Research Article

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Induction of autophagy improves hepatic lipid metabolism in glucose-6-phosphatase deficiency

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Background & Aims: Glucose-6-phosphatase (G6Pase α, G6PC) deficiency, also known as von Gierke's disease or GSDIa, is the most common glycogen storage disorder. It is characterized by a decreased ability of the liver to convert glucose-6-phosphate (G6P) to glucose leading to glycogen and lipid over-accumulation progressing to liver failure and/or hepatomas and carcinomas. Autophagy of intracellular lipid stores (lipophagy) has been shown to stimulate fatty acid β-oxidation in hepatic cells. Thus, we examined autophagy and its effects on reducing hepatic lipid over-accumulation in several cell culture and animal models of GSDIa.

Methods: Autophagy in G6PC-deficient hepatic cell lines, mice, and dogs was measured by Western blotting for key autophagy markers. Pro-autophagic Unc51-like kinase 1 (ULK1/ATG1) was overexpressed in G6PC-deficient hepatic cells, and lipid clearance and oxidative phosphorylation measured. *G6PC*$^{-/-}$ mice and GSDIa dogs were treated with rapamycin and assessed for liver function.

Results: Autophagy was impaired in the cell culture, mouse, and canine models of GSDIa. Stimulation of the anti-autophagic mTOR, and inhibition of the pro-autophagic AMPK pathways occurred both *in vitro* and *in vivo*. Induction of autophagy by ULK1/ATG1 overexpression decreased lipid accumulation and increased oxidative phosphorylation in G6PC-deficient hepatic cells. Rapamycin treatment induced autophagy and decreased hepatic triglyceride and glycogen content in *G6PC*$^{-/-}$ mice, as well as reduced liver size and improved circulating markers of liver damage in GSDIa dogs.

Conclusions: Autophagy is impaired in GSDIa. Pharmacological induction of autophagy corrects hepatic lipid over-accumulation and may represent a new therapeutic strategy for GSDIa.

Introduction

Glucose-6-phosphatase α, (G6Pase α, G6PC), is an ER-resident enzyme that is primarily expressed in the liver and kidney. It catalyzes the formation of free glucose from glucose-6-phosphate (G6P) in the final step of gluconeogenesis and glycogenolysis [1]. Loss-of-function mutations of G6PC lead to glycogen storage disease Ia (GSDIa, von Gierke's disease) [1]. This disease usually presents as hypoglycemia in infancy due to decreased ability by the liver to generate free glucose during the fasted state [2]. Improvements in dietary therapy aimed at preventing hypoglycemia have enabled patients with GSDIa to survive into adulthood [3]. However, these patients still develop derangements in hepatic metabolism such as increased *de novo* lipogenesis and glycogen synthesis, as well as decreased lipid β-oxidation and ketone production, that eventually can lead to steatohepatitis, liver failure, and development of hepatic adenomas as well as hepatocellular carcinoma [1,4,5].

(Macro)autophagy is a process in which cells engulf and degrade damaged intracellular components and cytoplasmic contents [6]. This process maintains organelle quality control [7] as

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Abbreviations: G6Pase α, glucose-6-phosphatase α; G6PC, glucose-6-phosphatase α catalytic subunit; GSDIa, glycogen storage disease type Ia; G6P, glucose-6-phosphate; mTOR, mammalian target of rapamycin; AMPK, 5' AMP-activated protein kinase; ULK1, Unc51-like kinase 1; NAFLD, non-alcoholic fatty liver disease; GSDII, glycogen storage disease type II; LC3, microtubule-associated protein 1A/1B-light chain 3; C-C3, cleaved caspase 3; Rap, rapamycin; AAV, adeno-associated virus; GFP, green fluorescent protein; RFP, red fluorescent protein; OCR, oxygen consumption rate; ALT, alanine amino transferase; KD, knockdown; KO, knockout.

well as degrades macromolecules such as proteins [8], glycogen [9], and lipid droplets [10], to provide substrates for cellular metabolism. Recently, impaired autophagy has been linked to several metabolic liver disorders such as non-alcoholic fatty liver disease (NAFLD) [11], α1-antitrypsin deficiency [12], and hemochromatosis [13]. Furthermore, autophagy has been shown to be impaired in other disorders of glycogen metabolism such as GSDII (Pompe's Disease) [14] and Lafora's disease [15]. Interestingly, mice with tissue-specific knockout of the key autophagy gene, *ATG7*, also showed lipid accumulation, decreased lipid turnover, and eventual development of hepatic tumors [10,16]. Induction of autophagy recently was shown to reduce hepatic lipid content and promote ketogenesis by degrading triglycerides to form free fatty acids in a process known as lipophagy [10,17–19]. Other studies also have suggested that autophagy participates in the degradation of glycogen via lysosomal α-glucosidase activity to generate free glucose in a manner that does not require G6PC [20,21]. In order to further examine the potential role of autophagy in the hepatic metabolism of GSDIa, we investigated basal autophagy in mouse, dog, and cell culture models of GSDIa. We found that autophagy was impaired in each of these models, and that restoration of autophagy led to reduced triglyceride content. Taken together, our data demonstrate a critical role for autophagy in the pathogenesis of GSDIa, and suggest that correction of the autophagy defect could be a novel strategy for the treatment of this disorder.

Materials and methods

Study approval

Animal studies were approved by the Duke University Institutional Animal Care and Use Committee (IACUC) under protocol numbers A231-11-09 and A083-11-04. All reasonable steps to reduce animal suffering were undertaken, and all animals received care according the criteria outlined in NIH publication 86-23.

Reagents

Detailed information concerning reagents used in this study is available in the Supplementary material.

Plasmids

Myc-hULK1 plasmid [22] (Addgene plasmid 31961) was a gift from D. Kim (University of Minnesota). GFP-RFP-LC3 (Addgene plasmid 21073) plasmid was a gift from T. Yoshimori (Osaka University) [12]. Constitutively active AMPK plasmid (pEBG-AMPK α1[1-312], Addgene plasmid 27632) was a gift from Dr. Reuben Shaw (Salk Institute) [23]. pEGFP-n1-TFEB (Addgene plasmid 38119) was a gift from Shawn Ferguson (Yale University) [24].

Animal models

G6PC$^{-/-}$ mice were identified, and G6PC transduced with AAV vector as previously described [25,26]. Transduction of G6PC into knockout (KO) mice was performed as previously reported. GSD1a affected puppies were identified, and AAV-G6Pase vector administered as previously described [27,28]. For more detailed information, please consult the Supplementary material.

Additional experiments

Western blotting, qPCR, immunofluorescence, and electron microscopy were performed as previously described [29]. Additional information regarding cell culture models and experiments, Western blotting, microscopy, oximetry, and metabolite analysis is available in the Supplementary material.

Statistics

Cell culture experiments were performed in triplicates and repeated three independent times using matched controls. Results were expressed as mean ± SEM. For all experiments details relating to number of samples, and statistical tests of significance are given in the legends of the appropriate figures.

Results

To determine the status of autophagy in GSDIa, we performed Western blotting to detect LC3-II, a marker for autophagosome number [30], in previously described *G6PC*$^{-/-}$ mice (referred hereafter as KO) [31] and littermate controls, and found that LC3-II levels were reduced in the KO mice (Fig. 1A; Supplementary Fig. 1A). Furthermore, the levels of the pro-autophagic proteins ATG5 and Beclin-1 were reduced in the KO mouse livers (Supplementary Fig. 1B, C). Similarly, autophagy marker LC3-II also was reduced in the kidneys of these mice (Supplementary Fig. 1D).

We next compared hepatic LC3-II levels in dogs homozygous for a naturally occurring loss-of-function mutation in *G6PC* (GSDIa dogs) [32] that had previously failed gene therapy and had recurrent GSDIa, with asymptomatic heterozygote carrier dogs and found that LC3-II levels also were decreased (Fig. 1B). To further confirm these findings, we transfected immortalized mouse hepatocytes (AML-12 cells), with three individual siRNAs targeting G6PC, and observed a decrease in endogenous LC3 puncta following immunostaining in cells that underwent G6PC knockdown (KD) (Fig. 1C). To ensure that the AML-12 cells were an accurate model system for the GSDIa livers, we measured G6P levels, and found an increase in the KD cells (Supplementary Fig. 2A). Furthermore, we performed periodic acid/Schiff Base staining (PAS) and observed an increase in glycogen deposits following KD (Supplementary Fig. 2B), as well as increased lipid droplet accumulation when the cells were stained with the fluorescent lipid dye, bodipy 493/503 (Supplementary Fig. 2C). Previous work has also shown an increase in apoptosis in the livers of G6PC KO mice [33], and we reconfirmed that by finding increased protein levels of cleaved caspase 3 and PARP in KO mouse livers (Supplementary Fig 2D, E); increased apoptosis was noted in the KD cells through blotting for cleaved caspase 3 (Supplementary Fig. 2F). LC3-II protein levels were reduced 96 h following siG6PC treatment of AML-12 cells; a similar result was seen following KD in two other hepatic cell lines, HepG2, and HuH7 (Fig. 1D). In addition, the levels of the pro-autophagic protein Beclin-1, and mRNA levels of several pro-autophagic genes also were reduced in AML-12 cells following KD (Supplementary Fig. 3A, B). To eliminate the possibilities of aberrant off-target effects, we tested each siRNA individually in AML-12 and HepG2 cells and consistently observed lower LC3-II levels (Supplementary Fig. 3C, D). In addition we observed that the decrease in LC3-II levels was tightly coupled with temporal changes in G6PC protein levels, as 48 h after KD, neither G6PC nor LC3-II levels were significantly decreased (Supplementary Fig. 3E), but both were significantly decreased 72 h after KD (Supplementary Fig. 3F). To confirm that the loss of autophagy was in fact G6PC-dependant, we examined the livers of KO mice treated with AAV2/9-G6Pase in which restoration of G6Pase activity was previously confirmed [25], and found that LC3-II levels were significantly higher in the AAV-treated mice compared to control vector-treated KO mice or wild-type (WT) mice (Fig. 1E).