Inhibition of glycogen biosynthesis via mTORC1 suppression as an adjunct therapy for Pompe disease

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abstract

Pompe disease, also known as glycogen storage disease (GSD) type II, is caused by deficiency of lysosomal acid α-glucosidase (GAA). The resulting glycogen accumulation causes a spectrum of disease severity ranging from a rapidly progressive course that is typically fatal by 1–2 years of age to a more slowly progressive course that causes significant morbidity and early mortality in children and adults. Recombinant human GAA (rhGAA) improves clinical outcomes with variable results. Adjunct therapy that increases the effectiveness of rhGAA may benefit some Pompe patients. Co-administration of the mTORC1 inhibitor rapamycin with rhGAA in a GAA knockout mouse reduced muscle glycogen content more than rhGAA or rapamycin alone. These results suggest mTORC1 inhibition may benefit GSDs that involve glycogen accumulation in muscle.

Introduction

Pompe disease, also known as acid maltase deficiency and glycogen storage disease (GSD) type II, is an autosomal recessive metabolic myopathy caused by deficiency of lysosomal acid α-glucosidase (GAA). GAA is an exo-1,4- and -1,6-α-glucosidase that hydrolyzes glycogen to glucose. The resulting glycogen accumulation causes progressive damage to cardiac, respiratory, and skeletal muscle. The disease ranges from a rapidly progressive infantile course that is usually fatal by 1–2 years of age to a more slowly progressive and extremely heterogeneous course that causes significant morbidity and early mortality in children and adults [1,2].

Treatment of Pompe disease was primarily symptomatic until 2006 when enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA, alglucosidase alfa, Myozyme/C210, Genzyme Corporation) was approved [3]. rhGAA addresses the primary defect in Pompe disease by breaking down excess lysosomal glycogen. Clinical trials in infants demonstrated that rhGAA improves cardiomyopathy and prolongs survival [4–10], while clinical trials in adults demonstrated improved motor function and stabilization of pulmonary function [11,12]. The natural history, presentation, and progression of Pompe disease are extremely heterogeneous. Accordingly, response to treatment is also variable and a minority of patients treated with rhGAA has shown marginal or no improvement. ERT plus an adjunct therapy that increases the effectiveness of rhGAA may benefit some patients with Pompe disease. An ideal therapy would reduce glycogen biosynthesis in all tissues except liver, since it is desirable that liver glycogen reserves not be affected because they are critical to the maintenance of whole-body glucose homeostasis. Given the contribution of respiratory insufficiency to the morbidity and mortality in Pompe disease [13], some patients may benefit from adjunct therapies to ERT that reduce glycogen accumulation in the diaphragm and other respiratory muscles. If successful for Pompe disease, such an approach to glycogen biosynthesis inhibition may have potential application in other diseases that involve glycogen storage in muscle.

While small molecule inhibitors have been developed to decrease the synthesis of glycolipids that accumulate in some lysosomal storage diseases [14], it is not clear that a similar “substrate reduction” strategy could be applied to reduce glycogen synthesis in glycogen storage diseases such as Pompe. It has been shown in GAA⁻/⁻ mice that substrate reduction can be achieved by genetically knocking out the glycogen synthase (GS) gene [15] or by using intramuscular viral vector delivery of shRNAs to GS and glycogenin in the gastrocnemius of GAA⁻/⁻ mice [16]. While these studies have been informative, a practical clinical paradigm that
affects glycogen synthesis in muscle has thus far not been forthcoming.

We have examined the biochemical pathways regulating glycogen accumulation in muscle tissue of GAA−/− mice [17] and evaluated the efficacy of small molecule inhibitors of these pathways combined with ERT. It has been reported that in cultured muscle cells GS activity can be partially regulated by the mammalian target of rapamycin (mTOR) [18–21]. mTOR is a conserved serine/threonine kinase that exists in several distinct multiprotein complexes such as mTORC1 (containing raptor) and mTORC2 (containing rictor). Some functions of mTOR could be independent of both mTORC1 and mTORC2 [22]. mTORC1 is regulated by the nutrient status of the cell [23,24]. Rapamycin bound to FKBP12 inhibits mTORC1. Unexpectedly, we found the ubiquitous mTORC1 pathway in vivo robustly regulates glycogen synthesis in skeletal muscle but does not affect glycogen synthesis in liver. We also found that the mTORC1 pathway has a minimal effect on glycogen synthesis in heart and diaphragm. To our knowledge these organ-specific differences in the mTORC1 pathway have not previously been reported. Our findings suggest that mTORC1 inhibition with rapamycin may benefit GSDs that involve glycogen accumulation in skeletal muscle.

**Results**

We presumed that mTORC1 might partially regulate GS by affecting its phosphorylation state (Fig. 1A). GS activity is regulated by phosphorylation at several sites, including Ser 641, which is known to repress GS activity [25]. Treating both wild-type (data not shown) and GAA−/− mice with the mTORC1 inhibitor rapamycin increased GS phosphorylation at Ser 641/645 in skeletal muscle, indicating that mTORC1 at least partially regulates GS activity in muscle (Fig. 1B). This result suggested that rapamycin might reduce glycogen synthesis and thereby complement the glycogen reduction in muscle seen with rhGAA treatment in Pompe disease.

To test this hypothesis, we evaluated the effects of rapamycin in combination with increasing doses of rhGAA on glycogen levels in GAA−/− mice. Tissue glycogen levels were determined in 3- to 4-month-old mice (Fig. 2, Baseline). Cohorts of mice were then given vehicle, rhGAA, or rapamycin plus rhGAA for 12 weeks. Biochemical analysis of glycogen levels in collected tissues demonstrated that rapamycin in combination with rhGAA improved glycogen clearance in heart, skeletal muscle, and diaphragm. The mice were not fasted before sacrifice because we wanted to assess natural liver glycogen levels. In mice that have not been fasted, the majority of glycogen in liver is found in the cytosol and not the lysosome. It is fortunate that inhibiting the mTORC1 pathway with rapamycin did not affect liver glycogen levels (Fig. 2), confirming that rapamycin does not inhibit liver gluconeogenesis and that whole-body glucose homeostasis is not affected by rapamycin, as previously reported [26,27].

The rate of glycogen accumulation in skeletal muscle decreases as GAA−/− mice age. To determine if rapamycin alone can slow glycogen accumulation, we began rapamycin treatment in young mice, in which lysosomal glycogen accumulates rapidly. We therefore evaluated rapamycin alone and in combination with rhGAA in young GAA−/− mice. Glycogen levels were determined at the start of the study in 5-week-old GAA−/− mice (Fig. 3, Baseline). Cohorts of mice were then administered vehicle, rapamycin, rhGAA, or rapamycin plus rhGAA for 12 weeks (Fig. 3). Biochemical analysis of glycogen levels in collected tissues demonstrated that rapamycin alone reduced glycogen accumulation in quadriceps and triceps, confirming our finding that the mTORC1 pathway regulates glycogen synthesis in skeletal muscle. Rapamycin alone did not inhibit glycogen accumulation in heart and had a small effect in diaphragm, implying that as a monotherapy rapamycin would not effectively treat these critical tissues. However, rapamycin combined with rhGAA did improve glycogen reduction in diaphragm. It is worth noting that rhGAA alone effectively reduces glycogen in cardiac muscle. The tissue-specific effects of rapamycin therefore appear to complement those of rhGAA, implying that their simultaneous use should reduce glycogen stores in all the tissues affected by Pompe disease. We have treated 9-month-old Pompe mice for 12 weeks with vehicle or rapamycin and observed that glycogen in relevant tissues did not increase over the course of the study in the vehicle treated group (data not shown). Glycogen levels were found to be similar between rapamycin and vehicle treated animals, most likely because in this model there is a natural decline in glycogen biosynthesis in older mice.

Administration of multiple doses of rhGAA to GAA−/− mice in these experiments results in high antibody titers to rhGAA. A similar antibody response occurs in patients with Pompe disease that have been given multiple doses of rhGAA. It has been proposed that the effectiveness of rhGAA may be impaired by a high, sustained antibody response to rhGAA [28]. In the current experiments, rapamycin dramatically reduced the antibody titers to rhGAA in GAA−/− mice (Fig. 4A). Even though rapamycin alone slowed glycogen accumulation in skeletal muscle in the absence of anti-rhGAA antibodies (Fig. 3), it was plausible that the improved glycogen clearance observed with the combination of rapamycin
and rhGAA was due to the rapamycin-mediated reduction of anti-rhGAA antibodies (rapamycin is an immunomodulatory agent and is clinically indicated for the prophylaxis of organ rejection in patients receiving a renal transplant) [29]. To evaluate this possible mechanism of action of rapamycin, we tolerized GAA<sup>−/−</sup> mice to rhGAA with a recombinant adeno-associated virus (AAV) vector encoding an enzymatically inactive mutant GAA (D404N) under the transcriptional control of a liver-restricted promoter as previously described [30] (Fig. 4A). GAA<sup>−/−</sup> mice were tolerized to rhGAA and then dosed with rhGAA or rhGAA plus rapamycin for 12 weeks. In the total absence of anti-rhGAA antibodies in the tolerized mice, the combination of rapamycin plus rhGAA reduced glycogen more than rhGAA alone (Fig. 4B). The efficacy of rapamycin in GAA<sup>−/−</sup> mice therefore appears to be due to the ability of rapamycin to inhibit glycogen synthesis rather than to its suppression of anti-rhGAA antibodies.

We also investigated whether the glycogen-reducing and immunosuppressive attributes of rapamycin were separable activities. First, titrating the rapamycin dose downward revealed that rapamycin abated glycogen accumulation at 0.25 mg/kg/day but...
was not effective at 0.025 mg/kg/day in GAA/C0 mice (Fig. 5A). We did not observe rapamycin side effects, e.g., failure to eat, abnormal body posture, or lack of grooming, at any of the doses used.

To assess immune suppression as a function of rapamycin dose, C57Bl/6 mice were treated with an adenovirus vector encoding $\alpha$-galactosidase A under the transcriptional control of a CMV promoter followed by rapamycin for 28 days. Rapamycin at 0.025 mg/kg/day reduced the antibody titer to $\alpha$-galactosidase A. (Fig. 5B). These data indicate that the lowest dose of rapamycin needed to suppress glycogen accumulation in skeletal muscle is also immunosuppressive.

To determine whether the observed glycogen-lowering effects are unique to rapamycin, we evaluated the rapamycin analogs AP23573 and CCI-779. When used in combination with rhGAA in

![Fig. 4. Rapamycin improves glycogen reduction in GAA/C0 mice tolerized to rhGAA. Groups (n = 8–10) of 3- to 4-month-old GAA/C0 mice were treated with rapamycin in food (0.05% w/w) and rhGAA (as described in Fig. 3) for 12 weeks. Four weeks before the start of the study some groups were tolerized to GAA by intravenous injection of an AAV vector encoding an inactive GAA (D404N). (A) Anti-GAA antibody titer in serum at the end of the study. (B) Tissue glycogen levels of the cohorts in (A) tolerized to GAA. Results are representative of two independent studies. Values are means ± SEM. Student's t test comparing indicated treatment groups; *P < 0.05, **P < 0.01, ***P < 0.001.

![Fig. 5. The lowest dose of rapamycin that abates glycogen accumulation is immune suppressive. (A) 5-week-old GAA/C0 mice (n = 10) were treated with daily intraperitoneal injections of rapamycin with the indicated dose for 12 weeks. Baseline group was sacrificed at start of study to establish starting glycogen levels. Rapamycin did not abate glycogen accumulation at 0.025 mg/kg. (B) Groups of 3- to 4-month-old C57Bl/6 mice were administered 6E10 drcp of Ad2/CMV-$\alpha$-galactosidase. Vehicle (−) or rapamycin at the concentrations shown was given daily by intraperitoneal injection for 28 days (formulated as described in Fig. 1). Serum was collected after 28 days and assayed for antibodies to $\alpha$-galactosidase. Rapamycin suppressed antibodies at 0.025 mg/kg compared to vehicle. Values are means ± SEM. One way ANOVA followed by Newman–Keuls comparing indicated treatment groups; *P < 0.05, **P < 0.01, ***P < 0.001.
young GAA\textsuperscript{−/−} mice, both analogs were as potent as rapamycin in reducing glycogen (Fig. 6). As with rapamycin, the AP23573 and CCI-779 analogs administered alone also abated glycogen accumulation in triceps and quadriceps but not in heart and diaphragm. The effects of rapamycin on glycogen accumulation therefore appear to be characteristic of this molecular class and act through the mTORC1 pathway.

**Discussion**

The presentation and progression of Pompe disease are extremely heterogeneous. Response to treatment is also variable. ERT plus an adjunct therapy that increases the effectiveness of rhGAA may benefit some patients with Pompe disease. Experiments described in this report suggest that increased phosphorylation of GS in skeletal muscle via mTORC1 inhibition with rapamycin represents a potential novel therapeutic approach for glycogen storage diseases that involve glycogen accumulation in muscle. In a GAA\textsuperscript{−/−} mouse model of Pompe disease, rapamycin combined with rhGAA improves glycogen clearance in skeletal muscle and diaphragm. Mechanistically, these results support a hypothesis whereby each dose of rhGAA reduces glycogen in skeletal muscle cells, which are simultaneously prevented from synthesizing new glycogen by the actions of rapamycin. Additional studies are needed to determine if rapamycin abates glycogen accumulation in neurons and anterior horn cells.

There is growing interest in tissue-specific mTOR functions and how they affect whole-body growth and metabolism [31]. We found unexpectedly in vivo that the mTORC1 pathway robustly regulates glycogen synthesis in skeletal muscle but does not affect glycogen synthesis in liver and has a minimal effect on glycogen

![Fig. 6. Rapamycin analogs improve glycogen clearance in combination with rhGAA. The CCI-779 and AP23573 analogs in the absence of rhGAA are not effective in heart and diaphragm. Groups of 5-week-old GAA\textsuperscript{−/−} mice (n = 10) were treated with rhGAA (20 mg/kg) as described in Fig. 3 for 12 weeks. The rapamycin analogs were formulated and administered as described in Fig. 2. Baseline groups were sacrificed at start of study to establish starting glycogen levels. (A) AP23573 and rhGAA given alone. Rapamycin, AP23573, and CCI-779 given in combination with rhGAA. (B) A separate study in which CCI-779 was given alone. Values are means ± SEM. One way ANOVA followed by Newman–Keuls comparing indicated treatment groups; *P < 0.05, **P < 0.01, ***P < 0.001.](image)
synthesis in heart and diaphragm. Additional studies into mTORC1 pathway differences in these critical organs may suggest additional new therapeutic approaches.

The rapamycin treatment regimen used in this study reduced the antibody response to rhGAA in GAA⁻/⁻ mice. Although anti-rhGAA antibodies do not affect the efficacy of rhGAA in GAA⁻/⁻ mice, the immune-suppressing attributes of rapamycin may be beneficial in the clinic because in some patients the antibody response to ERT may mitigate the treatment outcome [28]. Other immune suppression treatments are currently in use in Pompe patients, but there is no evidence that they have the added benefit of rapamycin’s inhibition of glycogen biosynthesis [32].

Analysis of Pompe skeletal muscle reveals accumulation of autophagic vesicles; it has been proposed that the glycogen-filled lysosomes may lead to a failure in productive autophagy [33]. Rapamycin is known to increase autophagy in some cells, so it was possible that rapamycin would increase unproductive autophagy and exacerbate glycogen accumulation in skeletal muscle. Our results clearly show that rapamycin treatment decreases glycogen accumulation. These results are consistent with the observation that rapamycin does not increase autophagy in skeletal muscle [34].

In conclusion, these preclinical results suggest a potential role for adjunct therapy with rapamycin in Pompe patients treated with rhGAA. Well designed clinical studies will be necessary to determine if such an approach leads to measureable advantages over rhGAA alone in the management of Pompe disease.

Materials and methods

Reagents and antibodies

Recombinant human GAA purified from CHO cells was from Genzyme Corporation (Cambridge, MA, USA). The Amplex Red glucose assay kit was from Molecular Probes (Eugene, OR, USA). Aspergillus niger amyloglucosidase and other chemical reagents were purchased from Sigma (St. Louis, MO, USA). The phosphorylation site specific (pGS Ser 641/645) glycogen synthase polyclonal antibody (Cat. No. ab 2479) was purchased from Abcam (Cambridge, MA, USA). The phosphorylation site specific (pGS Ser 641/645) glycogen synthase polyclonal antibody (Cat. No. ab 2479) was purchased from Abcam (Cambridge, MA, USA). The glycogen synthase monoclonal antibody (Cat. No. MAB3106) was purchased from Millipore (Billerica, MA, USA). BCA kit for protein determinations and ECL supersignal detection kit were purchased from Pierce (Rockford, III). AP23573 and CCI-779 were obtained from Selleck Chemicals. Food containing rapamycin was prepared by Test Diet (Richmond, IN). One-half inch pellets of PicoLab Rodent Diet 20 5053 contained rapamycin at 0.05% w/w.

Animal studies

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH Publication No. 86-23) and by Genzyme’s IACUC committee. The figure legends specify the number of animals in each experiment. Each bar in each figure represents the mean of 5–12 animals. To minimize a hypersensitivity reaction to the rhGAA, mice were pretreated with 5 mg/kg of diphenhydramine (delivered intraperitoneally) 10 min before injecting the third dose of rhGAA.

Preparation of tissue homogenates

Tissues were collected and frozen on dry ice and stored at −80 °C. Tissues were homogenized in Qiagen’s TissueLyser II (Cat. No. 85,300) as follows: weighed tissues were placed in a tube with a stainless steel bead with 3 × (vol/wt) homogenization buffer designed to inhibit phosphatases and proteases (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 25 mM β-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 2 mM EDTA, and Roche Complete protease inhibitor cocktail (Cat. No. 1,697,498)). The TissueLyser II (Qiagen) was run for 10 min at 30 Hz. Lysates were frozen at −80 °C and thawed. The thawed lysates were centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. The supernatants were aliquoted and stored at −80 °C. Protein determination of the lysates was performed by BCA as described by the manufacturer.

Western blot analysis of tissue homogenates

Typically 10–100 µg of tissue homogenate was boiled in 2× sample buffer (BioRad) containing β-mercaptoethanol. The lysate was then applied to a 4–15% or 10–20% Tris/glycine/SDS-polyacrylamide precast gel (Criterion, BioRad). Proteins were transferred to nitrocellulose with a semidyblot apparatus (BioRad). The blots were blocked with 3% milk or 2% BSA and then the appropriate antibody added at a final concentration of 0.2–1.0 µg/ml and incubated overnight at 4 °C. The appropriate secondary antibody conjugated to HRP was incubated with the blot and then the blot was treated with the ECL SuperSignal detection kit as described by the manufacturer (Pierce).

Measurement of tissue glycogen

Two methods were used for determining glycogen levels. The first method was performed as described previously [1]. For the second method, 3 µl of tissue lysate was diluted with 147 µl water and then dried under vacuum in a Savant speed vac without heat. Trifluoroacetic acid (200 µl of 4 N) was added and incubated at 100 °C for 4 h. The samples were centrifuged and dried under vacuum without heat. The dried samples were dissolved in 18 µl 2-deoxy-α-glucose and then analyzed with HPAE-PAD on a Dionex BioLC with a CarboPac PA10 column (isocratic elution with 20% 200 mM NaOH, 80% water) coupled to an ED40 electrochemical detector.

Measurement of anti-rhGAA and anti-α-gal antibody titer

To determine anti-rhGAA and anti-α-gal antibody titers, ELISA plates (Corning, Oneonta NY) were coated overnight at 4 °C with 5 µg/ml rhGAA or 5 µg/ml anti-α-gal in 250 mM sodium acetate pH 4.9. Plates were washed and blocked with 0.5% bovine serum albumin in phosphate buffered saline for a minimum of 1 h at 37 °C. After removal of the blocking solution and plate wash, serum samples were diluted with Perkin Elmer ELISA wash buffer 2-fold serially in duplicate across the plate with a starting dilution of 1:200 and incubated for 1 h at 37 °C. A 1:20,000 dilution of the horseradish peroxidase-conjugated goat anti-mouse IgG, IgM, IgA secondary antibody (Zymed, Cat. No. 62-6420) was applied to the plates and incubated for 1 h at 37 °C. The ELISA was developed using TMB One Component Microwell Substrate (BioFX Labs, Owings Mills MD) in the dark for 20 min. The reaction was stopped with 100 µl Stop Reagent (BioFX Labs, Owings Mills MD) and the plates read in a plate reader ( Molecular Devices Spectra Max Plus) at 450 nm and 650 nm. Titers are expressed as the reciprocal of the minimum serum dilution giving an OD450 ≤ 0.1.

Statistical analysis

Data are expressed as means ± SEM. Data were analyzed by Student’s t test and one way ANOVA with Newman–Keuls. A probability value of P < 0.05 was considered to be statistically significant.
Acknowledgments

We thank R. Gotschall, M. Troutt, S. Bercury, J. Foley, N. Pande, A. McVie-Wylie, W. Canfield, L. Curtin, E. Lee, and the members of Genzyme's Departments of Comparative Medicine and Pharmacology and Toxicology for their technical assistance and valuable input into the design of the studies. We thank J. Hollenstein who assisted with editing and preparation of the manuscript. The GAA-/- mice were kindly provided by N. Raben (N.I.H.).

References


