Acid sphingomyelinase-ceramide system in steatohepatitis: A novel target regulating multiple pathways

Carmen Garcia-Ruiz1,2,3,4,* Jose M. Mato2,5, Dennis Vance6, Neil Kaplowitz7, José C. Fernández-Checa1,2,3,4,*

1Department of Cell Death and Proliferation, Instituto Investigaciones Biomédicas de Barcelona, CSIC, Barcelona, Spain; 2Liver Unit-Hospital Clinic-IDIBAPS, Barcelona, Spain; 3Centro de Investigación Biomédica en Red (CIBERehd), Barcelona, Spain; 4University of Southern California Research Center for Alcohol Liver and Pancreatic Diseases and Cirrhosis, Keck School of Medicine, USC, Los Angeles, CA, USA; 5CIC bioGUNE, Parque Tecnológico de Bizkaia, Bizkaia, Spain; 6Department of Biochemistry and Group on the Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta, Canada; 7Division of Gastrointestinal and Liver Diseases, Keck School of Medicine, University of Southern California Research Center for Liver Diseases, University of Southern California, Los Angeles, USA

Summary

Steatohepatitis (SH) is an intermediate stage of fatty liver disease and is one of the most common causes of chronic liver disease worldwide that may progress to cirrhosis and liver cancer. SH encompasses alcoholic and non-alcoholic steatohepatitis, the latter being of particular concern as it is associated with obesity and insulin resistance and has become a major cause of liver transplantation. The molecular mechanisms governing the transition from steatosis to SH are not fully understood. Here we discuss emerging data indicating that the acid sphingomyelinase (ASMase), a specific mechanism of ceramide generation, is required for the activation of key pathways that regulate steatosis, fibrosis and lipotoxicity, including endoplasmic reticulum stress, autophagy and lysosomal membrane permeabilization. Moreover, ASMase modulates alterations of the methionine cycle and phosphatidylcholine homeostasis, two crucial events involved in SH that regulate methylation reactions, antioxidant defence and membrane integrity. These new findings suggest that targeting ASMase in combination with restoring methionine metabolism and phosphatidylcholine levels may be of utility in the treatment of SH.

Introduction

Fatty liver disease covers a spectrum of disorders that begins with steatosis that can progress to steatohepatitis, ultimately culminating in hepatocellular carcinoma (HCC). Steatohepatitis is characterized by steatosis, hepatocellular death, inflammation and fibrosis and encompasses alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) [1]. ASH is a disease with a high-mortality [2], NASH, which has a high prevalence in Western countries (3–16%) due to its association with obesity and insulin resistance (I/R), is a global health concern and a major cause of liver transplantation [3]. Despite recent progress, the mechanisms underlying the transition from steatosis to ASH/NASH are still poorly understood. In line with the “two-hit” hypothesis [4], the accumulation of lipids in the cytoplasm of hepatocytes, mostly in form of free fatty acids (FA) and triglycerides (TG), is considered a first step and a sensitizing factor to secondary hits that promote ASH/NASH.

Although the predominant view of the two-hit hypothesis postulates that fat quantity determines disease progression, recent findings have shown that the type of fat rather than its amount sensitizes the fatty liver to second hits promoting steatohepatitis [5,6]. Nutritional and genetic models of hepatic steatosis with selective types of fat demonstrated that cholesterol, particularly mitochondrial cholesterol (mChol), rather than TG/FA accumulation, sensitized hepatocytes to TNF/Fas-mediated steatohepatitis. Subsequent studies confirmed this concept [7–9], and paved the way for translational lipidomic studies in patients, describing the association of a dysregulated cholesterol metabolism with NASH [10,11]. Other studies delineated the association of NASH with changes in hepatic lipid composition [12,13].
Review

Currently there is no approved therapy for ASH/NASH and the existing treatments are inefficient. Uncovering the molecular mechanisms and interplay of key metabolic players may lead to the design of novel therapeutic combinations. Acid sphingomyelinase (ASMase), which generates ceramide by sphingomyelin (SM) hydrolysis, mediates TNF/Fas-induced hepatocellular apoptosis and promotes liver fibrogenesis [14–16]. Recent evidence demonstrates that ASMase regulates key mechanisms involved in steatosis, fibrosis and lipotoxicity, including endoplasmic reticulum (ER) stress, autophagy and lysosomal membrane permeabilization (LMP), which contribute to ASH and NASH (Fig. 1) [6,17,18]. A disturbed methionine metabolism, exemplified by decreased S-adenosyl-L-methionine (SAM, also abbreviated as SAMe or AdoMet) and/or increased S-adenosylhomocysteine (SAH) and homocysteine (Hcy) levels, and phosphatidylcholine (PC) depletion control key liver functions and have been described in experimental models of steatohepatitis and in patients [19–26]. Moreover, emerging data link ASMase activation with modulation of SAM and PC homeostasis, suggesting that targeting ASMase and restoring methionine metabolism and PC levels may be a promising strategy for the treatment of ASH/NASH.

Key Points

- ASMase is activated during ASH and NASH and is required for the activation of key molecular pathways mediating ASH/NASH, such as ER stress, autophagy and lysosomal membrane permeabilization (LMP)
- Persistent ER stress is a principal pathway that mediates insulin resistance, lipogenesis and steatosis. Autophagy has been described as a novel pathway causing lipid degradation, hence preventing steatosis, and has emerged as a mediator of liver fibrosis. LMP is a central process mediating lipotoxicity by a cross-talk between mitochondria and lysosomes
- Decreased S-adenosyl-L-methionine (SAM) and phosphatidylcholine (PC) levels are key factors contributing to ASH/NASH by altering methylation reactions, antioxidant defences and membrane integrity. Their repletion was shown to be protective in experimental models but their impact in humans with ASH/NASH remains to be further confirmed
- Emerging evidence indicates a reciprocal regulation between ASMase, decreased SAM and perhaps also with low PC content. In particular, ASMase activation leads to SAM depletion and a decreased SAM/ S-adenosylhomocysteine (SAH) ratio further activates ASMase. In addition to the altered methionine metabolism, ASMase is activated by oxidative stress and inflammatory cytokines
- Therapies targeting ASMase, in association with the replenishment of SAM and PC levels, may be a novel approach for the treatment of ASH/NASH, as they may have the additional advantage of inhibiting ER stress, autophagy and LMP

Fig. 1. ASMase in steatohepatitis: One target, multiple pathways. ASMase becomes activated in liver samples of patients and experimental models of ASH and NASH. Recent evidence shows that ASMase is required for endoplasmic reticulum (ER) stress, autophagy and lysosomal membrane permeabilization (LMP) in ASH/NASH, which in turn mediates insulin resistance (I/R), lipogenesis, fibrosis and regulates steatosis. LMP is a novel pathway of lipotoxicity that cross-talks with mitochondria to induce apoptosis/necrosis. Moreover, ASMase activation depletes hepatic S-adenosyl-L-methionine (SAM) and phosphatidylcholine (PC), which are key intermediates in liver physiology via methylation of multiple substrates (DNA, proteins and lipids), maintenance of antioxidant and GSH defence, and membrane integrity. Thus, ASMase contributes to liver steatosis, liver injury, inflammation and fibrosis, characteristic of ASH/NASH. ASMase targeting may be a novel target of potential relevance for ASH/NASH by disabling multiple pathogenic pathways.

Pathways regulating lipid metabolism, fibrosis and lipotoxicity in steatohepatitis

Endoplasmic reticulum stress

The ER controls protein synthesis and folding. Misfolded proteins cause ER stress and trigger the unfolded protein response (UPR), a complex signalling network, aimed to restore protein homeostasis by reducing protein synthesis and expanding protein folding. The UPR comprises three transducers, inositol requiring (IRE) 1α, PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6α, which activate a wide range of downstream targets [27–29]. Glucose-regulated protein 78 (GRP78, also known as BiP) is the master regulator, controlling activation of the UPR. Upon physiological conditions GRP78 binds these transducers and prevents their activation; however, accumulation of misfolded proteins sequesters GRP78 releasing IRE1α, PERK, and ATF6α to initiate the UPR.
ER stress and the UPR become activated during metabolic alterations in the liver and their persistent activity contributes toASH/NASH by triggering apoptosis, steatosis and I/R [28–32]. In addition to activating XBP-1, IRE1α recruits TNFR-associated receptor 2 leading to JNK activation. Bak and Bax are pro-apoptotic Bcl2 family members, which interact with the IRE1α pathway and mediate, in part, ER stress-induced apoptosis. Pro-caspase-12 also associates with activated IRE1α, allowing its proteolytic cleavage to active caspase-12. CHOP is an important mediator of ER stress-induced apoptosis, as it activates pro-apoptotic genes including the death receptor 5 (DR5), BCL2-like protein 11 (BIM), and Tribbles homolog 3 (TRIB3). Additionally, ER stress-mediated, Ca2+ release recruits and activates the mitochondrial apoptotic pathway [33]. Moreover, c-Jun N-terminal kinase (JNK) activation by ER stress is a critical mediator of I/R by interfering with insulin signalling, due to insulin receptor serine phosphorylation. ER stress inhibition by chemical chaperones restores insulin signalling and alleviates I/R [34]. Finally, an emerging role of ER stress in liver fibrogenesis involves the XBP-1 and IRE1α pathway [33]. Moreover, c-Jun N-terminal kinase (JNK) activation by ER stress is a critical mediator of I/R by interfering with insulin signalling, due to insulin receptor serine phosphorylation. ER stress inhibition by chemical chaperones restores insulin signalling and alleviates I/R [34]. Finally, an emerging role of ER stress in liver fibrogenesis involves the XBP-1 and IRE1α pathway [33].

Overall, ER stress is a key mechanism contributing to apoptosis, steatosis and fibrosis. 

**Autophagy**

Autophagy is a complex and highly regulated proteolytic pathway, involved in the turnover of organelles and cellular debris, which are targeted to lysosomes for degradation [39]. During autophagy, cytoplasmic materials (e.g., organelles or protein aggregates) are sequestered in a double membrane structure, the phagophore (also known as isolation membrane), that lengthens to create an autophagosome, which then fuses with lysosomes to form an autolysosome where its content is degraded. Activated during nutrient starvation, autophagy is considered as a protective mechanism by channelling cellular components for energy supply. Autophagy disruption is involved in the progression of many liver diseases, including ASH/NASH [39]. Besides the dual role of autophagy in cell death, promoting survival or death, depending on the context, recent evidence described a novel role of autophagy in lipid metabolism and hepatic steatosis, called lipophagy [40]. Until recently, the breakdown of TG and lipid droplets in hepatocytes had been thought to occur through the activation of lysosomal lipases. However, pharmacological or genetic inhibition of autophagy in hepatocytes results in hepatic steatosis. Moreover, mice with liver-specific deletion of Atg7 (Atg7 hep-/-), a key protein that regulates autophagosome formation, exhibited defective autophagy and increased TG and cholesterol storage underlying hepatic steatosis [40]. Autophagy induction by the liver-specific overexpression of Atg7 in ob/ob mice improves the metabolic state and reduces steatosis [41]. This new concept fostered the view that impaired autophagy causes hepatic steatosis. Furthermore, by providing FA for the mitochondrial β-oxidation and ATP production, lipophagy regulates not only lipid stores but also cellular energy homeostasis. While attractive, the link between impaired autophagy and hepatic steatosis needs to be further confirmed [42]. For instance, Atg7 hep-/- mice, fed a HFD, have a reduced hepatic lipid content [43]. Moreover, mice with liver-specific deletion of FIP200, a core subunit of the Atg1 complex, are protected from starvation and HFD-induced hepatic steatosis [44]. The view of autophagy as a lipogenic mechanism is also supported by the role of fasting in both processes. Fasting triggers autophagy and it is also known to cause hepatic steatosis. Thus, the role of autophagy in lipid metabolism and hepatic steatosis is complex and may be regulated by several factors, such as genetic background, experimental conditions or aging.

In addition to regulating lipid metabolism, autophagy promotes fibrogenesis by activating HSCs to a myofibroblastic phenotype that leads to the generation of collagen and degradation of matrix architecture. The most characteristic feature of hepatic stellate cells in normal liver is the presence of perinuclear membrane-bound droplets filled with retinyl esters. Recent findings have established that the loss of lipid droplets during HSC activation results from increased autophagic vacuoles and autophagic flux [45,46]. Pharmacologic or genetic autophagy inhibition attenuates HSC activation and fibrogenesis. Importantly, mice with a genetic deletion of Atg7 in HSCs have reduced fibrosis following sustained liver injury [47]. The link between lipophagy and HSC activation implies that autophagy increases energy production by liberating FA from retinyl esters to serve as an energy source for HSCs.

**Lysosomal membrane permeabilization**

Lysosomes are membrane bound organelles, containing specialized hydrolytic enzymes, used for digestion and removal of protein and organelles. In addition to their central role in the turnover of macromolecules, lysosomes play a critical role in cell death regulation [48,49]. A key step, engaging lysosomes in cell death, is the permeabilization of lysosomes, which allows the release of lysosomal content for cell death initiation. Regulation of lysosomal membrane permeabilization (LMP) is thus an important mechanism modulating the participation of lysosomes in cell death pathways, and LMP has emerged as a promising new target of relevance in cancer cell biology and therapy. LMP is caused by a wide range of stimuli, including ROS, saturated FA, sphingosine or cell death effectors such as Bax. LMP releases lysosome contents to the cytosol, including cathepsins (e.g. cathepsin B, CTSB), which in turn target mitochondria, leading to apoptosis. Cathepsin B can cause caspase-independent cell death or recruit mitochondria by BH3-interacting domain death agonist (BID) cleavage. Of relevance for steatohepatitis, palmitic acid (PA), one of the most abundant saturated FA in western diets, causes lipotoxicity
Review

by multifactorial mechanisms, including an ER-mitochondrial cross-talk [50]. Accordingly, it has been described that PA induces LMP in hepatocytes, causing the release of cathepsin B, preceding mitochondrial dysfunction and cytochrome c release [51]. Consequently, cathepsin B antagonism, by pharmacological inhibition or genetic silencing, protected hepatocytes against PA-mediated lipoapoptosis. Thus, LMP is an important pathway promoting saturated FA-mediated lipotoxicity. As discussed below, ASMase is required for LMP and PA-mediated hepatocellular death.

Methionine metabolism and phosphatidylcholine homeostasis in steatohepatitis

Reduced SAM levels and hyperhomocysteinemia

Methionine is primarily metabolized in the liver. Cirrhosis delays plasma clearance of methionine and alcohol alters hepatic methionine metabolism [20]. Methionine metabolism begins with its conversion to SAM by methionine adenosyltransferase I/III (MAT-I/III), the products of the MAT1A gene expressed almost exclusively in the liver [19,52]. SAM is essential in liver physiology as it functions as methyl donor and glutathione (GSH) precursor (Fig. 2). The methylation of acceptors (e.g., DNA, proteins, and lipids), catalysed by methyl transferases, converts SAM to SAH, which is hydrolysed to Hcy and adenosine by SAH hydrolase (SAHH). Methylation of Hcy by methionine synthase (MS) or betaine homocysteine methyltransferase (BHMT), using 5-methyltetrahydrofolate (5MTHF) or betaine as methyl donors, respectively, yields methionine, closing the cycle. The 5MTHF-dependent MS reaction links the folate cycle to the methionine cycle. SAM is catabolized by the glycine N-methyltransferase (GNMT) in a reaction that catalyses the methylation of glycine to form sarcosine. Besides remethylation to methionine, Hcy can be converted to cystathionine by cystathionine-β synthase.

Fig. 2. Hepatic methionine metabolism. Diet-derived methionine is transformed by MAT1A to SAM, which is then used for methylation reactions generating SAH and homocysteine. Homocysteine is remethylated to methionine by betaine or 5MTHF in reactions catalysed by BHMT and methionine synthase (MS). Betaine is derived from diet or mitochondria by diffusion following choline oxidation. MS links the methionine cycle to the folate cycle. Homocysteine escapes the methionine cycle via its transformation to cystathionine in the transsulfuration pathway, which provides cysteine for the synthesis of glutathione (GSH). SAM is transported to mitochondria to promote the methylation of mitochondrial components and the transport of GSH from cytosol by maintenance of mitochondrial membrane-fluidity. Several alterations in the methionine metabolism are associated with steatohepatitis, indicated by the red and blue arrows denoting decreases and increases, respectively. Decreased GNMT leads to increased SAM which contributes to steatohepatitis (see text for details).

SAM is a versatile metabolite that promotes survival in hepatocytes and apoptosis in liver cancer cells [53]. It has been reported that alcohol decreases SAM levels in rats, mice and humans, leading to increased SAH levels [20,54–57]. The consequent alteration in the SAM/SAH ratio impairs hepatocellular functions, including methylation reactions and compromises the antioxidant defence due to GS depletion, which sensitizes cells to TNF and oxidant-induced hepatocellular injury [19,58]. The mechanisms, whereby alcohol depletes SAM, involve MAT1/III inactivation by postranslational modifications [59], and impaired Hcy remethylation due to methionine synthase inhibition. Additionally, alcohol-induced perturbation of the methionine metabolism leads to increased Hcy levels [28,31]. The rise in Hcy is associated with alcohol-induced ER stress, which in turn contributes to steatosis and cell death. However, as discussed below, Hcy-induced ER stress requires ASMase.

SAM is synthesized in the cytosol and in nuclei but a significant bulk (about 30%) is found in mitochondria (mSAM), where it plays a critical role in the methylation of mitochondrial components, in the synthesis of ubiquinone [57,60,61] and in the maintenance of membrane dynamics needed for the transport of mitochondrial GSH (mGSH) [62–64]. Chronic alcohol intake inhibits the transport of mSAM, leading to mSAM depletion independently of the changes on membrane fluidity induced by alcohol [65].

To explore the role of hepatic SAM in liver disease, Mato and colleagues generated MAT1A−/− mice [66]. MAT1A−/− mice displayed reduced hepatic SAM and GSH levels. Although histologically normal, 3-month-old MAT1A−/− mice have hepatic hyperplasia and develop fatty liver on a choline deficient (CD) diet. Additionally, these mice develop spontaneous NASH and HCC at a later age. These findings established a pivotal role for SAM in liver function, and demonstrate that MAT1A deletion determines the progression of NASH to HCC. Consistent with the role of BMHT in maintaining Hcy and SAM homeostasis, BMHT−/− mice exhibit hyperhomocysteinemia and a decreased liver SAM/SAH ratio together with lower PC and SM content [67]. BMHT−/− mice develop liver steatosis at an early age that progresses to HCC. These results indicate that BHMT has an important role in Hcy and methionine homeostasis, thus regulating susceptibility to NASH and HCC. Moreover, GNMT−/− mice, which have increased SAM levels, develop liver steatosis, fibrosis, and HCC, presumably due to aberrant DNA and histone methylation and abnormal PC/PE ratio [68]. The excess SAM content in GNMT−/− mice reroutes phosphatidylethanolamine (PE) towards PC and TG synthesis via the PEMT pathway (see below) [69], while SAM depletion by nicotinamide administration or feeding of a methionine deficient (MD) diet prevents steatosis and fibrosis in GNMT−/− mice [69,70]. The findings in the MAT1A−/− mice (with low hepatic SAM) and in the GNMT−/− mice (with high hepatic SAM) highlight the importance of appropriate levels of SAM in liver physiology.

Interestingly, recent findings have shown that GNMT regulates liver cholesterol by stabilizing NPC2 (Niemann-Pick disease type C2), an endolysosomal protein involved in intracellular cholesterol trafficking [71]. Accordingly, GNMT−/− mice exhibit decreased NPC2 expression and increased hepatic free cholesterol. Given the role of cholesterol in ASH/NASH and the relevance of mChol in cell death and HCC [5,7,8,10,12,72], it is conceivable that, in addition to the alteration of DNA and histone methylation and abnormal PC/PE ratio, the accumulation of mChol may contribute to the phenotype of GNMT−/− mice. Whether or not the protection by nicotinamide administration or MD feeding in GNMT−/− mice [69,70] regulates NPC2 expression and hepatic free cholesterol homeostasis remains to be established.

Reduced phosphatidylcholine levels

The glycerolipids PC and PE are the most abundant phospholipid species in eukaryotic bilayers and are essential for the structure and function of cell membranes. PC and PE are synthesized de novo from choline and ethanolamine, respectively, in the two branches of the Kennedy pathway, based on the formation of high-energy intermediates, CDP-choline and CDP-ethanolamine, which are often referred to as the CDP-choline and CDP-ethanolamine pathway, respectively [73]. During PC synthesis, flux through the CDP-choline pathway is controlled by CTP: phosphocholine cytidylyltransferase (CCT). CCT exists in two isoforms, CCTα and CCTβ, of which CCTα is the predominant isofrom present in liver [74]. Hepatic PC can also be produced via the phosphatidylethanolamine N-methyltransferase (PEMT) that catalyses the methylation of PE to PC using SAM as methyl donor [75]. Thus, PEMT links the methionine cycle to PC homeostasis (Fig. 3). Although the methylation of PE via PEMT is the only pathway for conversion of PE to PC, it accounts for 20–30% of hepatic PC synthesis [76]. However, this pathway is essential for the generation of specific molecular species of PC. In addition to the CDP-choline pathway, another important source of hepatic PC comes from lipoproteins [77].

PC homeostasis is an important factor for ASH/NASH. Patients with hepatic steatosis have 25% less PC in the liver compared to normal subjects [12]. In addition, a functional polymorphism (V175M substitution) within the PEMT gene has been associated with NASH in humans [78,79]. This polymorphism decreases the specific activity of PEMT, suggesting that impaired hepatic PC biosynthesis might play a key role in human NASH. PEMT deletion impairs VLDL secretion, causing hepatic steatosis [80,81]. To explore the role of PC in NASH, Li et al. fed PEMT−/− mice a CD diet [22]. In contrast to wild type littermates fed a CD diet, PEMT−/− mice developed NASH, exhibiting liver failure, panlobular macrovesicular steatosis, hepatocellular ballooning, Mallory’s hyaline, and panacin inflation. Liver injury resulted from a loss of membrane integrity, caused by a decreased PC/PE ratio [22]. Liver samples from patients with NASH also exhibited decreased PC/PE ratio. Interestingly, increased PC levels and subsequent higher PC/PE ratio in the hepatic ER of obese mice have been shown to disrupt Ca2+ homeostasis by inhibiting the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) pump, which triggers ER stress and impaired glucose tolerance [30]. Considering the contrast between MAT1A−/− and GNMT−/− mice regarding SAM levels and PC/PE ratio, it is clear that both abnormally high and low levels of SAM and PC/PE may contribute to ASH/NASH.

Liver-specific CCTα (LCCCTα)−/− mice have impaired apoB-100 secretion and exhibit decreased hepatic PC levels and increased TG accumulation on a regular diet [62]. Interestingly, LCCCTα−/− mice display increased MAT1A and BMHT expression, likely as a compensatory mechanism to meet methylation demand in the context of PC limitation [83]. Feeding LCCCTα−/− mice a high fat diet (HFD) leads to severe NASH within one week, with liver...
Fig. 3. Pathways of hepatic PC generation and role in hepatic steatosis. Liver PC is principally generated from choline via the CDP-choline arm of the Kennedy pathway. An additional pathway of PC generation is the methylation of PE by PEMT using SAM as a methyl donor. PC regulates hepatic steatosis, as PC is essential for VLDL secretion. Moreover, as shown in certain contexts, such as in the liver of LCCTa null mice, PC prevents the unconventional proteolytic processing of SREBP in the ER by proteases SP1/SP2 that lead to the activation of lipogenesis pathways. PC is a substrate for the synthesis of SM catalysed by SMS in the Golgi. PC depletion results in a secondary increase of ceramide levels.
Acid sphingomyelinase in steatohepatitis

Pathways of ceramide generation and its role in metabolism and steatohepatitis

Ceramide is a critical component of membrane bilayers and in addition regulates apoptosis, cellular senescence, stress response, inflammation and metabolism [84,88–91]. Cells generate ceramide by several mechanisms. De novo synthesis occurs in the ER via the condensation of serine and palmitoyl-CoA, catalysed by serine palmitoyltransferase (SPT), and the subsequent acylation of sphingosine with fatty acyl chains of different lengths by ceramide synthases (Cers), which exhibit specificity to different fatty acids, giving rise to heterogeneous ceramide species [92]. In addition to this de novo pathway, ceramide can be produced by SM hydrolysis through activation of SMases, of which the Mg²⁺-dependent, membrane-bound neutral SMase, with a pH optimum of 7.5, and ASMase, with a pH optimum of 4.8, (further classified as lysosomal and secretory ASMase) mediate apoptosis and stress signalling [16,84,88,89].

The role of de novo ceramide synthesis in liver metabolism and pathophysiology has been investigated primarily within the context of hepatic I/R, which is closely associated with NASH and obesity and is a major risk factor for dyslipidemia, hypertension and cardiovascular diseases [90,91]. Studies using lard oil infusions have suggested that, specifically saturated fatty acids activate TLR-4 signalling through the adaptor protein MyD88, leading to the activation of IκB kinase, upregulation of de novo ceramide biosynthesis and ceramide-induced activation of protein phosphatase 2A, which directly inhibits insulin signalling at the level of protein kinase B (Akt) phosphorylation [91,93]. In this model, TLR-4 receptor signalling and ceramide synthesis are both critical for saturated fat-induced hepatic insulin resistance. In contrast, unsaturated fat-induced insulin resistance is not dependent on the TLR-4 receptor or ceramide synthesis [93,94]. However, this model has been disputed by recent findings, showing that both saturated and unsaturated fatty acids induce hepatic insulin resistance independently of TLR-4 signalling and ceramide de novo biosynthesis in vivo through DAG accumulation and subsequent PKC activation [95]. Moreover, mice deleted for CerS2, which preferentially synthesizes very long-chain ceramides (C22–C24), exhibit a compensatory increase in levels of C16 and sphinganine in the liver [96]. These changes in ceramide homeostasis translate into increased rates of hepatocyte apoptosis and proliferation that progress to the widespread formation of nodules of regenerative hepatocellular hyperplasia in aged mice. Progressive hepatomegaly and non-invasive hepatocellular carcinoma are also observed from approximately 10 months of age [96]. The role of de novo ceramide synthesis in the saturated fatty acid (e.g. palmitic acid) mediated lipotoxicity in hepatocytes remains to be established. While palmitic acid is a precursor of ceramide synthesized in the ER, the link between palmitic acid, de novo ceramide generation and ER stress in hepatotoxicity is controversial and poorly understood. Our observations, however, indicate that myristic acid potentiates palmitic acid-induced de novo ceramide synthesis, ER stress and lipotoxicity in primary mouse hepatocytes by a mechanism involving the N-myristoylation and subsequent activation of dihydroceramide synthase (Martinez et al., manuscript in preparation).

Information on the role of SMase in steatohepatitis is scant and limited to the study of FAN (factor associated with NSMase activation). FAN⁺/⁻ mice fed a methionine and choline deficient (MCD) diet exhibit steatohepatitis, decreased PC/PE ratio and increased ceramide generation, indicating that FAN is dispensable in this model of NASH [97]. However, recent evidence demonstrates that ASMase promotes ASH/NASH and that it regulates key players in lipid metabolism.

ASMase promotes hepatocellular apoptosis, liver fibrosis and ASH/NASH

Several mechanisms can contribute to the activation of ASMase in steatohepatitis. As an enzyme mediating stress response and
Review

apoptosis, ASMase is activated by TNF, ROS and oxidative stress, which are critical players in steatohepatitis (see below). The generation of ASMAse−/− mice has been a useful tool to study its role in cellular stress, infection, and apoptosis [98–101]. ASMAse deficiency models Niemann-Pick type A disease, a lysosomal storage disease characterized by the accumulation of SM, cholesterol, and glycosphingolipids in lysosomes of affected organs, particularly brain and liver [102,103]. ASMAse is expressed in almost every cell type and is located mainly within the endosomal/lysosomal compartment, although it has also been found in specific micro-domains in the plasma membrane functioning as a signalling platform for cell surface receptors (e.g. Fas) [104]. ASMAse mediates TNF-induced hepatocellular apoptosis and TNF/Gal-mediated fulminant liver failure and Fas-induced lethal hepatitis [14,104,105], involving the recruitment of mitochondria through ganglioside GD3 generation, which elicits apoptosis by a dual mechanism of ROS generation and NF-xB inhibition [106,107]. Moreover, Cu2+-induced hepatocellular apoptosis, characteristic of Wilson’s disease, requires ASMAse [108]. ASMAse inhibition with desipramine in rats with a mutation in the Atp7b gene, a genetic model of Wilson’s disease, protects against Cu2+-induced hepatocyte death and liver failure. In addition to its role in cell death, ASMAse regulates HSC activation and liver fibrogenesis. Selective stimulation of ASMAse, but not NSMase, occurs during the transdifferentiation of primary mouse HSCs to hepatic stellate cells, coinciding with the processing of the downstream effectors cathepsin B and D [15]. ASMAse antagonism blunted cathepsin B/D processing and prevented the activation and proliferation of mouse and human HSCs. Moreover, it has been shown that amitriptyline, an ASMAse inhibitor, reduces established hepatic fibrosis induced by CCl4 in mice [109].

ASMAse is implicated in alcoholic liver disease [110]. Alcohol feeding activates ASMAse and liver biopsies from patients with acute alcoholic hepatitis exhibit increased ASMAse mRNA levels [6,111,112]. Moreover, ASMAse−/− mice are resistant to alcohol-induced lipogenesis, macrosteatosis, mChol loading and subsequent LPS sensitization and concanavalin A-mediated liver injury [6]. This inability of alcohol to stimulate mChol trafficking was due to the lack of StARD1 expression, a protein essential for the regulation of cholesterol movement to the mitochondrial inner membrane that is regulated by ER stress. Moreover, ASMAse inhibition with amitriptyline in wild type mice prevented alcohol-induced steatosis, liver injury and LPS sensitization, without compromising liver regeneration. Additionally, ASMAse is overexpressed in adipose tissue of ob/ob mice, in mice fed an MCD diet, and in liver and serum samples from patients with NASH [15,113–115]. Moreover, ASMAse deletion, superimposed on a genetic background of LDL receptor deficiency (LDLr−/−/ASMAse−−), prevented diet-induced hyperglycemia and steatosis [116]. These improvements in LDLr−/−/ASMAse−− mice were associated with a paradoxical increase in hepatic ceramide levels and de novo ceramide synthesis due to increased SPT expression. These findings contrast with the effects of ASMAse overexpression in diabetic db/db mice, which improves glucose metabolism [117]. ASMAse−/− mice, fed a HFD, were resistant to HFD-induced activation of lipogenic enzymes [17]. These findings translated into resistance to HFD-mediated steatosis, with similar findings observed when mice were fed a MCD diet. Furthermore, ASMAse inhibition with amitriptyline protected wild type mice against HFD-induced steatosis, liver injury, inflammation and fibrosis. Collectively, these findings indicate that ASMAse plays a critical role in both ASH/NASH.

ASMAse regulates ER stress

Consistent with the resistance to alcohol or HFD-induced lipogenesis and steatosis, ASMAse deficiency prevents the expression of ER stress markers after alcohol or HFD feeding [6,17]. This outcome did not reflect an inherent defect of ASMAse−/− mice to sense ER stress, as tunicamycin induced the expression of ER stress markers and led to increased TG levels, resulting in steatosis [6]. This resistance to alcohol-mediated ER stress was observed despite an increase in plasma Hcy to levels similar to those found in alcohol-fed wild type littermates, regardless of whether alcohol was administered orally or intragastrically, suggesting that resistance to alcohol-induced ER stress in the absence of ASMAse is independent of hyperhomocysteinemia (Baulies et al., unpublished observations) [6]. Consistent with these findings, Boini et al. recently showed that ASMAse ablation prevented Hcy-induced glomerular injury in Cbs−/− mice, a genetic model of hyperhomocysteinemia and increased plasma Hcy levels [118,119]. In this model, ASMAse silencing prevented Hcy-induced ceramide generation, indicating that ASMAse activation by Hcy is a major pathway of glomerular ceramide generation. The findings in alcohol-fed ASMAse−/− mice (intragastrically or orally) are in line with data in mice fed a chow diet supplemented with Hcy, which exhibited increased plasma Hcy levels (3–7 fold) but no pathophysiologic changes or ER stress [120]. Moreover, supplementing MCD diet with Hcy attenuates MCD-induced hepatic UPR activation and liver injury. These results suggest that Hcy at pathophysiologic concentrations plays a minor role in ER stress and steatosis and that the associations between Hcy and UPR are not causally related. HFD feeding increased the expression of ER stress markers in livers of ASMAse−/− mice but not in ASMAse−/− mice [17]. The requirement of ASMAse for alcohol or HFD elicited ER stress argues that ASMAse-induced ceramide production per se triggers ER stress. Treatment of hepatocytes with exogenous ASMAse, but not NSMase, caused ER stress by a mechanism involving disruption of ER Ca2+ homeostasis [6], consistent with the resistance to alcohol or HFD-induced liver injury, as tunicamycin induced the expression of ER stress markers and led to increased TG levels, resulting in steatosis [6]. This resistance to alcohol-mediated ER stress was observed despite an increase in plasma Hcy to levels similar to those found in alcohol-fed wild type littermates, regardless of whether alcohol was administered orally or intragastrically, suggesting that resistance to alcohol-induced ER stress in the absence of ASMAse is independent of hyperhomocysteinemia (Baulies et al., unpublished observations) [6]. Consistent with these findings, Boini et al. recently showed that ASMAse ablation prevented Hcy-induced glomerular injury in Cbs−/− mice, a genetic model of hyperhomocysteinemia and increased plasma Hcy levels [118,119]. In this model, ASMAse silencing prevented Hcy-induced ceramide generation, indicating that ASMAse activation by Hcy is a major pathway of glomerular ceramide generation. The findings in alcohol-fed ASMAse−/− mice (intragastrically or orally) are in line with data in mice fed a chow diet supplemented with Hcy, which exhibited increased plasma Hcy levels (3–7 fold) but no pathophysiologic changes or ER stress [120]. Moreover, supplementing MCD diet with Hcy attenuates MCD-induced hepatic UPR activation and liver injury. These results suggest that Hcy at pathophysiologic concentrations plays a minor role in ER stress and steatosis and that the associations between Hcy and UPR are not causally related. HFD feeding increased the expression of ER stress markers in livers of ASMAse−/− mice but not in ASMAse−/− mice [17]. The requirement of ASMAse for alcohol or HFD elicited ER stress argues that ASMAse-induced ceramide production per se triggers ER stress. Treatment of hepatocytes with exogenous ASMAse, but not NSMase, caused ER stress by a mechanism involving disruption of ER Ca2+ homeostasis [6], consistent with the release of Ca2+ from the ER to the cytosol caused by ceramide [30,32,121], supporting the concept that aberrant lipid composition in the ER regulates SERCA, and hence ER Ca2+ homeostasis and subsequent ER stress susceptibility. However, whether ASMAse activation modulates SERCA activity in the ER, by perturbing ER membrane physical properties, remains to be established. In support for an endolysosomal/ER relationship, previous findings in Niemann Pick type C (NPC) disease have shown that stimulation of ER Ca2+ release by SERCA to the cytosol overcomes reduced lysosomal Ca2+ levels in NPC1−/− cells, and corrects endocytic transport [122]. Lysosomal SM accumulation through ASMAse deficiency inhibits the activity of a principle lysosomal Ca2+ channel (TRPML1) and blocks lysosomal Ca2+-dependent membrane trafficking [123]. Since NPC1 disease is characterized by increased accumulation of endolysosomal cholesterol and sphingolipids, it is conceivable that NPC1, whose physiological role is to participate in the transfer of cholesterol from endolysosomes to the ER to mediate the effect of ASMAse as an ER stress trigger.
ASMase regulates autophagy: The differential role of autophagy in steatosis and fibrosis

Mouse coronary arterial smooth muscle cells from ASMase−/− mice exhibit a defect in the fusion of autophagosomes with lysosomes due to impaired lysosomal function [18]. Knockdown of ASMase suppressed the induction of autophagy in leukemia HL-60 cells induced by amino acid deprivation [124] and ASMase is required for the upregulation of Atg5 expression and autophagy induction in HepG2 cells [125]. ASMase can regulate autophagy by several mechanisms. Decreased SM in lysosomes by ASMase can regulate the TRPLM1/lysosomal Ca2+/dynamin axis. Dynamin is a multi-subunit microtubule motor protein complex, involved in the trafficking of autophagosomes with lysosomes to form autolysosomes that translates to an increased number of autophagosomes [126–128]. Ceramide, an ASMase intermediate, participates in lysosome fusion to cell plasma membranes, endosomes, phagosomes and other organelles, and it is known to regulate cytoskeleton and microtubule assembly [129–132]. Moreover, ceramide can directly interact with LC3I, which may facilitate the targeting of lysosomes to autophagosomes [133].

In line with these findings, hepatocytes from ASMase−/− mice exhibit defective autophagic flux and, intriguingly, this outcome occurs despite resistance to HFD-induced steatosis [17]. This dissociation between autophagy and hepatic steatosis, as described above, is consistent with the finding that autophagy disruption by shRNA-mediated suppression of Atg7 failed to cause hepatic steatosis in lean mice [41]. These findings demonstrate that the role of autophagy in lipid metabolism and steatosis is more complex than thought and may be modulated by other factors. ER stress and autophagy are mutually regulated and defective autophagy triggers ER stress [41]. Accordingly, suppression of autophagy with chloroquine/brefeldin triggered ER stress in primary hepatocytes of ASMase+/+ mice but not ASMase−/− mice, indicating that ASMase is required for autophagy suppression-mediated ER stress. Hence, in the context of impaired autophagy, due to the lack of ASMase, a defective onset of ER stress-induced lipogenesis may be more significant in the regulation of hepatic steatosis than autophagy regulation by fat digestion. Overall, these findings indicate that, while the impact of autophagy regulation by ASMase in steatosis may be minor, ASMase-induced autophagy activation may stand as a novel mechanism contributing to liver fibrosis, based on the emerging link between autophagy and HSC activation (see above).

ASMase regulates lysosomal membrane permeabilization

ASMase deficiency leads to lysosomal cholesterol accumulation (LCA), as illustrated in macrophages from ASMase−/− mice [134]. As the primary phenotype of ASMase deficiency are increased SM levels in lysosomes, the localization of cholesterol in this compartment reflects the high affinity of SM to bind cholesterol, which decreases the efflux of cholesterol out of lysosomes [134]. Further, this trafficking defect impairs the esterification of cholesterol by acyl-CoA:cholesterol acyltransferase, further contributing to LCA in ASMase−/− macrophages. Interestingly, enrichment of wild type macrophages with exogenous SM reproduces LCA, due to decreased cholesterol efflux [134]. The characteristic LCA in ASMase−/− cells is common to other lysosomal storage diseases, such as NPC disease. In fibroblasts treatment with U18666A, which disrupts intracellular cholesterol trafficking and reproduces the NPC phenotype, induces LCA and protects against LMP-mediated apoptosis [135]. Moreover, NPTC−/− Chinese hamster ovary cells are less sensitive to LMP and stauroporine-induced apoptosis than wild type cells [135]. Hepatocytes from ASMase−/− mice exhibit LCA, resulting in the protection against amphiphilic lysosomotropic detergents, which induce cell death and caspase activation following LMP [17]. As LMP has been shown to contribute to PA-induced apoptosis [51], ASMase−/− hepatocytes are also resistant to PA-induced lipotoxicity, an effect that was reversed by depleting LCA with the oxysterol 25-hydroxycholesterol [17]. Thus, LCA resulting from ASMase deficiency modulates the autophagy-lysosomal degradation pathway and ameliorates LMP and the subsequent lipotoxicity of saturated fatty acids. Overall, these findings indicate that ASMase regulates ER stress, autophagy and LMP (Fig. 4), and spotlight ASMase as an important player in lipid metabolism and steatohepatitis.

Interplay between methionine metabolism, acid sphingomyelinase and phosphatidylcholine homeostasis

While disruption of the methionine cycle and PC depletion are well documented in ASH/NASH, the interactions between these metabolic perturbations and ASMase activation have been less recognized.

Mutual regulation between disruption of methionine metabolism and ASMase activation

Ceramide C2 reduces the expression of liver MAT1A [136]. Further, ASMase-induced ceramide generation mediates the down-regulation of MAT1A mRNA expression and a decrease in MATI/II protein levels induced by TNF, resulting in SAM depletion and TNF-mediated liver failure [104]. Although ASMase triggers the accumulation of glycosphingolipids, in particular ganglioside GD3 as shown previously [14,106], the role of ASMase in silencing MAT1A was independent of glycosphingolipid generation. Rather, ASMase-induced ceramide generation shortened the half-life of MAT1A mRNA, in line with previous findings, showing that ceramide C2 reduced the half-life of Bcl2 mRNA through a conserved AU-rich element in its 3’ untranslated region, a mechanism whereby recruitment of the exosome by AU-binding proteins affected 3’–to-5’ mRNA degradation [137,138]. Recent findings showed the stabilization of MAT1A mRNA by AUF1 upon its binding to AU-rich elements in the 3’ untranslated region of MAT1A [139]. However, it remains to be established whether ASMase-induced ceramide generation interferes with AUF1 binding to destabilize MAT1A mRNA. Additionally, findings in mice, fed a MCD diet, showed increased ceramide generation due to selective ASMase activation [114]. The effect of MCD diet was due to the lack of methionine but not choline in the diet, as these effects were reproduced by feeding a MD but not a CD diet, and were accompanied by increased Hcy and decreased SAM/SAH [114]. Moreover, the increase in ceramide levels, caused by the MCD diet, was independent of FAN [140], discarding a role for NSMase activation in the generation of ceramide by MCD diet. Thus, these results establish a self-sustained cycle in which disruption of the methionine cycle and ASMase activation engage due to increased Hcy and lower SAM/SAH (Fig. 4).
A likely mechanism contributing to the relationship between ASMase expression and decreased SAM levels is the methylation status of ASMase in its CpG sites [100]. The epigenetic regulation of ASMase by methylation has been shown in lymphoblasts from patients with deficient ASMase expression and upon incubation with the demethylating agent 5-aza-2'-deoxycytidine [141]. Consistent with a role for SAM in regulating ASMase expression, liver samples from 3-month-old MAT1A/C0/C0 mice exhibit increased basal expression and activity of ASMase, without changes in NSMase or acid ceramidase (unpublished observations), which preceded disease progression of MAT1A/C0/C0 mice typically seen at older age (8 months). ASMase activation in this model is accompanied by the disruption of lipid metabolism and alterations in DNA methylation, all of which could contribute to steatohepatitis progression of MAT1A/C0/C0 mice. Wilson's disease provides another example of the potential interplay between ASMase and SAM levels via DNA methylation. This autosomal recessive genetic disorder is characterized by Cu2+ accumulation in tissues, which manifests in neurological symptoms, anaemia and liver disease. As mentioned above, Cu2+ activates ASMase to cause erythrocyte and hepatocellular apoptosis that were prevented by ASMase inhibition with desipramine [108]. Further, Cu2+ binds noncompetitively to SAHH resulting in its inhibition by releasing NAD+ [142] and altered methionine metabolism has been described in Long-Evans Cinnamon rats, a model of Wilson’s disease [143]. However, it remains to be established whether altered DNA methylation in this model contributes to ASMase activation and if ASMase inhibition by desipramine modulates methionine metabolism.

Besides methylation, ASMase is also subject to redox regulation due to the presence of critical cysteine residues that regulate its activity [100]. Hence, oxidative stress induced by alcohol or HFD is a primary mechanism leading to ASMase activation. Moreover, as mentioned above, ASMase is activated by TNF, an important player in steatohepatitis, in a cascade that involves the proteolytic processing of pro-ASMase within TNF receptosomes [144]. Thus, while ASMase activation and SAM depletion via MAT1A silencing may engage in a vicious cycle, targeting SAM depletion per se may not necessarily translate to the prevention of ASMase activation, as other mechanisms can activate ASMase independently of SAM depletion, such as inflammatory cytokines (e.g. TNF) or oxidative stress.

ASMase regulates PC homeostasis

In addition to ASMase activation, MCD or MD diets deplete PC levels (30–40%) and decrease PC/PE ratio in liver homogenates and mitochondria [114]. Although the decrease in PC could be the consequence of a reduced methylation of PE to PC by PEMT, due to decreased SAM levels, it could also reflect the inhibition of de novo PC synthesis by ceramide. For example, previous findings indicated that ceramide C2 or ceramide C6 directly inhibited
the CCT step of PC synthesis, resulting in increased phosphatidylcholine levels [145–147]. Moreover, it has been shown in vitro that sphingosine inhibits CCT activity in rat liver [148]. As ceramide can be deacylated by ceramidases to sphingosine, it is conceivable to imagine an inhibitory effect of ASMase on CCT by ceramide as well as sphingosine generation (Fig. 4). Glucocorticoids increase PC levels by stimulating CCT activity, accounting for the increase in surfactant synthesis in lung [149,150]. Interestingly, betamethasone enhances CCT activity and PC levels in rat lung, effects that were accompanied by increased SM content and ASMase inhibition [151]. As described above, LCTTx−/− mice not only exhibit reduced hepatic PC and increased DAG levels, but also display increased liver ceramide content [25]. Since strategies that reversed PC levels or decreased DAG accumulation (CDP-choline or lysophosphatidylcholine) in LCTTx−/− mice fed a HFD, failed to decrease ceramide content, this outcome suggests that the source of ceramide generation in this model is not linked to de novo SM synthesis in the Golgi (Fig. 3), indicating an alternative route for ceramide generation. Although the specific pathway, contributing to the described increased hepatic ceramide content in LCTTx−/− mice, remains to be determined, an attractive possibility would be the activation of ASMase, establishing a link between low PC and ASMase activation. Accordingly, ASMase−/− mice exhibit increased PC levels in the liver and brain [102], which contribute to the protection of ASMase−/− mice against MCD-induced hepatic steatosis [17]. Although further work is needed to characterize the functional relationship between ASMase and PC, it appears that PC reduction and ASMase activation engage in a vicious cycle of potential significance in steatohepatitis.

Future perspectives

The lack of approved therapies contributes to the growing number of patients who will face ASH/NASH and progressive liver dysfunction in the near future. Disturbed methionine metabolism is a key hallmark of ASH/NASH, and alterations in PC homeostasis contribute to disease progression. Targeting these specific pathways with exogenous SAM, betaine or replenishment of PC levels has shown promising results in experimental models [20,25,31,152,153]. However, while SAM treatment has shown beneficial effects in patients with moderate alcoholic cirrhosis [24,154], the impact of betaine or polyenylphosphatidylcholine in patients with NASH has been less promising [21,26]. Choline supplementation protected Pemt−/−/Ldlr−/− mice against high fat high cholesterol diet induced steatohepatitis. However, protection was not due to restoration of hepatic PC levels or PC/PE ratio but was associated with decreased hepatic cholesterol accumulation and normalization of the expression of cholesterol-metabolizing enzymes [155]. Moreover, complex diseases, such as steatohepatitis, require combination therapies and the elucidation of interplays of metabolic pathways individually recognized to play a role in this process may open new therapeutic avenues. In this scenario, decreased SAM content by reduced MAT1A expression and low PC levels activate ASMase, which in turn, represses MAT1A and impairs PC de novo biosynthesis, establishing a feed-forward loop. Moreover, ASMase is required for the activation of pathways that regulate steatosis, fibrosis and lipotoxicity. This emerging functional link provides a rationale to restore SAM and PC levels in combination with ASMase inhibition that may be of potential relevance in ASH/NASH treatment. Moreover, in addition to the contribution of SAM/PC depletion to ASMase activation, ASMase becomes activated in response to other stimuli, including inflammatory cytokines (e.g. TNF) or oxidative stress, which are known key players in steatohepatitis. While further work remains to be done as to whether ASMase activation precedes the depletion of SAM/PC or not, both processes are mutually regulated in a vicious cycle of potential relevance to chronic liver disease. Targeting just SAM/PC may not guarantee ASMase inactivation. Although a new generation of ASMase inhibitors are being developed, tricyclic antidepressants, which are already in the clinic, have been shown to efficiently antagonize ASMase by preventing its proteolytic activation. Thus, it may be constructive to test these in new experimental models that combine alterations in SAM/SAdh or decreased PC levels with ASMase deficiency to more accurately evaluate the potential of applying this approach to patients.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

Review


...The ratio of Metabolic and inflammatory markers in non-alcoholic fatty liver disease...
Review


