Suppression of mTORC1 activation in acid-α-glucosidase-deficient cells and mice is ameliorated by leucine supplementation

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Shemesh A, Wang Y, Yang Y, Yang G-S, Johnson DE, Backer JM, Pessin JE, Zong H. Suppression of mTORC1 activation in acid-α-glucosidase-deficient cells and mice is ameliorated by leucine supplementation. Am J Physiol Regul Integr Comp Physiol 307: R1251–R1259, 2014. First published September 17, 2014; doi:10.1152/ajpregu.00212.2014.—Pompe disease is due to a deficiency in acid-α-glucosidase (GAA) and results in debilitating skeletal muscle wasting, characterized by the accumulation of glycogen and autophagic vesicles. Given the role of lysosomes as a platform for mTORC1 activation, we examined mTORC1 activity in models of Pompe disease. GAA-knockdown C2C12 myoblasts and GAA-deficient human skin fibroblasts of infantile Pompe patients were found to have decreased mTORC1 activation. Treatment with the cell-permeable leucine analog l-leucyl-l-leucine methyl ester restored mTORC1 activation. In vivo, Pompe mice also displayed reduced basal and leucine-stimulated mTORC1 activation in skeletal muscle, whereas treatment with a combination of insulin and leucine normalized mTORC1 activation. Chronic leucine feeding restored basal and leucine-stimulated mTORC1 activation, while partially protecting Pompe mice from developing kyphosis and the decline in muscle mass. Leucine-treated Pompe mice showed increased spontaneous activity and running capacity, with reduced muscle protein breakdown and glycogen accumulation. Together, these data demonstrate that GAA deficiency results in reduced mTORC1 activation that is partly responsible for the skeletal muscle wasting phenotype. Moreover, mTORC1 stimulation by dietary leucine supplementation prevented some of the detrimental skeletal muscle dysfunction that occurs in the Pompe disease mouse model.

mTORC1: α-glucosidase; lysosome; muscle; Pompe disease; leucine

ACID ALPHA-GLUCOSIDASE (GAA) deficiency (Pompe disease) is a relatively rare autosomal recessive inherited disease that affects ~1 in 40,000 individuals (2). Pompe disease is a type II glycogen storage disease that results from mutations in the GAA gene that prevent or reduce the normal breakdown of glycogen in lysosomes. Physiological and metabolic defects occur primarily in glycogen-laden cardiac and skeletal muscle. Disease severity depends on the specific mutation and residual GAA enzymatic activity. The infantile onset form presents as hypotonia and muscle weakness with massive accumulation of glycogen in skeletal and heart muscle, and cardiorespiratory failure leading to death. Older patients with the slowly progressive form develop severe skeletal muscle weakness and eventually respiratory failure without significant involvement of cardiac muscle. Recent studies have observed that GAA knockout (KO) mice display marked accumulation of autophagic vesicles with altered macroautophagy function (8, 15, 18). In addition, GAA deficiency causes lysosome enlargement concomitant with the lysosomal accumulation of glycogen (25).

Raptor is a critical subunit of the mTORC1 complex, and the phenotype of skeletal muscle-specific raptor knockout mice is similar to that of GAA knockout mice (3). Several studies have shown that amino acids induce the translocation of cytosolic mTORC1 to the lysosome (20, 21), which is induced by conformational changes in the vacuolar H+ -ATPase in response to intralysosomal amino acids (32). On the other hand, the mTORC1 activator Rheb is present on the lysosome membrane, and growth factor stimulation results in its activation (29). Thus, the combination of growth factor activation of Rheb and amino acid-stimulated recruitment of mTORC1 to the lysosome is thought to result in the full activation of mTORC1. Since GAA deficiency in skeletal muscle results in lysosome swelling, we hypothesized that mTORC1 activation might be compromised due to impairment of normal lysosomal function.

In this study, we took advantage of two cellular models of GAA deficiency. RNAi knockdown of GAA in murine C2C12 myoblasts and primary fibroblasts from infantile human subjects with Pompe disease and a mouse knockout model of GAA deficiency. These models were chosen to compare the relative effects of insulin and amino acids on mTORC1 activation in human and mouse cell lines with that of in vivo Pompe deficiency in mice. In these model systems, we demonstrate that GAA deficiency results in a reduction in growth factor (insulin) and amino acid-induced activation of mTORC1. However, leucine supplementation partially rescues mTORC1 activation in GAA-deficient cultured cells. Moreover, chronic dietary leucine supplementation in a Pompe disease mouse model prevents the decline in skeletal muscle mTORC1 activation by leucine and is able to partially protect against muscle wasting and functional deterioration. These data point to a loss of mTORC1 activity as an important pathophysiological component of Pompe disease.

MATERIALS AND METHODS

GAA-deficient cell lines. Two-acid-α-glucosidase-knockdown C2C12 myoblast cell lines were generated by infection with lentiviruses packaged with either of two pLKO.1-GAA shRNA lentiviral vectors (Clone IDs NM_008064.2–1454s1c1 and NM_008064.2–1264s1c1;
Phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific, Waltham, MA) were added to the incubation medium with ice-cold modified RIPA buffer (25 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, NE). Proteins were solubilized and IRDye 800CW was purchased from LI-COR Biosciences (Lincoln, NE). The secondary antibodies except anti-GAA (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (MBL International, Woburn, MA) were purchased from Cell Signaling Technology (Beverly, MA). The secondary antibodies were near-infrared fluorescent dye conjugates IRDye 680RD, IRDye 800CW, and IRDye 700CW, and the RNA was purified from the upper aqueous phase using RNase-free zirconium oxide beads in a bullet blender (Next Advance, Averill Park, NY) for 4 min at 4°C at speed setting 8, and the supernatant was incubated at room temperature for 5 min to dissociate nucleoproteins. Chloroform was added to extract nucleic acids, and the RNA was purified from the upper aqueous phase using RNAasy mini kit (Qiagen, Valencia, CA). Purified RNA was reverse-transcribed using SuperScript VILO cDNA synthesis kit (Life Technologies).

**Western blot analysis**. Cells were washed twice with PBS and incubated in an amino acid- and growth factor-free RPMI-1640 medium (US Biological, Salem, MA) for 1 h to lower mTORC1 activity to similar levels in all cell lines. Following two washes with PBS, the medium was replaced with that mentioned in figure legends. To expose the cells to amino acids, DMEM was the replacing medium (Life Technologies) that contains 15 amino acids (0.4 mM glycine, 0.4 mM arginine, 0.2 mM cysteine, 0.2 mM histidine, 0.8 mM isoleucine, 0.8 mM leucine, 0.8 mM lysine, 0.2 mM methionine, 0.4 mM phenylalanine, 0.4 mM serine, 0.8 mM threonine, 0.08 mM tryptophan, 0.4 mM tyrosine, and 0.8 mM valine). In all but the insulin dose-response experiment, 100 nM or 10 nM insulin (Sigma Aldrich, St. Louis, MO) was supplemented to replacement media bathing C2C12 myoblasts or human skin fibroblasts, respectively, 10 mM leucine (Sigma Aldrich) or 100 μM L-leucyl-L-leucine methyl ester (LL-01e) was supplemented where indicated. Cells were incubated with the replacing medium for 10 min and then processed for immunoblotting. This time frame was chosen on the basis of previous reports (20, 32).

**Protein degradation assay**. Cells were washed twice with PBS and lysed with ice-cold modified RIPA buffer (25 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol) containing phosphatase and protease inhibitor cocktails (Thermo Fisher Scientific, Rockford, IL). Whole cell lysates were centrifuged at 13,000 g for 5 min at 4°C. Supernatants were moved to a new tube, and protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA). Cell extracts were subjected to reducing SDS-PAGE and blotted using standard procedures. All primary antibodies except anti-GAA (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GAPDH (MBL International, Woburn, MA) were purchased from Cell Signaling Technology (Beverly, MA). The secondary antibodies were near-infrared fluorescent dye conjugates IRDye 680RD, and IRDye 800CW was purchased from LI-COR Biosciences (Lincoln, NE).

**Pompe mice**. The GAA-deficient Pompe mouse model (6neo/6neo) on the C57BL/6J background, as described by Raben et al. (16), was provided by Dr. Steven Walkley (Albert Einstein College of Medicine). Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Control WT and Pompe mice were maintained on a normal chow diet (NCD; 10% kilocalories from fat) with or without 1.5% leucine in their drinking water for 12 wk. All mice were maintained on a 12:12-h light-dark cycle with free access to food and water, and protocols were performed in accordance with and approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

**mTORC1 activation assay of in vivo skeletal muscle**. WT or Pompe mice were fasted for 12 h and infused through an intravenous line with saline or leucine (30 mg·kg·min⁻¹) for 1 h, then intraperitoneally injected with 1 U/kg insulin or saline for 30 min. These timeframes were chosen on the basis of previous studies examining insulin- and leucine-stimulated mTORC1 activation in skeletal muscle (24). Skeletal muscle was collected, snap-frozen, and stored at −80°C. The muscle tissue was pulverized, extracts were prepared, and then immunoblotted for T389-S6K1 phosphorylation.

**Physiological parameters**. Body composition parameters that include total body weight, total water, fat, and fat-free mass was determined by quantitative nuclear magnetic resonance noninvasive imaging, as described previously (33). Spontaneous locomotor activity was determined by indirect calorimetry using the Columbus Instruments Oxymax system, as described previously (33). Kyphosis was measured by X-ray image analysis, as described by Lima et al. (11). Running capacity was determined as described by Zong et al. (34). Briefly, the mice were acclimatized to a treadmill (Columbus Instruments) by running at 10 m/min for 15 min over three consecutive days. On the fourth day, groups of mice were run at 10 m/min on a 15° grade incline, progressing to 15 m/min. The total distance run to exhaustion was determined.

**Protein degradation assay**. Protein degradation was measured by urinary 3-methylhistidine (3-MH) on consecutive 24-h urine collections, as described by Rathmacher et al. (19).

**Skeletal muscle glycogen and serum creatine kinase levels**. WT and Pompe mice untreated or supplemented with dietary leucine were fasted for 12 h, and sera were collected and stored at −20°C. Creatine kinase activity was determined using MaxDiscovery creatine kinase enzymatic assay kit (Bio Scientific, Austin, TX). Glycogen content of skeletal muscle that was dissected, snap frozen, and stored at −80°C was determined using glycogen colorimetric assay kit (BioVision, Sherwood Park, AB, Canada).

Fig. 1. Acid-alpha-glucosidase (GAA) knockdown in C2C12 cells and GAA deficiency in human infantile Pompe patient fibroblasts. A: C2C12 myoblasts were infected with a control empty lentivirus or lentiviruses encoding two independent GAA shRNAs. The relative expression of GAA mRNA was determined by quantitative RT-PCR. B: lysates from the shRNA knockdown C2C12 cell line (KD#1) and infantile Pompe disease patient fibroblast 1 cell line were immunoblotted for the GAA protein. Expression levels were compared with control C2C12 myoblasts and fibroblasts from a healthy individual. These are representative immunoblots independently performed three times. *P < 0.05 by Student’s t-test.
**Statistical analysis.** Results are presented as means ± SE. The difference between means was analyzed using two-sided Student’s t-test and was set to be significant if \( P < 0.05 \). For multiple comparisons, the data were analyzed by two-way ANOVA followed by the Tukey’s multiple-comparison test. Statistical analyses were set to be significant if \( P < 0.05 \) using Prism program version 6.0 (GraphPad). Identical letters in figures indicate bars that are not statistically significant from each other.

**RESULTS**

Previous studies in cultured cell systems have shown that mTORC1 activation is dependent upon lysosome association (12, 20). To examine mTORC1 signaling in a cell culture model of GAA deficiency, we generated two independent shRNA lentiviral knockdown C2C12 cell lines with ~20% of wild-type (WT) GAA transcript, resulting in considerably reduced protein levels (Fig. 1, A and B). Human skin fibroblasts of a healthy individual and a compound heterozygote infantile Pompe disease patient were also assessed for GAA protein levels (Fig. 1B). Similar to that observed in C2C12 cells, the processed forms of GAA were present in human fibroblasts of a healthy individual but were substantially reduced in the infantile Pompe disease fibroblast cells. Having established GAA-deficient cells (GAA-KD), we next examined mTORC1 activation by amino acids and insulin. As expected, insulin stimulation resulted in a dose-dependent increase in mTORC1 activation in control C2C12 cells, as indicated by the increase in T389-S6K1 phosphorylation (Fig. 2A, lanes 1–4; and B). Insulin was also capable of increasing T389-S6K1 phosphorylation in the two independent C2C12 GAA shRNA knockdown cell lines, but the extent of mTORC1 activation was reduced (Fig. 2A, lanes 5–8 and 9–12).

The amino acid leucine is a known activator of mTORC1 (10) and was able to enhance the mTORC1-specific phosphorylation site (T389) of S6K1 (6) in wild-type and GAA-KD myoblasts (Fig. 3A, lanes 2 and 7). Insulin also induced T389-S6K1 phosphorylation in both control and the GAA-KD cells (Fig. 3A, lanes 3 and 8). However, the addition of an amino acid mixture provided by DMEM with insulin displayed a greater increase in T389-S6K1 phosphorylation in control vs. GAA-KD cells (Fig. 3A, lanes 4 and 9). Moreover, the combination of the amino acid mixture with leucine and insulin was able to further increase mTORC1 activation in the GAA-KD cells but still was less than that of control cells (Fig. 3A, lanes 5 and 10). When quantified and averaged over several independent experiments, the GAA-KD cells had a trend of reduced leucine or insulin stimulation of mTORC1 that was not statistically significant, whereas the combination of amino acids plus insulin or the combination of amino acids with leucine and insulin was reduced in the GAA-KD cells (Fig. 3B).

Leucine is transported into cells by the 1-3-amino acid transport system, whereas the dimeric leucine methyl ester derivative (LL-OMe) is membrane-permeable (26). As shown in Fig. 3, C and D, insulin and amino acids or the combination of insulin plus amino acids and leucine, or LL-OMe in DMEM media stimulated T389-S6K1 phosphorylation to a similar extent in wild-type cells. Although leucine did not augment insulin stimulation of T389-S6K1 phosphorylation in GAA-KD cells, LL-OMe was able to further increase insulin activation of mTORC1 in the GAA-KD cells to a similar extent as in the control WT cells.

To confirm and extend our analysis of the effects of amino acids and insulin on mTORC1 activation in human cells, we obtained human skin fibroblasts of a healthy individual and of two infantile Pompe patients. As observed in the GAA-KD cells, insulin- and amino acid-stimulated T389-S6K1 phos-
phosphorylation was substantially reduced in both infantile patient fibroblasts compared with control fibroblasts (Fig. 4, A and B, lanes 2 and 6). In these cells, insulin combined with leucine stimulation was able to further enhance mTORC1 activation in both Pompe patient fibroblasts, albeit the extent of mTORC1 activation was less than the control fibroblasts (Fig. 4, A and B, lanes 3 and 7). The combination of insulin plus LL-OMe treatment was more effective in increasing mTORC1 activation in all three cell lines (Fig. 4, A and B, lanes 4 and 8). Quantification over several experiments indicated that LL-OMe in combination with amino acids and insulin was able to fully restore mTORC1 activation in the Pompe-infantile fibroblast cell line but only partially restored mTORC1 activation in the Pompe-infantile fibroblast 2 cell line, suggesting the presence of other cell context-dependent factors.

Having determined that mTORC1 activation in GAA-KD myoblasts can be augmented by leucine/LL-OMe enrichment, we next examined the effect of acute leucine and insulin stimulation on mTORC1 activation in skeletal muscle of the GAA knockout Pompe mouse model (16) at 16 wk of age. In the basal state, the T389-S6K1 phosphorylation was reduced in the extensor digitorum longus (EDL) muscle of Pompe mice compared with WT control mice (Fig. 5, A and B). Acute leucine stimulation (1 h) increased T389-S6K1 phosphorylation, but to a greater extent in wild-type compared with Pompe EDL skeletal muscle. Acute insulin stimulation also increased T389-S6K1 phosphorylation, but was not statistically different between wild-type and Pompe skeletal muscle. Similarly, the combination of leucine plus insulin resulted in a similar extent of T389-S6K1 phosphorylation in skeletal muscle between WT and Pompe mice.

To determine whether the reduction of mTORC1 activation was dependent upon the extent of muscle wasting in Pompe mice, we next determined T389-S6K1 phosphorylation in WT and Pompe mice at 2, 4, and 10 mo of age (Fig. 6). Basal mTORC1 activity was not statistically different between WT and Pompe EDL muscle at 2 mo of age, whereas acute leucine stimulation was reduced approximately twofold. At 4 and 10 mo of age, both basal and acute leucine-stimulated T389-S6K1 phosphorylation was reduced also approximately twofold in the Pompe mice. Together, these data demonstrate that the decrease in leucine-stimulated mTORC1 activity is persistently reduced over a 10-mo age span in the EDL skeletal muscle of GAA-deficient mice.

As a previous case report indicated that nocturnal intragastric feeding was able to prevent muscle wasting in two children with GAA deficiency (23), we speculated that chronic dietary leucine supplementation might be capable of elevating the basal level of mTORC1 activity, as well as restoring the acute leucine stimulation. To test this hypothesis, we supplemented the drinking water of WT and Pompe mice with leucine for 12 wk. Chronic dietary leucine supplementation resulted in a similar

Fig. 3. Leucine activation of mTORC1 is reduced in GAA knockdown C2C12 myoblasts. Control empty lentivirus-infected cells (WT) and GAA-KD C2C12 myoblasts were placed in serum and amino acid-free medium for 1 h. The cells were either left untreated or incubated with leucine (10 mM) and/or insulin (100 nM) in the absence or presence of an amino acid mixture (DMEM) for 10 min. A: cell lysates were immunoblotted with a pT389S6K1, S6K1, and GAPDH antibodies. B: quantification of pT389S6K1/total S6K1. These are representative immunoblots independently performed three times. *P < 0.05 by Student’s t-test. C: WT and GAA-KD cells were either left untreated or incubated with an amino acid mixture (DMEM) and insulin (100 nM) in the absence or presence of leucine (10 mM) or LL-OMe (100 μM) for 10 min. Cell lysates were immunoblotted with pT389S6K1, S6K1, and GAPDH antibodies. D: quantification of pT389S6K1/total S6K1. These are representative immunoblots independently performed three times. *P < 0.05 by Student’s t-test.
extent of basal T389-S6K1 phosphorylation in WT and Pompe EDL skeletal muscle (Fig. 7). Acute leucine infusion increased mTORC1 activation equally in both WT and Pompe skeletal muscle. Moreover, acute insulin stimulation with or without leucine also increased T389-S6K1 phosphorylation that was again similar between WT and Pompe mice skeletal muscle. Together, these data demonstrate that mTORC1 activation is reduced by GAA deficiency in vivo but that chronic dietary leucine supplementation enhances basal and leucine-stimulated activation to similar extents.

Fig. 4. mTORC1 activation is reduced in human skin fibroblasts of infantile Pompe disease patients. A and B: control human fibroblasts and two fibroblast cell lines from infantile Pompe disease patients were either left untreated or incubated with an amino acid mixture (DMEM) and insulin (10 nM) in the absence or presence of leucine (10 mM) or LL-OMe (100 μM) for 10 min. Cell lysates were immunoblotted with pT389-S6K1, S6K1, and GAPDH antibodies. C: quantification of pT389S6K1/total S6K1. These are representative immunoblots independently performed three times. *P < 0.05 by Student’s t-test.

Fig. 5. mTORC1 activation is reduced in skeletal muscle of the (6neo/6neo) GAA knockout Pompe mouse model. A: control wild-type and Pompe mice at 16 wk of age were maintained on a normal chow diet. The mice were then fasted for 12 h and either infused with saline or leucine (30 mg·kg⁻¹·min⁻¹) for 60 min and then injected with either saline or insulin 1 U/kg. The mice were euthanized, and the extensor digitorum longus (EDL) muscle was isolated, extracted, and immunoblotted for pT389-S6K1, total S6K1, and GAPDH. A representative immunoblot is shown. B: quantification of pT389S6K1/total S6K1 from a total of 6–10 independent mice. Normalization for the WT control mice immunoblotting intensity was used to compare samples from multiple independent immunoblots. *P < 0.05, **P < 0.02 by Student’s t-test.

Fig. 6. Basal and leucine-stimulated mTORC1 activation is reduced in skeletal muscle of Pompe mice at 2 to 10 mo of age. Control wild-type and Pompe mice at 2, 4, and 10 mo (MO) of age were maintained on a normal chow diet. The mice were then fasted for 12 h and either infused with saline or leucine (30 mg·kg⁻¹·min⁻¹) for 60 min. The mice were euthanized and the extensor digitorum longus (EDL) muscle was isolated, extracted, and immunoblotted for pT389S6K1, total S6K1, and GAPDH. The bar graph represents the quantification of pT389S6K1/total S6K1 of 2 mo (n = 5–8), 4 mo (n = 8–12), and 10 mo (n = 5–8) old independent mice. Nonidentical letters indicate measurements that were statistically different from each other at P < 0.05 determined by two-way ANOVA followed by the Tukey’s multiple comparisons.
Weight and fat-free mass were similar to that of WT control Pompe mice supplemented with dietary leucine, total body non-leucine-supplemented control WT mice. Importantly, in contrast to mice supplemented with leucine for 12 wk displayed an increase in total body weight and fat-free mass compared with control WT mice (Fig. 8A). WT mice supplemented with leucine for 12 wk displayed an increase in total body weight due to decreases in both fat and lean mass (n = 6–16). A: mice were weighed and analyzed by NMR for fat and lean mass (n = 6–16). B: mice were subject to microCT to determine the degree of spinal cord kyphosis and were partially restored following dietary leucine supplementation (Fig. 9D). Preserved skeletal muscle function was confirmed both by the ability of leucine-supplemented Pompe mice to increase their spontaneous locomotor activity to near-normal levels and improvement in long-distance running capacity (Fig. 10, A and B).

If reduced mTORC1 activity were part of the pathophysiology of skeletal muscle dysfunction in Pompe disease, then restoration of mTORC1 activity would be expected to correct some of these defects. Consequently, we examined the effect of dietary leucine supplementation on several pathologic features of Pompe mice. Sixteen-week-old male Pompe mice had reduced total body weight due to decreases in both fat and fat-free mass compared with control WT mice (Fig. 8A). WT mice supplemented with leucine for 12 wk displayed an increase in total body weight and fat-free mass compared with non-leucine-supplemented control WT mice. Importantly, in Pompe mice supplemented with dietary leucine, total body weight and fat-free mass were similar to that of WT control mice fed a normal diet. Similarly, the spinal cord kyphosis that develops in these mice was significantly blunted following a leucine-supplemented diet (Fig. 8B). In parallel, accumulation of skeletal muscle glycogen was reduced nearly by half in leucine-supplemented Pompe mice (Fig. 8C).

Skeletal muscle protein breakdown can be indirectly assessed by the levels of urinary 3-MH (1). Pompe mice had higher levels of 3-MH whether normalized for urinary creatinine levels or total urinary volume (Fig. 9, A and B). Leucine supplementation reduced 3-MH levels when normalized for urinary creatinine in WT mice but was not significantly different when normalized for total urinary volume. However, in either case, leucine supplementation of Pompe mice decreased 3-MH levels, indicating a decrease in skeletal muscle protein breakdown. Similarly, serum creatine kinase levels were elevated in the Pompe mice and were reduced following dietary leucine supplementation (Fig. 9C). In addition, muscle weights of the gastrocnemius and EDL were decreased in Pompe mice and were partially restored following dietary leucine supplementation (Fig. 9D). Preserved skeletal muscle function was confirmed both by the ability of leucine-supplemented Pompe mice to increase their spontaneous locomotor activity to near-normal levels and improvement in long-distance running capacity (Fig. 10, A and B).

DISCUSSION

There are currently two prevailing hypotheses proposed to account for the pathology of striated muscle deterioration that occurs in Pompe disease. According to the lysosomal rupture hypothesis, GAA deficiency results in lysosomal glycogen accumulation due to the reduced ability to hydrolyze the glycogen polymer into individual glucose monomers. The lysosomes swell, presumably due to the generation of an osmotic gradient between the lysosome and cytoplasm. Skeletal muscle contraction applies mechanical stress on the swollen lysosomes, resulting in their rupture and the release of digestive enzymes that, in turn, damage the contractile myofibril and its function (4). However, other tissues can display clinical manifestations even though they are noncontractile. For example, increased glycogen content in phrenic motor neurons of GAA-KO mice has been shown to reduce efferent phrenic nerve inspiratory burst amplitudes (7). It is also pos-

Fig. 7. Dietary leucine supplementation restores basal and leucine-stimulated mTORC1 activation. A: Control wild-type and Pompe mice at 4 wk of age were either maintained on a normal chow diet with water or leucine-supplemented water (1.5%) for 12 wk. The mice were then fasted for 12 h and either infused with saline or leucine (30 mg·kg⁻¹·min⁻¹) for 60 min, then injected with either saline or insulin at 1 U/kg. The mice were euthanized, and the EDL muscle was isolated, extracted, and immunoblotted for T389-S6K1 phosphorylation, total S6K1, and GAPDH. A representative immunoblot is shown. B: quantification of pT389-S6K1/total S6K1 from a total of 6–10 mice.

Fig. 8. Dietary leucine supplementation protects Pompe disease mice from changes in body composition, skeletal muscle glycogen, and spinal cord kyphosis. Control wild-type and Pompe mice at 4 wk of age were either maintained on a normal chow diet with water or leucine-supplemented water (1.5%) for 12 wk. A: mice were weighed and analyzed by NMR for fat and lean mass (n = 6–16). B: mice were subject to microCT to determine the degree of spinal cord kyphosis (n = 13–24). C: mice were euthanized, and the gastrocnemius white skeletal muscle was extracted and assayed for total glycogen content (n = 5–7). Nonidentical letters (a, b, c) indicate measurements that were statistically different from each other at P < 0.05 as determined by two-way ANOVA followed by the Tukey multiple-comparison test.
that were statistically different from each other at 5–8 wk.

Nonidentical letters (a, b, c) indicate measurements progressing to 15 m/min. The total distance run to exhaustion was determined by 11–17 mice per group. Nonidentical letters (a, b, c) indicate measurements that were statistically different from each other at 5–8 wk, determined by two-way ANOVA followed by the Tukey multiple-comparison test.

Control WT and Pompe mice at 4 wk of age were either maintained on a normal chow diet with water or leucine-supplemented water (1.5%) for 12 wk.

Control WT and Pompe mice at 4 wk of age were either maintained on a normal chow diet with water or leucine-supplemented water (1.5%) for 12 wk.

Running capacity in Pompe disease mice. Control wild type and Pompe mice at 4 wk of age were either maintained on a normal chow diet with water or leucine-supplemented water (1.5%) for 12 wk.

Nonidentical letters indicate measurements that were statistically different from each other at 5–8 wk, determined by two-way ANOVA followed by the Tukey multiple-comparison test.

A: spontaneous locomotor activity determined over a 72-h period (n = 4). B: amount of 3-MH per milliliter urine collected over a 24-h period (n = 5–8). C: serum creatine kinase levels (n = 6–7). D: muscle weights of the gastrocnemius (GAS) and EDL muscles (n = 4–6). Nonidentical letters indicate measurements that were statistically different from each other at P < 0.05 determined by two-way ANOVA followed by the Tukey multiple-comparison test.
the presence of growth factors through a signaling pathway involving Akt and the GTPase TSC1/2 (5, 29). In addition, lysosomal targeting of mTORC1 requires the lysosomal proton pump vacuolar-ATPase (32). Thus, normal lysosomal function is necessary for the coordinate activation of mTORC1 by amino acids and growth factors.

On the basis of these data, we postulated that mTORC1 activation would be affected by GAA deficiency. Consistent with this prediction, in two independent C2C12 GAA-KD cell lines and in two independent infantile Pompe patient fibroblasts, there was a significant reduction in the ability to simulate mTORC1 activation. These data are consistent with a recent report of reduced S6K1 phosphorylation in Pompe disease patient fibroblasts (14). Despite the reduction of mTORC1 activation, the combination of an amino acid mixture with leucine and insulin was able to activate mTORC1, although to a lower extent than in WT cells. However, the diffusible methyl-ester form of leucine, LL-OMe, was able to restore mTORC1 activation to nearly WT levels at concentrations two orders of magnitude lower than leucine. LL-OMe is toxic in vivo (27), but dietary leucine supplementation is readily tolerated in mice (31).

Although the model cultured cells of GAA deficiency displayed reduced acute insulin- and leucine-stimulated mTORC1 activation, in vivo, we only observed a deficiency in acute leucine-stimulated mTORC1 activity in skeletal muscle of the Pompe mouse model. At present, we do not know the basis for this difference, but it underscores numerous observations that results obtained in cell culture systems do not necessarily reflect the more complex interactions that occur in vivo.

Nevertheless, in vivo, we observed that chronic leucine treatment prevented the decline in acute leucine-stimulated mTORC1 activation and ameliorated several phenotypic characteristics of muscle wasting in the Pompe disease mouse model, including a reduction in glycogen content, spinal cord kyphosis, and skeletal muscle protein breakdown, and improved spontaneous locomotor activity and running capacity. Although voluntary ingestion of high-protein diets has some clinical benefit (31), high-protein diets are poorly adhered to and are not continuous, particularly during sleeping, when mTORC1 activity is low.

However, in our experiment, leucine was provided in water that mice drank throughout the day, thus providing a more continuous activation of mTORC1 as observed by an increased basal 389T-S6K1 phosphorylation in EDL muscle. These data are also consistent with two case studies in which either nasogastric tube feeding and/or gastrostomy was used to provide continuous nocturnal high-protein diet feeding that substantially prevented the development of skeletal muscle pathologies (23). Together, these data suggest that near-continuous high-protein (and/or leucine) supplementation to maintain mTORC1 in a chronically active state may serve to limit the degree of skeletal muscle wasting that occurs in Pompe disease patients.

**Perspectives and Significance**

It is well established that TOR plays a central signaling role in the control of cellular macromolecular synthesis/degradation and energy balance that is conserved from yeast to mammals. An important aspect of these integrative responses is the ability of mTORC1 to serve as a nutrient sensor that is activated by amino acids at the lysosome. The data presented in this study indicate that in a lysosome-dysfunctional Pompe disease mouse model, mTORC1 activation by leucine is suppressed but can be restored by chronic long-term dietary leucine supplementation, resulting in improved skeletal muscle function. These findings may provide a relatively simple therapeutic intervention to reduce or slow the occurrence of the severe debilitating muscle weakness that occurs in Pompe disease patients.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

ACID-GLUCOSIDASE DEFICIENCY REDUCES MTORC1 ACTIVATION


