Sirtuin signaling controls mitochondrial function in glycogen storage disease type Ia

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Abstract

Glycogen storage disease type Ia (GSD-Ia) deficient in glucose-6-phosphatase- α (G6Pase- α) is a metabolic disorder characterized by impaired glucose homeostasis and a long-term complication of hepatocellular adenoma/carcinoma (HCA/HCC). Mitochondrial dysfunction has been implicated in GSD-Ia but the underlying mechanism and its contribution to HCA/HCC development remain unclear. We have shown that hepatic G6Pase- α deficiency leads to downregulation of sirtuin 1 (SIRT1) signaling that underlies defective hepatic autophagy in GSD-Ia. SIRT1 is a NAD⁺-dependent deacetylase that can deacetylate and activate peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), a master regulator of mitochondrial integrity, biogenesis, and function. We hypothesized that downregulation of hepatic SIRT1 signaling in G6Pase- α -deficient livers impairs PGC-1 α activity, leading to mitochondrial dysfunction. Here we show that the G6Pase- α -deficient livers display defective PGC-1 α signaling, reduced numbers of functional mitochondria, and impaired oxidative phosphorylation. Overexpression of hepatic SIRT1 restores PGC-1 α activity, normalizes the expression of electron transport chain components, and increases mitochondrial complex IV activity. We have previously shown that restoration of hepatic G6Pase- α expression normalized SIRT1 signaling. We now show that restoration of hepatic G6Pase- α expression also restores PGC-1 α activity and mitochondrial function. Finally, we show that HCA/HCC lesions found in G6Pase- α -deficient livers contain marked mitochondrial and oxidative DNA damage. Taken together, our study shows that downregulation of hepatic SIRT1/PGC-1 α signaling underlies mitochondrial dysfunction and that oxidative DNA damage incurred by damaged mitochondria may contribute to HCA/HCC development in GSD-Ia.

Keywords

Mitochondria; Glucose-6-phosphatase- α ; SIRT1/PGC-1 α signaling; Liver homeostasis

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Conflict of interest J.-H. Cho, G.-Y. Kim, B. C. Mansfield, and J. Y. Chou declare that they have no conflict of interest.

Animal rights All institutional and national guidelines for the care and use of laboratory animals were followed.

Introduction

Glycogen storage disease type Ia (GSD-Ia; MIM232200) is caused by a deficiency in glucose-6-phosphatase- α (G6Pase- α or G6PC) that is expressed primarily in the liver, kidney, and intestine (Chou et al. 2002, 2010). G6Pase- α catalyzes the hydrolysis of G6P to glucose in the terminal step of gluconeogenesis and glycogenolysis and is the key enzyme maintaining interprandial blood glucose homeostasis. G6Pase- α is also crucial to liver homeostasis. G6Pase- α -deficient livers exhibit elevated glycogen synthesis and enhanced glycolysis which are reflected in the clinical manifestations in GSD-Ia that include hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia (Chou et al. 2002, 2010). One of the most severe long-term complications in GSD-Ia is hepatocellular adenoma (HCA) that may undergo malignant transformation to carcinoma (HCC) (Chou et al. 2002, 2010). The glycogen shunt, a condition when glucose is shunted to glycogen and subsequently consumed through glycolysis, has been implicated in promoting cancer cell survival (Shulman and Rothman 2017). Moreover, the metabolic and non-metabolic changes mediated by enhanced glycolysis also promote cancer cell proliferation and invasion (DeBerardinis et al. 2008). More recently, we have shown that G6Pase- α deficiency leads to defective hepatic autophagy that is frequently associated with carcinogenesis (Cho et al. 2017).

Mitochondria are organelles that supply energy, provide building blocks for new cells, control redox homeostasis, regulate cell signaling, and control cell death (Vyas et al. 2016; Zong et al. 2016). The primary function of mitochondria is to produce energy in the form of ATP via oxidative phosphorylation (OXPHOS) (Sazanov 2015). Defective OXPHOS can lead to an overproduction of reactive oxygen species (ROS) that damage DNA, proteins, and lipid membranes (Sun et al. 2016). Consequently, mitochondrial dysfunction has been linked to many diseases (Wei et al. 2008; Gorman et al. 2016). Three studies have documented abnormal or dysfunctional mitochondria in GSD-Ia, demonstrating a decrease in mitochondrial number (Riede et al. 1980), the presence of swollen and deformed mitochondria (Cho et al. 2017), and an impairment in OXPHOS and mitochondrial ultrastructure (Farah et al. 2017). While these studies suggest a link of mitochondrial dysfunction to GSD-Ia, the underlying mechanisms remain unknown.

Mitochondrial homeostasis is tightly regulated by the coordinated actions of mitochondrial biogenesis and mitochondria-selective autophagy (mitophagy) (Palikaras and Tavernarakis 2014). The peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) serves as a transcriptional co-activator which plays a critical role in maintaining mitochondrial integrity, biogenesis, and function (Ventura-Clapier et al. 2008). Although multiple signaling pathways regulate PGC-1 α expression and activity, post-translational protein acetylation has emerged as a critical regulator of activity (Jeninga et al. 2010). The PGC-1 α activity can be activated via deacetylation by SIRT1, a NAD⁺-dependent deacetylase, and inhibited via acetylation by general control non-repressed protein 5 (GCN5), a lysine acetyltransferase (Jeninga et al. 2010).

We have shown that hepatic G6Pase- α deficiency causes downregulation of SIRT1 signaling and hypothesize that this will reduce PGC-1 α activity, leading to mitochondrial dysfunction

in GSD-Ia. In this study, we show that impairment in hepatic SIRT1/PGC-1 α signaling underlies mitochondrial dysfunction in GSD-Ia. We further show that the HCA/HCC lesions present in G6Pase- α -deficient livers accumulate markedly increased levels of damaged mitochondria and oxidative DNA damage.

Materials and methods

Animals

All animal studies were conducted under a protocol approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee. The liver-specific *G6pc*-deficient (L-*G6pc*^{-/-}) mice and the control L-*G6pc*^{+/+} and L-*G6pc*^{+/-} mice have been described (Cho et al. 2017). To overexpress SIRT1, mice were infused at 12 weeks with *G6pc* gene deletion (WP) via the retro-orbital sinus with Ad-SIRT1, a SIRT1-expressing adenovirus (Ad) vector at 3.3×10^9 plaque-forming unit (pfu)/kg and analyzed at 13 WP. Ad-GFP was used as a control. To reconstitute hepatic G6Pase- α activity, mice at 4 WP were infused via retro-orbital sinus with rAAV-G6PC (Yiu et al. 2010), a G6Pase- α expressing recombinant adeno-associated virus (rAAV) vector at 1×10^{12} viral particles (vp)/kg and analyzed at 12 WP. Liver samples were collected from mice following a 6-h fast.

Hepatocytes isolation and flow cytometry analysis

Hepatocytes were isolated from mice at 12 WP after 6 h of fasting using the collagenase perfusion method. Due to the high glycogen and fat levels in the livers of L-*G6pc*^{-/-} mice, hepatocyte preparations from both control and L-*G6pc*^{-/-} mice were purified further via 20% Percoll gradient to deplete these components along with dead or dying cells as previously described (Cho et al. 2017). The resulting hepatocyte lysates were then deproteinized and used to determine ATP levels. It is important to note that ATP levels estimated in the purified hepatocytes could be significantly lower than the levels determined in freshly isolated hepatocytes because of the potential loss of ATP during the extended hepatocyte isolation procedure. To determine functional mitochondria, 2×10^5 hepatocytes were incubated with 200 nM MitoTracker Red (Molecular probes, Grand Island, NY) for 30 min at 37°C, washed with PBS, and analyzed by flow cytometry using a Guava EasyCyte Mini System (Millipore, St Charles, MO).

Analysis of oxygen consumption

Basal oxygen consumption was measured using the Oxygen Consumption Rate Assay Kit (Cayman Chemical, Ann Arbor, MI) that utilizes a phosphorescent oxygen probe to measure oxygen consumption rates in living cells. In the assay, the extracellular oxygen consumption reagent is quenched by oxygen, and the rates of oxygen consumption during mitochondrial respiration are calculated from the changes in fluorescence signal over time. Briefly, hepatocytes (4×10^4 cells per well) in a 96-well plate were incubated at 37°C in 150 μ l of HepatoZYME-SFM and 10 μ l of MitoXpress probe and overlaid with 100 μ l of HS Mineral Oil. The oxygen consumption was measured using a FlexStation II Fluorimeter (Molecular Devices, Sunnyvale, CA) at Ex/Em = 380/650 nm every 2 min for 40 min.

Complex IV enzymatic activity measurement

Histochemical analysis of complex IV enzymatic activity was performed as described by Sotgia et al. (2012). Briefly, 10 μ m air-dried liver cryosections were incubated for 30 min at room temperature in 10 ml PBS solution containing 10 mg cytochrome c, 10 mg diaminobenzidine tetrahydrochloride (DAB), and 2 mg catalase (Sigma-Aldrich, St. Louise, MO). The complex IV enzyme activity was monitored by oxidation of DAB to a brown color. Sodium azide (1 mM) was used as a complex IV enzyme inhibitor.

Isolation of mitochondria and analysis of metabolites

Liver mitochondria were isolated using the Mitochondria Isolation Kit (Thermo Scientific, Waltham, MA) and levels of mitochondrial NADH were determined using the EnzyChrom NAD⁺/NADH assay kit from BioAssay Systems (Hayward, CA). The levels of ATP in deproteinized hepatocyte lysates and levels of acetyl-coA in deproteinized liver lysates were determined using the respective assay kit obtained from BioVision (Mountain View, CA).

Quantitative real-time RT-PCR and western blot analyses

The expression of mRNA was quantified by real-time PCR using the TaqMan probes in an Applied Biosystems 7300 Real-Time PCR system (Life Technologies, Carlsbad, CA). Data were normalized to Rpl19 RNA. Western blot analysis was performed as described previously (Cho et al. 2017). Rabbit monoclonal antibodies used from Cell Signaling Technology (Danvers, MA) were Acetylated-Lysine, GCN5, and p-H2AX-S139. Mouse monoclonal antibodies used from Abcam (Cambridge, MA) were Total OXPHOS Rodent WB Antibody Cocktail and PINK1 and from Santa Cruz Biotechnology (Dallas, TX) were β -actin and TFAM. Rabbit polyclonal antibodies used were ACLY (Cell Signaling Technology), MTATP6 (Abcam), SIRT1 (Millipore, Billerica, MA), and PGC-1 α (Novus Biologicals, Littleton, CO). The monoclonal antibody against human G6Pase- α has been described (Cho et al. 2017).

Immunoprecipitation

Immunoprecipitation was performed as previously described (Cho et al. 2017). To detect acetyl-PGC-1 α , liver lysates were immunoprecipitated with an antibody against the acetylated lysine and analyzed by western blot using the antibody against PGC-1 α .

Electron microscopy

Electron microscopy analysis of mouse liver sections was described previously (Cho et al. 2017). Briefly, mouse livers were fixed in 2.5% glutaraldehyde, treated with 1% osmium tetroxide and 2% uranyl acetate, serially dehydrated and serially infiltrated via Spurr's resin/ethanol, polymerized, and cut into 90 nm sections. The resulting grids were stained with uranyl acetate and lead citrate, imaged with a JEOL-1400 transmission electron microscope operated at 80 kV, and images acquired on an AMT BioSprint 29 camera. Quantitation of mitochondria in hepatocytes was performed using Image J software (NIH).

Immunohistochemical analysis

Mouse liver tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Grand Island, NY), embedded in paraffin, sectioned to 10 μ m thickness, and paraffin removed by Xylene (Fisher Scientific, Waltham, MA). Sections were then incubated in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 10 min at 100 °C. Endogenous peroxidases were quenched with 0.9% hydrogen peroxide in methanol, blocked with the Avidin/Biotin Blocking Kit (Vector Laboratories), then incubated with an antibody against 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Abcam), followed by the appropriate biotinylated secondary antibodies (Vector Laboratories). The resulting complexes were detected with an ABC kit using the DAB Substrate (Vector Laboratories). Sections were also counterstained with hematoxylin (Sigma-Aldrich) and visualized using a Zeiss Axioskop2 plus microscope equipped with 10X/0.45NA, 20X/0.5NA or 40X/0.75NA objectives (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The unpaired *t* test was performed by using the GraphPad Prism Program, version 4 (San Diego, CA). The values were considered statistically significant at *P* < 0.05.

Results

Livers of L-G6pc^{-/-} mice harbor dysfunctional mitochondria

Oxidative phosphorylation is more efficient than glycolysis in ATP production per glucose, but at high G6P levels, glycolysis can contribute significantly to ATP levels (DeBerardinis et al. 2008). In GSD-Ia, hepatic G6Pase- α -deficiency results in increased hepatic glycolysis. However, ATP levels were similar between hepatocytes isolated from L-G6pc^{-/-} and control mice at 12 WP (Fig. 1a), suggesting that the G6Pase- α -deficient hepatocytes exhibit impaired OXPHOS. Indeed, the basal oxygen consumption, an indicator of mitochondrial OXPHOS activity, was reduced in G6Pase- α -deficient hepatocytes, compared to controls (Fig. 1b). Moreover, levels of functional mitochondria detected by MitoTracker Red CMXRos staining in G6Pase- α -deficient hepatocytes at 12 WP were ~50% of controls (Fig. 1c). Electron microscopy of liver sections stained for active mitochondria showed ~30% fewer mitochondria per hepatocyte in L-G6pc^{-/-} mice (Fig. 1d). This suggests that the loss of functional mitochondria in G6Pase- α -deficient liver might impair OXPHOS.

In OXPHOS, mitochondria generate ATP via five protein complexes (I to V) in the electron transport chain (ETC) (Fosslien 2001). OXPHOS impairment can result from either substrate limitation or defective ETC (Fosslien 2001). Hepatic levels of mitochondrial NADH, an electron donor for OXPHOS, were similar between control and L-G6pc^{-/-} mice at 12 WP (Fig. 1e). However, hepatic levels of cytochrome c oxidase 1 (MTCO1) in complex IV, a gene encoded by mitochondrial DNA, were statistically lower in L-G6pc^{-/-} mice (Fig. 1f), suggesting that defective ETC impairs hepatic OXPHOS.

We have recently shown that hepatic G6Pase- α deficiency leads to down-regulation of SIRT1 signaling and defective hepatic autophagy in GSD-Ia (Cho et al. 2017). PGC-1 α can be activated via deacetylation by SIRT1 and inhibited via acetylation by GCN5 (Jeninga et

al. 2010). Consistent with this, hepatic levels of SIRT1 were significantly lower in *L-G6pc*^{-/-} mice at 12 WP, while hepatic levels of GCN5 were unchanged (Fig. 2a). At 12 WP, total hepatic levels of PGC-1 α protein were similar between control and *L-G6pc*^{-/-} mice (Fig. 2b), but the ratios of acetylated PGC-1 α (inactive form) to total PGC-1 α were significantly higher in the livers of *L-G6pc*^{-/-} mice in marked contrast to those of controls (Fig. 2b).

PGC-1 α can activate nuclear respiratory factor 1 and 2, leading to the induction of mitochondrial transcription factor A (TFAM), a key transcriptional activator of mitochondrial DNA replication and gene expression (Austin and St-Pierre 2012). The *L-G6pc*^{-/-} mice displayed decreased hepatic expression of TFAM (Fig. 2c), consistent with a reduced PGC-1 α activity. Hepatic levels of MTATP6, the mitochondrial DNA encoded subunit 6 of ATP synthase in complex V were also decreased in *L-G6pc*^{-/-} mice (Fig. 2c). Protein acetylation can also be regulated by the substrate acetyl-CoA (Jeninga et al. 2010; Wellen et al. 2009). High levels of acetyl-CoA have been reported in GSD-Ia patients (Jones et al. 2009). In *L-G6pc*^{-/-} mice at 12 WP, hepatic levels of ATP-citrate lyase (ACLY) (Fig. 2a) that catalyzes the synthesis of acetyl-CoA from citrate, and acetyl-CoA (Fig. 2d) were both increased, compared to controls. Collectively, attenuated SIRT1 expression and elevated acetyl-CoA correlate with increased hepatic levels of acetyl-PGC-1 α in *L-G6pc*^{-/-} mice.

SIRT1 overexpression normalizes hepatic levels of acetyl-PGC-1 α , MTCO1, MTATP6, and complex IV activity

To demonstrate that downregulation of hepatic SIRT1 signaling underlies suppressed PGC-1 α activity and attenuated expression of mitochondrial ETC components, we examined the effects of SIRT1 overexpression. Ad-SIRT1-mediated hepatic SIRT1 overexpression significantly reduced levels of acetyl-PGC-1 α and increased levels of MTCO1 and MTATP6 in the livers of *L-G6pc*^{-/-} mice, compared to the controls (Fig. 3a). Supporting this, enzyme histochemical analysis showed that SIRT1 overexpression in *L-G6pc*^{-/-} mice also restored mitochondrial complex IV enzymatic activity (Fig. 3b). We have previously shown that SIRT1 restoration failed to normalize metabolic alterations associated with GSD-Ia (Cho et al. 2017). We now show that SIRT1 overexpression is unable to normalize hepatic acetyl-CoA contents and serum triglyceride levels in the *L-G6pc*^{-/-} mice (Fig. 3c). Collectively, inactivation of hepatic PGC-1 α activity via downregulation of SIRT1 signaling leads to the hepatic mitochondrial dysfunction seen in the *L-G6pc*^{-/-} mice.

Hepatic G6Pase- α restoration corrects mitochondrial dysfunction

We have recently shown that rAAV-G6PC-mediated restoration of hepatic G6Pase- α expression corrects impaired SIRT1 signaling associated with GSD-Ia (Cho et al. 2017). We therefore treated *L-G6pc*^{-/-} mice at 4 WP with rAAV-G6PC and examined mitochondrial function of the treated mice at 12 WP. We showed that restoration of hepatic G6Pase- α expression normalized hepatic levels of acetyl-PGC-1 α , MTCO1, MTATP6 (Fig. 4a), and mitochondrial complex IV activity (Fig. 4b). The results demonstrate that restoration of hepatic G6Pase- α expression normalizes hepatic OXPHOS and that G6Pase- α plays a critical role in liver homeostasis and mitochondrial function.

HCA/HCC lesions accumulate damaged mitochondria and exhibit oxidative DNA damage

In agreement with Mutel et al. (2011), none of the *L-G6pc*^{-/-} mice developed tumors at the pre-tumor stage of 12 WP, 30% developed HCA/HCC at the tumor-developing stage of 53 WP, and 100% developed HCA/HCC at tumor-bearing stage of 78 WP. To investigate the chronological changes of mitochondrial dysfunction in G6Pase- α -deficient livers, we examined protein levels of PINK1 (PTEN-induced putative kinase 1), a mitochondrial kinase that is selectively accumulated in the outer membrane of the damaged mitochondria (Ashrafi and Schwarz 2013). Notably, compared to controls, hepatic levels of PINK1 in *L-G6pc*^{-/-} mice were unchanged at 12 WP, began to increase at 24 WP, and became markedly elevated at 53 WP (Fig. 5a), indicating progressive accumulation of damaged mitochondria in G6Pase- α -deficient livers.

Remarkably, all HCA/HCC lesions analyzed displayed mitochondrial and DNA damage evident by increased protein levels of PINK1 and p-H2AX which is a hallmark of DNA double-strand break (Fig. 5b). At the pre-tumor stage of 12 WP, the decrease in functional mitochondria was reflected by reduced MTCO1 expression in complex IV (Fig. 1f). Intriguingly, the HCA/HCC lesions had reduced expression of additional ETC components, complex I, II, III along with a further decrease in MTCO1 (Fig. 5c), indicating that tumor lesions harbored more dysfunctional mitochondria. Damaged mitochondria are the sources of ROS that cause oxidative DNA damage (Sun et al. 2016). Indeed, immunohistochemical analysis revealed a markedly increased immunostaining of 8-OHdG, indicative of oxidative DNA damage in the HCA/HCC lesions of the *L-G6pc*^{-/-} mice (Fig. 5d). Damaged mitochondria frequently exhibit disruption of cristae, the fundamental structures for mitochondria and the site of OXPHOS, leading to impaired mitochondrial respiration (Cogliati et al. 2016). In tumor-bearing *L-G6pc*^{-/-} mice, disrupted cristae were detected primarily in HCA/HCC lesions (Fig. 5e), indicating the presence of damaged mitochondria in tumor regions. Collectively, these results show that HCA/HCC developed in the G6Pase- α -deficient liver is characterized by marked mitochondrial and oxidative DNA damage.

Discussion

GSD-Ia is a metabolic disorder caused by a deficiency in G6Pase- α which catalyzes the hydrolysis of G6P to glucose in the terminal step of gluconeogenesis and glycogenolysis of the liver, kidney, and intestine (Chou et al. 2002, 2010). GSD-Ia patients manifest impaired glucose homeostasis and a severe long-term complication of HCA that may undergo malignant transformation to HCC (Chou et al. 2002, 2010). The etiology of tumor development in GSD-Ia remains unclear. To study the mechanism underlying HCA/HCC development in GSD-Ia, we generated *L-G6pc*^{-/-} mice which develop tumors (Cho et al. 2017). Using the *L-G6pc*^{-/-} mice, we showed that hepatic G6Pase- α -deficiency downregulates the SIRT1 deacetylase leading to hepatic autophagy impairment, which could contribute to tumor development in GSD-Ia (Cho et al. 2017). Mitochondria play multifunctional roles in malignant tumor progression (Vyas et al. 2016; Zong et al. 2016). Previous studies have linked abnormal or dysfunctional mitochondria with GSD-Ia (Riede et al. 1980; Cho et al. 2017; Farah et al. 2017), but the underlying mechanism is unknown. PGC-1 α is a transcriptional factor that plays a critical role in maintaining mitochondrial

integrity, biogenesis, and function (Ventura-Clapier et al. 2008). PGC-1 α activity can be activated via protein deacetylation (Jeninga et al. 2010). Our hypothesis was that in the G6Pase- α -deficiency of GSD-Ia, SIRT1 downregulation impairs PGC-1 α activity, leading to mitochondrial dysfunction. Here, we provide evidence supporting this hypothesis. We further show that HCA/HCC development in the G6Pase- α -deficient livers correlates with a progressive accumulation of damaged mitochondria along with markers of oxidative DNA damage.

We provide evidence showing that G6Pase- α -deficient livers exhibited increased levels of the inactive, acetyl-PGC-1 α and mitochondrial dysfunction reflected by reduced basal oxygen consumption, fewer functional and total mitochondria per hepatocyte, and decreased levels of MTCO1 in complex IV and MTATP6 in complex V in the mitochondria. An increase in ACLY activity and levels of acetyl-CoA that would reinforce acetylation-based inactivation of PGC-1 α , were also present. Importantly, hepatic SIRT1 overexpression normalized levels of acetyl-PGC-1 α , restored the attenuated expression of ETC components, and increased mitochondrial complex IV activity, demonstrating that downregulation of SIRT1-PGC-1 α signaling underlies mitochondrial dysfunction in GSD-Ia.

The HCA/HCC lesions isolated from *L-G6pc*^{-/-} mice exhibit marked mitochondrial and oxidative DNA damage evidenced by increased expression of PINK1 and p-H2AX, reduced expression of mitochondrial ETC components, increased 8-OHdG staining, and disrupted cristae. This most likely reflects the impairment in hepatic autophagy/mitophagy previously reported and impairment of mitochondrial biogenesis, which can lead to a progressive accumulation of damaged mitochondria that generate excess ROS leading to the oxidative DNA damage and genomic instability observed. Collectively, the results suggest that HCA/HCC development in GSD-Ia is mediated, at least in part, by oxidative DNA damage incurred by damaged mitochondria.

Previous studies have shown that restoration of hepatic G6Pase- α expression in *L-G6pc*^{-/-} mice corrects metabolic abnormalities associated with GSD-Ia, normalizes SIRT1 signaling, and rectifies hepatic autophagy deficiency. We now show that restoration of hepatic G6Pase- α expression also normalizes PGC-1 α signaling and mitochondria function in GSD-Ia. Therefore, G6Pase- α plays a key role in liver homeostasis, and impaired hepatic autophagy and mitochondrial dysfunction caused by hepatic G6Pase- α deficiency may contribute to HCA/HCC development in GSD-Ia. The finding that hepatic SIRT1 overexpression normalizes mitochondrial dysfunction and autophagy impairment suggests that restoration of SIRT1 signaling offers a potential therapeutic strategy to slow down or prevent HCA/HCC development in GSD-Ia. In the present study, SIRT1 overexpression was mediated by Ad-SIRT1 and the action was transient. We have now constructed rAAV-SIRT1, a SIRT1 expressing recombinant adeno-associated virus vector to examine whether longer-term restoration of SIRT1 signaling can slow down or prevent HCA/HCC development in GSD-Ia.

Acknowledgements

We thank Dr. Pierre Chambon for the gift of the AlbCreERT2 mice. Microscopy imaging was performed at the Microscopy & Imaging Core (National Institute of Child Health and Human Development, NIH) with the assistance of Chip Dye.

Funding This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health; and by the Children's Fund for Glycogen Storage Disease Research.

Abbreviations

GSD-Ia	Glycogen storage disease type Ia
G6Pase-α	Glucose-6-phosphatase- α
HCA	Hepatocellular adenoma
HCC	Hepatocellular carcinoma
SIRT1	Sirtuin 1
PGC-1α	Peroxisome proliferator-activated receptor- γ coactivator 1 α
G6P	Glucose-6-phosphate
OXPHOS	Oxidative phosphorylation
ETC	Electron transport chain
GCN5	General control non-repressed protein 5
WP	Weeks post <i>G6pc</i> gene deletion
rAAV	Recombinant adeno-associated virus
MTCO1	Mitochondrially encoded cytochrome c oxidase 1
MTATP6	Mitochondrial gene encoded subunit 6 of mitochondrial ATP synthase
TFAM	Mitochondrial transcription factor A
ACLY	ATP-citrate lyase
PINK1	PTEN-induced putative kinase 1
8-OHdG	8-hydroxy-2'-deoxyguanosine

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Fig. 1. Impaired hepatic mitochondrial function in L-G6pc^{-/-} mice. Control and L-G6pc^{-/-} mice at 12 WP were used. **a** ATP levels ($n = 7$). **b** Basal oxygen consumption in control and G6Pase- α -deficient hepatocytes ($n = 4$). **c** Quantitative flow cytometric analysis of hepatocytes stained with MitoTracker Red CMXRos ($n = 5$). **d** Electron micrographs of hepatocytes and quantification of mitochondrial numbers in the livers of control and L-G6pc^{-/-} mice ($n = 3$). L denotes lipid droplet. Scale bar, 10 μ m. **e** Mitochondrial NADH levels ($n = 6$). **(f)** Western-

blot analysis of hepatic ETC components and quantification by densitometry ($n = 6$). Data represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$

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Fig. 2. Downregulation of PGC-1 α signaling in L-G6pc^{-/-} mice. Control and L-G6pc^{-/-} mice at 12 WP were used. **a** Western-blot analysis of SIRT1, GCN5, ACLY, and β -actin, and quantification by densitometry ($n = 4$). **b** Western-blot analysis of PGC-1 α either after immunoprecipitation (IP) of liver lysates with an antibody against the acetylated lysine or in whole liver lysates, and quantification by densitometry ($n = 4$). β -actin was used as a loading control. **c** Western-blot analysis of hepatic TFAM, MTATP6 and β -actin, and quantification by densitometry ($n = 4$). **d** Hepatic levels of acetyl-CoA ($n = 4$). Data represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$

Fig. 3. SIRT1 overexpression corrects hepatic mitochondrial dysfunction in L-G6pc^{-/-} mice. Control and L-G6pc^{-/-} mice at 12 WP were treated with 3.3×10^9 pfu/kg of Ad-GFP or Ad-SIRT1, and analyzed at 13 WP. **a** Western-blot analysis of PGC-1α after immunoprecipitation (IP) of liver lysates with an antibody against the acetylated lysine, and western blot analysis of the indicated proteins in liver lysates from control and L-G6pc^{-/-} mice treated with either Ad-GFP or Ad-SIRT1, and quantification by densitometry ($n = 4$). **b** Histochemical analysis of complex IV enzymatic activity in liver sections. Na⁺-azide, an

inhibitor of complex IV activity, was shown to abolish complex IV enzymatic activity in the liver section of Ad-SIRT1-treated L-*G6pc*^{-/-} mice. Scale bar, 100 μ m. **c** The levels of hepatic acetyl-CoA ($n = 5$) and serum triglyceride ($n = 9$) in control and L-*G6pc*^{-/-} mice treated with either Ad-GFP or Ad-SIRT1. Data represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$

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Fig. 4. Hepatic G6Pase- α restoration normalizes mitochondrial function. L-G6pc^{-/-} mice were treated with 1×10^{12} vp/kg of rAAV-G6PC at 4 WP and analyzed at 12 WP. **a** Western-blot analysis of PGC-1 α after immunoprecipitation (IP) of liver lysates with an antibody against the acetylated lysine, western blot analysis of the indicated proteins in liver lysates, and quantification by densitometry ($n = 4$). **b** Histochemical analysis of complex IV enzymatic activity in liver sections. Na⁺-azide, an inhibitor of complex IV activity, was shown to abolish complex IV enzymatic activity in the liver section of rAAV-G6PC-treated L-G6pc^{-/-} mice. Scale bar, 100 μ m. Data represent the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$

Fig. 5. The HCA/HCC lesions accumulate damaged mitochondria and exhibit oxidative DNA damage. **a** Western-blot analysis of the indicated proteins in the livers of control and L-G6pc^{-/-} mice at 12, 24 and 53 WP, and quantification by densitometry ($n = 3$). **b** Western-blot analysis of PINK1, p-H2AX and β -actin in the non-tumor (N) liver tissues and tumor (T) lesions of the L-G6pc^{-/-} mice, and quantification by densitometry ($n = 5$). **c** Western blot analysis of hepatic ETC components in littermate control (C) and tumor-bearing L-G6pc^{-/-} mice and quantification of protein levels by densitometry ($n = 4$). The non-tumor (N) and tumor (T) region of the same mouse were examined. The asterisk denotes a non-specific band. **d** Immunohistochemical analysis of 8-OHdG in non-tumor liver tissues, HCA and HCC from the same L-G6pc^{-/-} mouse. Scale bar, 100 μ m. **e** Electron micrographs of non-

tumor liver tissues, HCA and HCC from the same *L-G6pc*^{-/-} mouse. N denotes nucleus. Scale bar, 5 μ m. The insets represent higher magnification views. Scale bar, 1 μ m. Data represent the mean $\pm$ SEM. **P* < 0.05, ***P* < 0.005

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