Research Article

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Methionine and S-adenosylmethionine levels are critical regulators of PP2A activity modulating lipophagy during steatosis

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Background & Aims: Glycine N-methyltransferase (GNMT) expression is decreased in some patients with severe non-alcoholic fatty liver disease. *Gnmt* deficiency in mice (*Gnmt*-KO) results in abnormally elevated serum levels of methionine and its metabolite S-adenosylmethionine (SAMe), and this leads to rapid liver steatosis development. Autophagy plays a critical role in lipid catabolism (lipophagy), and defects in autophagy have been related to liver steatosis development. Since methionine and its metabolite SAMe are well known inactivators of autophagy, we aimed to examine whether high levels of both metabolites could block autophagy-mediated lipid catabolism.

Methods: We examined methionine levels in a cohort of 358 serum samples from steatotic patients. We used hepatocytes cultured with methionine and SAMe, and hepatocytes and livers from *Gnmt*-KO mice.

Results: We detected a significant increase in serum methionine levels in steatotic patients. We observed that autophagy and lipophagy were impaired in hepatocytes cultured with high methionine and SAMe, and that *Gnmt*-KO livers were characterized by an impairment in autophagy functionality, likely caused by defects at the lysosomal level. Elevated levels of methionine and SAMe activated PP2A by methylation, while blocking PP2A activity restored autophagy flux in *Gnmt*-KO hepatocytes, and in hepatocytes treated with SAMe and methionine. Finally, normalization of methionine and SAMe levels in *Gnmt*-KO mice using a methionine deficient diet normalized the methylation capacity, PP2A methylation, autophagy, and ameliorated liver steatosis.

Conclusions: These data suggest that elevated levels of methionine and SAMe can inhibit autophagic catabolism of lipids contributing to liver steatosis.

Keywords: Autophagy; SAMe; Methionine; Liver; Steatosis; *Gnmt*.

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Abbreviations: GNMT, glycine N-methyltransferase; SAMe, S-adenosylmethionine; MTOR, the mammalian target of rapamycin; PP2A, protein phosphatase 2A; LD, lipid droplets; MAT, methionine adenosyltransferase; SAH, S-adenosylhomocysteine; DG, diglyceride; TG, triglycerides; PEMT, phosphatidylethanolamine N-methyltransferase; AHCY, S-adenosylhomocysteine hydrolase; NAFLD, non-alcoholic fatty liver disease; Deaza, deazaadenosine; LC3, microtubule-associated light chain 3; AST, aspartate transaminase; ALT, alanine transaminase; H&E, hematoxylin and eosin; SQSTM1/p62, sequestosome 1; Chlo, chloroquine; N/L, ammonium chloride and leupeptin; LAMP2, lysosome-associated membrane protein type 2; O.A, oleic acid; 4EBP, eukaryotic translation initiation factor 4E-binding protein 1; PE, phosphatidylethanolamine; PC, phosphatidylcholine; MDD, methionine deficient diet; MDM, medium deficient in methionine; SAH, S-adenosyl-L-homocysteine.

Introduction

Liver steatosis results from an excessive delivery of free fatty acids from adipose tissue into the liver, and from a misbalance in *de novo* lipid synthesis and catabolism [1]. During autophagy, cellular proteins and organelles are sequestered by autophagosomes and degraded after fusion with lysosomes in response to a lack of nutrients or to stress [2]. Autophagy can also hydrolyze triglycerides (TG) in a process known as lipophagy [3], and

inhibition of autophagy in hepatocytes leads to lipid accumulation [3]. Conversely, genetical [4] or pharmacological [5] promotion of autophagy alleviates hepatic steatosis in non-alcoholic fatty liver disease (NAFLD) in mice. These studies suggest that defects in autophagy might be involved in the development of fatty liver [3,6], and that modulation of autophagy could be beneficial in NAFLD [5].

S-adenosylmethionine (SAMe), the principal biological methyl donor, is synthesized from methionine and ATP in a reaction catalyzed by the enzyme methionine adenosyltransferase (MAT). SAMe, upon transfer of its activated methyl group to an acceptor molecule such as glycine, is converted to S-adenosylhomocysteine (SAH) [7]. Glycine *N*-methyltransferase (GNMT) is one of the key enzymes involved in methionine and SAMe metabolism [8], and it has been proposed that GNMT maintains intracellular concentrations of SAMe within a narrow range [9]. There are several genetic conditions that lead to abnormally elevated plasma concentrations of methionine and SAMe that have been related with liver steatosis, such as GNMT, S-adenosylhomocysteine hydrolase and cystathionine β-synthase deficiency [10]. For example, *Gnmt*-KO mice, characterized by highly elevated SAMe and methionine levels, develop steatosis, fibrosis and hepatocelullar carcinoma [11].

Recently, it was shown that *GNMT* mRNA levels were often repressed in advanced NAFLD patients [12]. Hypermethioninemias has been associated with liver steatosis [13], and dietary methionine restriction can reverse liver steatosis in the *ob*/*ob* mice [14]. In a recent study in yeast, it was shown that methionine-induced inhibition of autophagy could be due to its conversion into SAMe [15]. It is an interesting possibility that high levels of methionine and/or its metabolite SAMe could also contribute to hepatic steatosis development by blocking lipid degradation through inhibition of lipophagy.

We found that the abnormally elevated levels of methionine and SAMe in the absence of *Gnmt* induced protein phosphatase 2A (PP2A) methylation that led to an inhibition of autophagy flux, contributing to lipid accumulation. Strikingly, we found that this effect was independent of the mammalian target of rapamycin (MTOR), one of the critical inactivators of the autophagosome formation [16]. Altogether, these findings indicate that *Gnmt* deficiency, and/or hypermethioninemia and elevated SAMe levels could play a role in the pathogenesis of NAFLD by inhibiting lipophagy through PP2A methylation.

Material and methods

Human samples

Serum samples from non-steatotic and NAFLD patients were examined (non-steatotic = 92, NAFLD = 263). Clinical data were collected retrospectively using patient records described in a previous report [13]. Informed consent for all clinical investigations was obtained in accordance with the principles in the Declaration of Helsinki. The institutional review board of the Hospitals involved approved the protocol.

Animals

3-month-old male *Gnmt*-KO (n = 10) or WT (n = 10) mice were maintained on a methionine deficient diet (MDD) (S8946-E020 EF AIN 76A 0.15% L-methionine, from SSNIFF) for 21 days prior to analysis [17]. 3-month-old male wild-type (WT; n = 10) and *Gnmt*-KO (n = 10) mice were euthanized after 8 h and 24 h of food starvation. Experiments were performed following the guidelines of the CIC bioGUNE institutional review committee on animal use.

Hepatocyte isolation

Hepatocytes were isolated from mice livers by collagenase perfusion [18]. SAMe (Samyr®) was from Abbott. Purity of hepatocytes was determined by comparing their mRNA expression levels of *Desmin* (a hepatic stellate cell marker) and *F4/80* (a macrophage marker) with the expression levels in purified cultures of hepatic stellate cells and Kupffer cells (each set as 100% value) [19]. We observed a maximum of 2.4% of contamination with Kupffer cells and 0.1% of contamination with hepatic stellate cells (data not shown).

Methionine and SAMe measurements

SAMe and methionine were determined as described in previous reports [11].

Western blot analysis

Immunoblotting was performed with specific antibodies (Supplementary Table 2). In selected figures, images of gels were constructed by splicing non-contiguous portions of the same gel (indicated by dashed lines).

Autophagic flux

Autophagic flux was determined by analyzing LC3-II turnover by preventing lysosomal degradation, using leupeptin (100 μM) and ammonium chloride treatment (20 mM) (N/L)] [20] or using chloroquine (60 μM) (Chlo). LC3-flux was calculated by subtracting the densitometry value of normalized LC3-II in the sample treated with N/L or Chlo, by the value in the control sample (untreated). LC3-II flux was expressed relative to their respective controls.

Immunocytochemistry

Paraformaldehyde-fixed hepatocytes were incubated with antibodies against LC3 overnight (Supplementary Table 2). Then, the cells were incubated with CY3 or FITC-conjugated secondary goat antibodies and the DNA-binding fluorochrome DAPI (D9542, SIGMA). Samples were examined under Leica TCS-SP (UV) confocal laser microscope (60× objective). Quantification of LC3 puncta staining was performed using ImageJ software as detailed elsewhere [21]. LC3-flux was calculated by subtracting the number of LC3 puncta per cell in the sample treated with Chlo by the value of LC3 puncta per cell in the control sample (untreated with Chlo).

BODIPY staining

Hepatocytes in culture were incubated with BODIPY 493/503 (Molecular Probes) at a concentration of 1 mg/ml during 30 min prior to fixation (4% paraformaldehyde). Quantification of lipid bodies was performed using Frida Software as detailed elsewhere [22].

Histological analysis

Formalin-fixed liver sections were stained with hematoxylin–eosin or Periodic acid–Schiff. For Sudan Red staining, frozen liver tissue sections were used [11].

Quantification of lipids

Lysosome-enriched fraction was isolated from liver pieces (500 mg) [23]. Lipids were extracted from tissue, cell [24] and lysosome [25] homogenates, and TG were quantified using a commercial kit (37481 and 37484 from Menarini Diagnostics). Liver free cholesterol and cholesteryl esters, were separated by thin layer chromatography and quantified by optical densitometry as detailed elsewhere [25].