Research Article

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ASMase is required for chronic alcohol induced hepatic endoplasmic reticulum stress and mitochondrial cholesterol loading

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Background & Aims: The pathogenesis of alcohol-induced liver disease (ALD) is poorly understood. Here, we examined the role of acid sphingomyelinase (ASMase) in alcohol induced hepatic endoplasmic reticulum (ER) stress, a key mechanism of ALD.

Methods: We examined ER stress, lipogenesis, hyperhomocysteinemia, mitochondrial cholesterol (mChol) trafficking and susceptibility to LPS and concanavalin-A in *ASMase*$^{-/-}$ mice fed alcohol.

Results: Alcohol feeding increased *SREBP-1c*, *DGAT-2*, and *FAS* mRNA in *ASMase*$^{+/+}$ but not in *ASMase*$^{-/-}$ mice. Compared to *ASMase*$^{+/+}$ mice, *ASMase*$^{-/-}$ mice exhibited decreased expression of ER stress markers induced by alcohol, but the level of tunicamycin-mediated upregulation of ER stress markers and steatosis was similar in both types of mice. The increase in homocysteine levels induced by alcohol feeding was comparable in both *ASMase*$^{+/+}$ and *ASMase*$^{-/-}$ mice. Exogenous ASMase, but not neutral SMase, induced ER stress by perturbing ER Ca^{2+} homeostasis. Moreover, alcohol-induced mChol loading and StARD1 overexpression were blunted in *ASMase*$^{-/-}$ mice. Tunicamycin upregulated StARD1 expression and this outcome was abrogated by tauroursodeoxycholic acid. Alcohol-induced liver injury and sensitization to LPS and concanavalin-A were prevented in *ASMase*$^{-/-}$ mice. These effects were reproduced in alcohol-fed *TNFR1/R2*$^{-/-}$ mice. Moreover, ASMase does not impair hepatic regeneration following partial hepatectomy. Of relevance, liver samples from patients with alcoholic hepatitis exhibited increased expression of ASMase, StARD1, and ER stress markers.

Conclusions: Our data indicate that ASMase is critical for alcohol-induced ER stress, and provide a rationale for further clinical investigation in ALD.

Keywords: Ceramide; ER stress; Mitochondrial cholesterol; Homocysteine; Alcohol induced liver disease; Mitochondrial GSH.

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Abbreviations: ALD, alcohol induced liver disease; ASMase, acid sphingomyelinase; BHMT, betaine homocysteine methyl transferase; CBS, cystathionine-β-synthase; DGAT2, diacylglycerol transferase 2; ER, endoplasmic reticulum; FAS, fatty acid synthase; Hcy, homocysteine; MCD, methionine and choline deficient; mChol, mitochondrial cholesterol; mGSH, mitochondrial GSH; MS, methionine synthase; MAT1A, methionine adenosyl transferase 1A; NSMase, neutral sphingomyelinase; PH, partial hepatectomy; SM, sphingomyelin; Smgs1, sphingomyelin synthase1; Sgms2, sphingomyelin synthase2; StARD1, steroidogenic acute regulatory domain protein; SREBP, sterol regulatory element binding protein; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response.

Introduction

Alcohol-induced liver disease (ALD) is a major cause of chronic liver disease and related death in western countries. Unfortunately, little progress has been made in ALD treatment due to the incomplete understanding of ALD pathogenesis and progression from hepatic steatosis to advanced stages, i.e., alcoholic hepatitis (AH) [1,2]. Alcohol triggers lipogenesis and liver injury by several mechanisms, including perturbed NAD^+/NADH, acetaldehyde generation, adiponectin downregulation, hyperhomocysteinemia, and mitochondrial dysfunction [2–7]. Endoplasmic reticulum (ER) stress has emerged as a key event in ALD, contributing to steatosis and liver injury [4]. Early work indicated a critical role for TNF in ALD and increased hepatic TNF levels and TNFR1 expression have been reported in patients with ALD

[8,9]. Moreover, alcohol intake has been shown to sensitize to TNF-induced hepatocellular death [10,11].

Ceramide has been extensively studied in relation to stress and cell death [12,13]. In addition to *de novo* synthesis in the ER, ceramide can be generated by sphingomyelin (SM) hydrolysis by sphingomyelinases (SMases) [14]. Although the neutral SMase (NSMase) has been shown to induce apoptosis [15,16], this outcome may be dependent on the topology/sidedness of NSMase-induced ceramide generation [17]. Moreover, acid SMase (ASMase) mediates apoptosis in response to cell death receptors, chemotherapy or ionizing radiation [18–20]. ASMase is activated in TNF receptosomes by proteolytic cleavage of pro-ASMase due to sequential activation of caspase-8 and caspase-7 [21]. In addition to its role in cell death, ASMase has emerged as a critical player in liver fibrosis by regulating lysosomal cathepsins B/D [22].

Chronic alcohol feeding has been shown to activate ASMase, contributing to hepatic steatosis [23,24]. However, the role of ER stress in these studies was not examined, specifically if ASMase was required for the engagement of this key pathway in ALD. Further, these reports did not address the role of ASMase in alcohol-induced mitochondrial cholesterol (mChol) trafficking, which contributes to TNF susceptibility [25], or liver regeneration. To critically examine the contribution of ASMase to ALD, we analyzed the response of *ASMase*$^{-/-}$ mice to oral alcohol feeding. We show that ASMase is required for alcohol-induced ER stress and consequent activation of lipogenic pathways. Moreover, *ASMase*$^{-/-}$ mice are resistant to alcohol-induced mChol loading and mitochondrial GSH (mGSH) depletion. Of relevance, ASMase does not impair liver regeneration after partial hepatectomy and liver samples from patients with acute AH display increased expression of ASMase and ER stress markers. Overall, ASMase is a critical player in ALD by inducing ER stress.

Materials and methods

Animals and treatments

All procedures were approved by the ethics committee of the University of Barcelona and conducted according to institutional guidelines. *ASMase*$^{-/-}$ mice (C57BL/6 strain, a generous gift from R. Kolesnick and E. Gulbins) have been characterized previously [19]. *TNFR1*$^{-/-}$, *TNFR2*$^{-/-}$, and double *TNFR1/R2*$^{-/-}$ mice (C57BL/6 strain) were generated and characterized as described [26]. Male knockout mice and control littermates (*ASMase*$^{+/+}$ or TNFRs$^{+/+}$) (8–10 weeks old) were pair fed control or ethanol-containing liquid diets (Test Diet, cat no: LD101 and LD101A, respectively), providing 36% of calories from ethanol (or maltose), 35% of calories from fat, 18% of calories from protein, and 11.5% of calories from carbohydrate, as described previously [10]. The ethanol diet was introduced gradually by increasing the ethanol content of the diet by 1% (vol/vol) every 2 days until mice consumed diets containing 5% (vol/vol) ethanol. All mice were then fed the liquid diet containing 5% ethanol for 4 weeks. In some cases, betaine (1.5% wt/vol) was administered to wild type mice during alcohol feeding as described [27]. Moreover, to address the role of cholesterol on SREBP-2-mediated gene expression, wild type mice were fed a high cholesterol (HC, 2% cholesterol) diet for 2 days as described [25]. Sample processing for analyses is described in Supplementary Materials and methods.

Partial hepatectomy

To examine the impact of ASMase inhibition on liver regeneration, wild type mice were treated with amitriptyline i.p. for (2.5 mg/kg) for 10 days before partial hepatectomy. Mice were anesthetized with a 92:7 mg/kg mix of ketamine:xylazine and subjected to midventral laparotomy with 70% liver resection (left lower and upper and right upper lobes), and the weight of the excised liver was determined. Sham surgery entailed midventral laparotomy. Survival was higher than 80% and all deaths were due to post-surgery complications during the first 24 h post-PH. Hepatocyte proliferation was determined by PCNA staining at different time points and liver regeneration index was calculated as the ratio of the liver remnant to body mass and $\times 100$.

Human liver samples

Patients gave written consent in accordance with the Declaration of Helsinki, and the protocol, approved by ethical committees from the Hospital Clinic and Hopital Huriez, followed ethical guidelines on handling of human samples. Liver samples were obtained from patients with AH by transyugular biopsies (n = 10) or from explanted livers from patients undergoing liver transplantation for alcoholic liver disease (n = 7). Normal livers were obtained from cadaveric liver donors (n = 4) or from resection for unrelated liver diseases (metastasis from distant organ cancer, n = 8). Patients characteristics are shown in Supplementary Fig. 1, and description for RNA isolation is shown in Supplementary Materials and methods.

Results

ASMase deficiency prevents alcohol-induced lipogenesis and macrosteatosis

We examined the effect of ASMase deficiency in the expression of lipogenic enzymes. Alcohol feeding to *ASMase*$^{+/+}$ mice increased the expression of transcription factor SREBP-1c and target enzymes, including DGAT-2 and FAS (Fig. 1A–C). Alcohol also increased expression of SREBP-2, (Fig. 1D). Interestingly, SREBP-1c, DGAT-2, and FAS induction by alcohol were blunted in *ASMase*$^{-/-}$ mice fed alcohol (Fig. 1A–C). However, *ASMase*$^{-/-}$ mice fed control diet exhibited increased expression of SREBP-2 that was increased by alcohol feeding (Fig. 1D).

These findings translated in increased TG and FFA levels and macrosteatosis in *ASMase*$^{+/+}$ mice, but not *ASMase*$^{-/-}$ mice (Supplementary Fig. 1A–C). Alcohol increased SM and cholesterol levels in *ASMase*$^{+/+}$ mice (Supplementary Fig. 1D and E), which displayed higher filipin staining (Supplementary Fig. 1F). *ASMase*$^{-/-}$ mice fed control diet exhibited increased hepatic SM and cholesterol content compared to *ASMase*$^{+/+}$ mice (Supplementary Fig. 1D and E). Upon ethanol feeding, SM and cholesterol levels further increased in *ASMase*$^{-/-}$ mice (Supplementary Fig. 1D-F). ASMase deficiency did not perturb alcohol metabolism as blood alcohol levels were comparable in *ASMase*$^{+/+}$ mice and *ASMase*$^{-/-}$ mice fed alcohol (117 ± 12 mg/dl and 125 ± 13 mg/dl, respectively). Furthermore, CYP2E1 upregulation following alcohol feeding was similar in both types of mice (Supplementary Fig. 2). Although the liver/body weight increased in *ASMase*$^{-/-}$ mice, this increment in response to alcohol feeding was similar between *ASMase*$^{+/+}$ mice and *ASMase*$^{-/-}$ mice (Supplementary Fig. 2). The hepatomegaly seen in the null mice likely reflects the accumulation of lipids such as sphingomyelin and cholesterol as well as massive lysosomal proliferation reflected by increased LAMP2 expression and characteristic foam cells formation (not shown). Thus, these findings indicate that the genetic ablation of ASMase increases hepatic SM and cholesterol but prevents alcohol-induced TG, FFA accumulation and macrosteatosis.

ASMase is required for alcohol-induced ER stress

ER stress and the unfolded protein response (UPR) are critical adaptive mechanisms to metabolic alterations in the liver, especially in the context of lipid metabolism [28,29]. Alcohol intake has been shown to induce ER stress [4,27], which contributes to fatty liver [30]. As expected, alcohol-fed *ASMase*$^{+/+}$ mice showed