent findings have reported that sortilin deletion, a member of the vacuolar protein sorting 10 protein (VPS10P)-domain receptor family which functions both as a co-receptor and as a trafficking molecule, ameliorates HFD-mediated metabolic disruption by inhibiting ASMase [109]. Moreover, alcohol feeding increases ASMase expression and activity in experimental models and patients with acute AH [74,110]. Recent findings have shown that ASMase is required for alcohol-induced ER stress [74]. The link of ASMase and ER stress induction in the context of ALD is of interest. Alcohol feeding induces hyperhomocysteinemia, which is thought to contribute to ER stress [93,94]. However, ASMase null mice fed alcohol do not exhibit ER stress despite increased homocysteine (Hcy) to levels similar to those seen in wild littermates and were resistant to alcohol-induced steatosis, susceptibility to LPS and concanavalin A-mediated liver injury and mitochondrial cholesterol accumulation [74]. However, tunicamycin induces ER stress in ASMase null mice, indicating that the resistance of ASMase null mice to alcohol-induced ER stress is not due to an inherent defect in the ER stress response. The findings that Hcy requires ASMase to induce ER stress are consistent with recent observations that ASMase ablation prevents Hcy-induced glomerular injury in mice deficient in cystathionine β -synthase mice, a genetic model of hyperhomocysteinemia characterized by increased plasma Hcy levels [111]. Moreover, ASMase silencing prevents Hcy-induced ceramide generation, indicating that ASMase activation by Hcy is a major pathway of glomerular ceramide generation. Furthermore, mice fed a chow diet supplemented with Hcy exhibit increased plasma Hcy levels but no pathophysiological changes or ER stress [112]. Hence, these data suggest that Hcy at pathophysiological concentrations plays a minor role in ER stress and steatosis and that the association between Hcy and ER stress are not causally related. While these findings dissociate Hcy from ER stress, they also suggest that ASMase functions as an ER stress trigger. In line with this possibility, incubation of HepG2 cells with exogenous ASMase but not NSMase increases the expression of ER stress markers and this event is accompanied by disruption of ER Ca^{2+} homeostasis in response to thapsigargin [74], in agreement with previous data using cell permeable ceramide C_2 [113]. Although, this outcome supports the concept that altered lipid composition in the ER regulates ER Ca^{2+} homeostasis and subsequent ER stress susceptibility [114,115], it remains to be established whether ASMase activation modulates SERCA activity in the ER by disrupting ER membrane physical properties. Consistent

with these findings, ASMase null mice are refractory to alcohol-mediated cholesterol accumulation and subsequent mGSH depletion due to the lack of ER stress-mediated StARD1 expression [74]. Maintenance of mGSH homeostasis is associated with resistance to LPS and ConA-mediated liver damage in the absence of ASMase. In addition to the regulation of mitochondrial function, ASMase regulates other cell death pathways, including the lysosomal membrane permeabilization (LMP) as well as autophagy [116]. The ability of ASMase in promoting LMP is due to the decrease in lysosomal cholesterol levels secondary to the hydrolysis of lysosomal sphingomyelin pool by ASMase. To explore the potential relevance of the ASMase–ER stress–StARD1 pathway in ALD, ASMase inhibition by amitriptyline protected against alcohol-induced steatosis, ER stress and susceptibility to LPS/ConA-induced liver injury in wild type mice fed alcohol. Overall, these data indicate that ASMase is critical for alcohol-induced ER stress, and provide a rationale for further clinical investigation in ALD pointing to ASMase as a potential relevant target in the treatment of ALD by preventing alcohol-induced ER stress and subsequent downstream events.

Conclusions and future directions

Mitochondria are unique organelles responsible for life sustaining reactions and cell death pathways. They are the main producers of ROS/RNS species within MCR and are targets of alcohol toxicity. Essential for the metabolism of alcohol, mitochondria regenerate NAD^+ from NADH to oxidize alcohol, a process that generates ROS as subproducts. Cumulative evidence both in patients and in experimental models of ALD, indicate mitochondrial abnormalities at the morphological and functional levels caused by alcohol. Given the critical role of mGSH in controlling hepatocellular survival in the face of inflammation and oxidative stress, its depletion caused by alcohol is thought to be of significance in ALD. As cholesterol accumulation by alcohol downregulates mGSH, a better understanding of the cell biology and mechanisms responsible for cholesterol upregulation and trafficking to mitochondrial membranes, including ER stress, may open up novel avenues for intervention and treatment. In this regard, emerging data indicate that alcohol-induced ER stress is an important mechanism leading to mitochondrial cholesterol accumulation and hence mGSH depletion. The onset of ER stress appears to be independent of hyperhomocysteinemia and requires ASMase which upregulates the expression of the mitochondrial cholesterol transporter StARD1. Hence, targeting ASMase may constitute a novel approach in the treatment of ALD. The current evidence in experimental models may further stimulate clinical investigations on the potential of ASMase inhibition in patients with ALD.

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