Adjunctive β2-agonists Reverse Neuromuscular Involvement in Murine Pompe Disease

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Running title: CI-MPR Impacts Pompe Disease Therapy
NONSTANDARD ABBREVIATIONS

acid α-glucosidase (GAA)
adeno-associated virus (AAV)
cation-independent mannose-6-phosphate receptor (CI-MPR)
knockout (KO)
double knockout (DKO)
ABSTRACT

Pompe disease has resisted enzyme replacement therapy with acid α-glucosidase (GAA), which has been attributed to inefficient cation-independent mannose-6-phosphate receptor (CI-MPR) mediated uptake. We evaluated β2-agonist drugs, which increased CI-MPR expression in GAA knockout (KO) mice. Clenbuterol along with an adeno-associated virus vector increased Rotarod latency by 75% at 4 weeks, in comparison with vector alone (p<2x10^{-5}). Glycogen content was lower in the diaphragm (p=0.04), soleus (p=0.0006), extensor digitorum longus (EDL; p=0.002), cerebrum (p<5x10^{-5}), and cerebellum (p=0.03) following combination therapy, in comparison with vector alone. Glycogen remained elevated in the muscles following clenbuterol alone, equivalent to the levels in mock-treated control mice. Elderly GAA-KO mice have been resistant to correction with gene therapy; however, one year-old GAA-KO mice treated with combination therapy demonstrated two-fold increased wirehang latency, in comparison with vector or clenbuterol alone (p<0.001). The glycogen content of skeletal muscle decreased following combination therapy in elderly mice (p<0.05). Finally, CI-MPR-KO/GAA-KO mice did not respond to combination therapy, indicating its efficacy depended upon CI-MPR expression. In summary, β2-agonist treatment increased CI-MPR expression and enhanced efficacy from gene therapy in Pompe disease, and a similarly enhanced benefit might be expected in other lysosomal storage disorders that involve primarily the brain.

Keywords: Mannose-6-phosphate receptor, gene therapy, adeno-associated virus, acid alpha-glucosidase, acid maltase, glycogen storage disease type II.
INTRODUCTION

Pompe disease (Glycogen storage disease type II; acid maltase deficiency; MIM 232300) ranges in severity from a severe, infantile-onset cardiomyopathy to a late-onset myopathy, which is caused by deficiency acid α-glucosidase (GAA) varying from complete to partial. The current standard of care is enzyme replacement therapy (ERT) that requires frequent infusions and incurs high costs during life-long treatment. ERT in Pompe disease requires frequent infusions of rhGAA, often on a weekly basis, to correct glycogen storage in striated muscle via receptor-mediated uptake. The enzyme dosages required for ERT in Pompe disease are up to 100-fold greater than those for other lysosomal disorders [1], which can be attributed at least in part to the poor uptake of recombinant human (rh) GAA by skeletal muscle associated with low abundance of the cation-independent mannose-6-phosphate receptor (CI-MPR) [2; 3]. The paucity of CI-MPR in adult mammalian muscle has underscored the concept that CI-MPR is limiting for ERT in Pompe disease. Previously, low levels of CI-MPR were demonstrated in skeletal muscle of GAA-KO mice, specifically in muscles comprised primarily of type II myofibers [2]. Similarly, fibroblasts from a Pompe disease patient were deficient in CI-MPR recycling and the uptake of rhGAA was impaired [4]. Further evidence for the importance of CI-MPR was demonstrated by the increased efficacy of rhGAA modified to increase mannose-6-phosphate content [5-7]. The limiting effect of receptor-mediated uptake during ERT was confirmed by demonstrating that clenbuterol, a selective β2-agonist, enhanced CI-MPR expression and increased efficacy from ERT in mice with Pompe disease [3].

Patients with late-onset Pompe disease have severe pulmonary insufficiency that may progress to respiratory failure while receiving ERT [8]. Many individuals with late-onset Pompe disease have residual gait abnormalities despite adherence to ERT, indicating a relative lack of response in leg muscles [9]. Even among the majority of infants with Pompe disease who respond well when treated early in life [10], only partial efficacy from ERT has been observed. These children retain motor developmental delays and respiratory insufficiency [11]. Persistent neuromuscular involvement has included hypernasal speech and swallowing difficulties [12], or strabismus and ptosis [13], despite long-term ERT in infantile-onset Pompe disease. Many young children with Pompe disease require temporary or long-term assisted ventilation [11]. Each of these above clinical abnormalities was refractory to ERT, indicating a need for improved therapy.
Gene therapy has been developed in preclinical experiments to address the limitations of ERT, including partial efficacy and the need for frequent administration. The availability of novel adeno-associated virus (AAV) serotypes, including AAV serotype 8, has advanced liver-targeted gene therapy by improving liver tropism [14]. AAV2 vectors pseudotyped with AAV8 (AAV2/8) delivered genes to the liver approximately 100-fold more efficiently in mice, including GAA knockout (KO) mice, in comparison with traditional AAV2 vectors [14; 15]. Subsequently, a single administration of the AAV2/8 vector substantially corrected glycogen storage in the diaphragm and heart following the administration of a lower number of vector particles (1x10^{11} vector particles (vp), approximately 4x10^{12} vp/kg body weight) [16]. The aforementioned AAV vector contained a liver-specific regulatory cassette that induced immune tolerance to GAA, which was dependent upon activation of regulatory T cells [17].

Dose-limited immune responses interrupted a clinical trial of AAV vector-mediated gene therapy, when escalating vector dosages of an AAV serotype 2 vector (up to 2x10^{12} vp/kg) were administered to subjects with hemophilia B. Anti-AAV immune responses were encountered at the highest dose that abrogated efficacy [18]. This apparent threshold for triggering anti-AAV responses has stimulated strategies to reduce vector dose requirement, including the adoption of AAV2/8 vectors that feature higher tissue tropism and less immunogenicity [19; 20]. Thus, efficacy could be anticipated from a single dose of an AAV2/8 vector that might be efficacious without triggering neutralizing immune responses. Indeed, we demonstrated efficacy with an AAV2/8 vector for greater than one year in the follow-up of vector-treated dogs with glycogen storage disease type I, thereby demonstrating the long-term stability of AAV-mediated correction of the liver [21]. Moreover, preclinical experiments of liver-targeted gene therapy in Pompe disease have stably corrected biochemical abnormalities in GAA-KO mice, albeit at a dose greater than that administered in clinical trials to date (4x10^{12} vp/kg) [16].

The current study has evaluated the potential for CI-MPR up-regulation to enhance efficacy from low dose AAV vector-mediated gene therapy in Pompe disease (8x10^{11} vp/kg), thereby advancing clinical translation of potentially curative therapy for this and other lysosomal storage disorders. Similar to the situation for ERT, receptor-mediated uptake underlies liver-targeted gene therapy for Pompe disease. In this therapeutic model the liver is highly transduced with an AAV vector encoding GAA that creates a liver
depot for the continuous secretion of GAA into the bloodstream [16; 22; 23]. Given the evidence that CI-MPR expression was crucial to efficacy from GAA replacement in Pompe disease, we chose to enhance CI-MPR levels in GAA-KO mice in combination with liver-targeted gene therapy. This strategy differs from previous attempts to address the limiting role of CI-MPR expression during ERT in Pompe disease, which increased the mannose-6-phosphate content of rhGAA [6; 24]. We wished to increase CI-MPR in skeletal muscle to demonstrate the dependence of biochemical correction upon receptor-mediated uptake of GAA. Therefore, the vector-transduced liver depot was enhanced by the addition of a selective β2-agonist drug, clenbuterol. Clenbuterol was previously demonstrated to increase the expression of the insulin-like growth factor (Igf) 2 receptor (identical to CI-MPR) in muscle of mice [25].

In the current study clenbuterol was administered in combination with an AAV vector containing a liver-specific regulatory cassette (AAV-LSPhGAA). These new experiments demonstrated that clenbuterol and albuterol, selective β2-agonist drugs, enhanced CI-MPR expression and increased efficacy from a liver depot in GAA-KO mice, thereby confirming the efficacy of CI-MPR up-regulation during gene replacement therapy in Pompe disease [3].

**MATERIALS AND METHODS**

*Generation of muscle-specific CI-MPR-KO and DKO mouse models:*

CI-MPR-KO mice were generated using a muscle-specific promoter (muscle creatine kinase; CK) and the cre/loxP conditional knock out system as described previously [28]. The muscle-specific CI-MPR-KO mice were crossed with GAA-KO mice to generate muscle-specific CI-MPR-KO/GAA-KO (“double KO”; DKO) mice. This mouse colony was subsequently screened to be GAA -/-, M6PR flox/flox and MCK-Cre positive. DKO mice were genotyped and bred as described [3]. At the indicated time points post-injection, tissue samples were obtained and processed as described below. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.
In vivo evaluation of AAV vector-mediated efficacy

Rotarod testing was performed as described [15]. Wirehang testing was performed with a 0.5 cm mesh hardware cloth fixed to an 8 by 10 inch frame. Mice were placed on the wire mesh, which was slowly inverted 6 inches over a cage containing paper bedding. The latency, or time until the mouse fell off of the wire mesh, was recorded. Western blotting of hGAA was performed as described using the hGAA monoclonal antibody (courtesy of Genzyme Corp., Framingham, MA) and the CI-MPR antibody (catalog number GTX28093; Gene Tex, Irvine, CA) [22]. GAA activity and glycogen content were analyzed as described [15]. Histologic processing and staining of brain was performed using a modified paraffin processing and staining protocol as previously described [29].

Statistical analyses

Comparison of two groups was assessed by a homoscedastic Student T-test. A p-value <0.05 was considered to be statistically significant.

RESULTS

Enhancement of CI-MPR expression and efficacy from gene therapy with β2-agonist administration

The liver was transduced by administering a low number of AAV-LSPhGAA vector particles (2×10^{10} vp; 8×10^{11} vp/kg) to two groups of 3 month-old male GAA-KO mice, and clenbuterol (30 mg/l in drinking water) was administered to one group of 3 month-old vector-injected male mice. Neuromuscular function was evaluated by Rotarod testing, which quantifies the ability of mice to walk on a rotating rod and previously correlated with the biochemical correction of striated muscle in GAA-KO mice [15]. The effect of clenbuterol was evident, when Rotarod testing was performed 4 weeks following vector administration. The Rotarod latency was increased by 75% following vector administration and clenbuterol treatment, in comparison with vector administration alone (Figure 1A; p<2×10^{-5}). Rotarod latency also increased slightly following clenbuterol treatment alone, in comparison with mock treatment (p=0.02); however, vector administration did not increase Rotarod significantly in comparison with mock treatment of GAA-KO mice.
These data demonstrated a synergistic effect of combination therapy in GAA-KO mice upon motor function. The effect of clenbuterol was further demonstrated by a trend toward greater weight gain following combination therapy, in comparison with mock treatment (Figure 1B; p=0.06).

The efficacy from clenbuterol treatment was evaluated with regard to biochemical correction of GAA deficiency and glycogen storage in striated muscles and the brain. GAA activity was significantly higher in the heart following combination therapy, in comparison with vector administration alone (Figure 1C; p=0.03). The beneficial effect of clenbuterol administration upon biochemical correction was emphasized by lower glycogen content of multiple skeletal muscles and the brain. Glycogen content was lower in the diaphragm (p=0.04), soleus (p=0.0006), extensor digitorum longus (EDL; p=0.002), cerebrum (p<5x10^-5), and cerebellum (p=0.03) following combination therapy, in comparison with vector administration alone (Figure 1D). The glycogen content of soleus from vector-treated mice was too variable to demonstrate any improvement from combination therapy, although combination therapy did lower the glycogen content of soleus relative to mock treatment. Clenbuterol by itself did not significantly reduce the glycogen content of skeletal muscle, in comparison with mock-treated GAA-KO mice (Figure 1E).

The basis for glycogen clearance during clenbuterol treatment was demonstrated by Western blotting detection of CI-MPR. The signal for CI-MPR was higher in the EDL of clenbuterol-treated GAA-KO mice (Figure 2A), but not in the gastrocnemius (Figure 2B). The lack of increased CI-MPR expression in gastrocnemius correlated with the lack of increased biochemical correction from clenbuterol treatment in that muscle (Figure 1D). The signal for CI-MPR was higher in the cerebral and cerebellar hemispheres of brain following clenbuterol administration (Figure 2C-2D). Quantification of the relative intensity for the CI-MPR signal from each tissue revealed a significantly greater amount of CI-MPR in the EDL (p<0.05) and cerebrum (p<0.01), and a trend toward higher cerebellar content (p=0.06) following clenbuterol treatment (Figure 2E). Thus, the higher CI-MPR expression in skeletal muscle and the brain following clenbuterol treatment in conjunction with AAV2/8-LSPhGAA administration (Figure 2) correlated with biochemical correction (Figure 1).
Long-term efficacy from β2-agonist treatment as an adjunct to gene therapy

The next experiments were designed to determine if the added efficacy from β2-agonist therapy would be sustained during longer-term treatment. AAV-LSPhGAA was administered to two groups of adult GAA-KO mice at a slightly higher particle number (4x10¹⁰ vector particles), and clenbuterol (30 mg/l) was administered to one group. When mice were evaluated serially by Rotarod testing a gradual increase in latency was observed for each vector-treated group from 1 to 12 weeks (Figure 3A). Latency increased for mice treated with clenbuterol and vector, in comparison with either vector alone or with no treatment. Biochemical correction was evaluated in striated muscle, and surprisingly, GAA activity was not increased following combination therapy (Figure 3B). However, biochemical correction with the vector was enhanced by clenbuterol administration, as demonstrated by significantly lower glycogen content in the diaphragm, quadriceps, gastrocnemius, and EDL, in comparison with vector alone (Figure 3C). Combination therapy also significantly lowered the glycogen content of the cerebrum, in comparison with no treatment (Figure 3C). Thus, Rotarod and glycogen content data demonstrated the long-term efficacy from adjunctive therapy with clenbuterol.

A second β2-agonist drug, albuterol (30 mg/l in drinking water), was administered to an equivalent group of vector-treated mice, to determine if the beneficial effect might be achieved with a β2-agonist other than clenbuterol. Rotarod latency was not increased following administration of the combined albuterol and vector administration (Figure 3D). Biochemical correction was evaluated in striated muscle and the brain and GAA activity was not higher following combination therapy, in comparison with vector alone (Figure 3E). However, combination therapy significantly reduced the glycogen content of the diaphragm and of the cerebrum, in comparison with vector alone (Figure 3F). Thus, clenbuterol was more effective than albuterol, because clenbuterol significantly lowered glycogen content in the quadriceps and EDL of vector treated mice, whereas albuterol did not (Figure 3C versus Figure 3F).

Histology was evaluated to further demonstrate the effect of β2-agonist treatment upon the effect of gene therapy in GAA-KO mice at 18 weeks following vector administration (Figure 4). Glycogen staining was increased in the cerebellum of mock-treated GAA-KO mice (Figure 4A), in comparison with mice treated with vector alone (Figure 4B). Similarly, glycogen staining was reduced by combination therapy with
vector and albuterol (Figure 4C) or clenbuterol (Figure 4D), in comparison with mock treatment (Figure 4A). Combination therapy reduced glycogen staining in the hippocampus with the cerebrum (Figure 4G-H versus 4E) of vector-treated GAA-KO mice, in comparison with vector alone. A unique reduction of glycogen content was demonstrated in the quadriceps of mice treated with clenbuterol and vector (Figure 4L vs 4I-K).

**Muscle-specific knockout of CI-MPR impaired the response to AAV-LSPhGAA with clenbuterol**

The impact of CI-MPR depletion upon the liver depot strategy was further evaluated by administering AAV-LSPhGAA to DKO mice that lacked CI-MPR expression in striated muscle. The key role of CI-MPR was previously suggested by the lack of efficacy for either ERT [3] or muscle-restricted gene therapy [30] in DKO mice. The vector particle number administered was reduced to a low dose (2x10^{10} vp) [31], in order to evaluate the effect of CI-MPR depletion upon low dose gene therapy (Figure 5). The GAA activities of muscles from DKO and GAA-KO mice were similarly elevated following AAV-LSPhGAA administration, in comparison with GAA-KO mice treated similarly (Figure 5A), which suggested that another receptor in addition to CI-MPR was involved in GAA uptake into DKO muscle from the blood.

Given the lack of effect upon muscle GAA activity from depleting CI-MPR in DKO mice, it might be assumed that gene therapy had circumvented the effect of CI-MPR deficiency in the muscle of DKO mice. However, the clearance of glycogen from the skeletal muscle of DKO mice was impaired following administration of AAV-LSPhGAA (Figure 5B). The residual glycogen content in the diaphragm, gastrocnemius, soleus, and EDL was significantly higher in DKO mice, in comparison with GAA-KO mice, when analyzed 18 weeks following vector administration (Figure 5B; Table 1).

The mechanism of β2-agonist treatment was further evaluated in DKO mice, which should have a lesser response to combination treatment if CI-MPR modulated the effect of β2-agonists. The vector dose was increased 5-fold relative to the above-mentioned experiment in DKO mice (to 1x10^{11} vp), which should increase the likelihood of achieving efficacy (Figure 5). Rotarod latency was not significantly greater in DKO mice following combined treatment, in comparison with vector alone or mock treatment (not shown). Biochemical correction was evaluated in both the heart and skeletal muscle of DKO mice. GAA activity was not significantly greater following combination treatment, in comparison with vector alone (Figure 5C). The
glycogen content of the diaphragm was not reduced following combination treatment, in comparison with mock treatment; however, the glycogen content of the diaphragm increased slightly following administration of vector alone, in comparison with mock treatment (Figure 5D). Therefore the reduced glycogen content of the diaphragm from combination therapy, in comparison with vector alone, was from random variation. In contrast, glycogen content of the quadriceps of DKO mice was not reduced by combination therapy, in comparison with vector alone (Figure 5D). Thus, despite the markedly higher GAA activity following vector administration (Figure 5C), gene therapy with or without clenbuterol treatment did not consistently reduce glycogen content in the major muscles of DKO mice (Figure 5D).

The potential role of mitochondrial proliferation in the enhanced efficacy from β2-agonist treatment was evaluated by analyzing cytochrome C oxidase activity in the quadriceps of GAA-KO mice following combination treatment [32]. Clenbuterol treatment slightly reduced cytochrome c oxidase activity and albuterol had no effect, in comparison with vector alone (Figure 5E). Therefore, the lack of enhanced glycogen reduction in DKO mice supported the hypothesis that β2-agonist treatment was effective due to CI-MPR over-expression.

*Enhanced muscle strength and biochemical correction of skeletal muscle from adjunctive clenbuterol in elderly GAA-KO mice*

Combination therapy was evaluated in mice with advanced Pompe disease, because elderly GAA-KO mice were particularly resistant to correction with AAV vector-mediated gene therapy [26; 27]. Groups of 12 month-old GAA-KO mice of both sexes were treated with a high dose of AAV2/8-LSPhGAA (1x10^{11} vector particles) and clenbuterol, or each therapy individually, for 12 weeks prior to evaluation of biochemical correction in striated muscle. Muscle strength was increased by combination therapy, in comparison with vector alone, as reflected by doubling of the wirehang latency (160 +/- 46 seconds, versus 65 +/- 20 seconds; Figure 6A). Rotarod latency was not increased by the combination therapy at 18 weeks, in comparison with other groups (Figure 6A). Biochemical correction was evaluated to better understand the basis for improved muscle strength from combination therapy. The biochemical correction of heart was improved by either combination therapy or vector alone, as reflected by increased GAA activity (Figure 6B)
and decreased glycogen content (Figure 6C), in comparison with no treatment (p<0.01). The biochemical correction of quadriceps improved uniquely following combination therapy, as reflected by decreased glycogen content in comparison with vector alone (Figure 6C). The glycogen content of EDL trended lower following combination therapy, in comparison with vector alone (Figure 6C; p<0.07). Clenbuterol alone did not reduce the glycogen content of heart or skeletal muscle in elderly GAA-KO mice (not shown).

**DISCUSSION**

The upregulation of CI-MPR enhanced the response to gene therapy in GAA-KO mice treated with clenbuterol (and to a lesser extent albuterol), based upon increased Rotarod latency and lower glycogen content in skeletal muscle. Importantly, the glycogen content was reduced in the diaphragm and multiple skeletal muscles by either short-term or long-term combination therapy with an AAV vector and β2-agonist. The improved biochemical correction in diaphragm represents a critical component of efficacy, both due to the respiratory involvement in Pompe disease and to the resistance of this muscle to correction with gene therapy [33]. Both neuromuscular function and the biochemical correction of skeletal muscles were improved following treatment of GAA-KO mice with selective β2-agonist drugs and gene therapy.

Combination therapy achieved partial efficacy in elderly GAA-KO mice, which have been refractile to gene therapy [26; 27]. We confirmed that the effect of clenbuterol depends upon CI-MPR up-regulation, because combination therapy was much less efficacious in DKO mice than in GAA-KO mice. Part of the effect from clenbuterol might stem from improved trafficking of GAA to lysosomes, rather than improved uptake, because glycogen content was reduced by the addition of clenbuterol without further elevating GAA activity (Figure 1C-D).

The dependence of efficacy from gene therapy upon CI-MPR-mediated uptake of GAA was emphasized by the lack of glycogen clearance from the muscle of DKO mice that lacked CI-MPR in muscle, even when GAA activity was markedly higher following administration of a 5-fold higher dosage of vector (Figure 5). DKO muscle contained elevated GAA activity following vector administration, which most likely reflected uptake of GAA by non-muscle cells within DKO mouse skeletal muscle (for example, endothelial
cells and fibroblasts). GAA was presumably not taken up by the DKO muscle cells, which continued to accumulate high amounts of glycogen.

The vector dose administered in this study (8x10^{11} vp/kg body weight) was low enough to consider as part of a dose escalation in a “Phase 1/2” clinical trial of gene therapy designed to evaluate both safety and efficacy, given that this dose avoided neutralizing immune responses against the AAV vector in subjects with hemophilia B [18]. Furthermore, enhancing CI-MPR expression with clenbuterol uniquely increased the efficacy of a liver depot with regard to correction of skeletal muscle, which addressed a hurdle to therapy in advanced Pompe disease. The addition of clenbuterol to such a clinical trial would increase the likelihood of achieving efficacy, a key consideration when developing therapy for rare disorders affecting relatively few patients. Therefore, adjunctive therapy with β2-agonists might facilitate the translation of gene therapy to clinical applications in Pompe disease.

Clenbuterol has demonstrated hypertrophic effect upon skeletal muscle in rodent models by increasing expression of Igf-1 and Igf-2 [34; 35]. Clenbuterol administration was associated with greater muscle weight in the limb muscles, including gastrocnemius as seen in the current study [36-38]. The expression of the Igf-2 receptor, identical to CI-MPR, was higher in the hypertrophied masseter muscle following clenbuterol treatment [25]. Taken together, these data suggest that the mechanism for enhanced efficacy from replacement therapy by the addition of clenbuterol is the expression of CI-MPR by type II myofibers that were previously unresponsive to ERT [2; 24]. Consistent with this hypothesis, we demonstrated greater expression of CI-MPR in the EDL [3].

The treatment of Pompe disease might be enhanced by adjunctive therapies that improve the response to GAA replacement, such as β2-agonists; however, the translation of rodent studies to clinical trials will depend upon the response of humans to these drugs. One critical factor will be the effective concentration of β2-agonists. Limited data are available, but the effective concentration (EC50) for clenbuterol was lower for humans and other higher mammals than for rodents. For example, the EC50 for clenbuterol with regard to the relaxation of rat smooth muscle was approximately 10-fold higher than the EC50 for equine or human smooth muscle [39-41]. Furthermore, the EC50 in horses and humans were similar to the EC50, when clenbuterol was dosed as a bronchodilator [40; 42]. These studies suggest that
lower dosages of clenbuterol might be effective in humans, in comparison with the high dosages utilized in rodent studies.

The underlying hypothesis for this study stated that increased CI-MPR expression would improve the response to GAA replacement, which hinges upon the increased insulin-like growth factor signaling and muscle hypertrophy from treatment with β2-agonists [25]. Although the vast majority of data regarding the effects of β2-agonists upon muscle has been obtained from studies in rodents, several clinical trials have indicated that β2-agonists are well-tolerated and promote muscle hypertrophy in humans. Several studies of β2-agonists in patients with neuromuscular demonstrated increased muscle strength and/or increased muscle mass. The largest study enrolled 90 patients with fascioscapulohumerol muscular dystrophy (MD) in a randomized, placebo-controlled trial of albuterol for one year, and revealed increased grip strength and lean body mass [43]. Increased lean body mass reflected increased muscle mass. Similarly, a study in which boys with Duchenne MD took albuterol for 12 weeks demonstrated increased quadriceps strength [44]. A larger follow-up study in patients with Duchenne MD revealed increased lean body mass following albuterol treatment [45]. A study of clenbuterol in patients with chronic heart failure revealed that lean muscle mass increased after 12 weeks [46]. A study of clenbuterol in 14 subjects with amyotrophic lateral sclerosis revealed increased muscle strength and improved pulmonary function testing, reflected by forced vital capacity, at the three and 6 month time points [47]. Finally, a small study in which patients with late-onset Pompe disease took albuterol for three years revealed that the drug was well-tolerated and each patient had increased performance on muscle function testing [48]. Increased muscle mass or strength in the aforementioned studies could reflect muscle hypertrophy and increased CI-MPR expression from β2-agonist treatment in humans. The possibility that β2-agonists achieve muscle hypertrophy at standard dosages supports the further translation of adjunctive therapy with ERT for Pompe disease in clinical trials.

The blood brain barrier remains a significant obstacle to therapy in lysosomal storage disorders, either in the form of ERT or gene therapy. It has been hypothesized that low phosphorylation of lysosomal enzymes and low expression of the CI-MPR prevented the uptake of lysosomal enzymes and biochemical correction of the brain in lysosomal storage disorders [49]. The current study demonstrated that β2-agonist treatment increased CI-MPR expression and enhanced the efficacy from the administration of a low number
of AAV vector particles, in contrast with the administration of clenbuterol alone that failed to achieve significant biochemical correction. Clenbuterol crossed the blood-brain barrier to affect the brain in rodents [50]. We demonstrated a unique effect upon the brain by demonstrating increased CI-MPR expression in the cerebrum following 4 weeks of adjunctive clenbuterol treatment. We confirmed that β2-agonist treatment increased CI-MPR levels and enhanced the biochemical correction of the brain from GAA replacement with gene therapy, which was consistent with results from a recent study of β2-agonist treatment in combination with ERT in mice with Pompe disease [51]. Consistent with the latter study, glycogen content of the cerebellum was reduced by adding adjunctive β2-agonist treatment (Figure 1). By 18 weeks following vector treatment the differences between combination therapy and vector alone were blurred, although combination therapy reduced the glycogen content in the cerebrum (Figure 3C) and glycogen staining in the cerebellum (Figure 4C). Recently an in vitro model of the blood-brain barrier revealed that arylsulfatase A uptake across the barrier was partially dependent upon CI-MPR, further validating the role of CI-MPR in the entry of lysosomal enzymes into the central nervous system [52]. These data support the possibility that β2-agonists might be a useful adjunctive therapy for other lysosomal storage disorders such as mucopolysaccharidoses that feature severe brain involvement [53].

Considering the central role of mitochondria in substrate metabolism, a secondary aim of this study was to compare the effects of long-acting [clenbuterol t½ 36-39 h] vs. short-acting [albuterol t½ 1.6 h] β2-agonist treatment on mitochondrial function. Although clenbuterol-induced β2-adrenergic stimulation by a single injection has previously been shown to increase mRNA expression of the mitochondrial master regulator PGC-1α more than 30-fold [32], there is substantial evidence that chronic administration of long-acting β2-agonists induces a transition from slow to fast muscle fiber types [for review see [54]] and ultimately impairs mitochondria [55]. Hoshino et al. (2011) provided a mechanistic basis for this observation by demonstrating that clenbuterol treatment causes a down-regulation of PGC-1α concurrently with an up-regulation of its repressor protein RIP140, which reduces not only total mitochondrial content but also organellar oxidation rates of fat and pyruvate [55]. Our results support the contention that chronic clenbuterol administration may impair mitochondrial function, but we also extend previous findings by showing that short-acting β2-agonists (such as albuterol) do not reduce mitochondrial enzyme activities or
protein expression. More research is needed to further elucidate the therapeutic efficiency of short- vs. long-acting β2- agonists in Pompe disease, specifically ways to improve efficacy of short-acting forms while maintaining mitochondrial integrity as well as β-adrenergic receptor density following chronic treatment. Clenbuterol treatment was associated with approximately 20% lower cytochrome C oxidase activity in our study with GAA-KO mice, but the clinical significance of this suppression is currently unclear. Reductions of >70% in a respiratory chain complex, such as cytochrome C oxidase that represents complex IV, are deemed pathological.[56] Further investigation of this phenomenon is warranted to elucidate potential side-effects of chronic β2- agonist administration on aerobic capacity in humans. The potential risks must be balanced with the potentially therapeutic benefits from increasing CI-MPR expression in patients undergoing ERT for a lysosomal storage disorder, such as Pompe disease.

The efficacy of GAA replacement therapy was enhanced by increased CI-MPR expression from β2-agonist administration. This preclinical data promises that the response to ERT in Pompe disease might be improved by treatment with clenbuterol or a similarly active β2-agonist drug. Furthermore, it is likely that adjunctive therapy to increase CI-MPR expression will facilitate the translation of gene therapy for Pompe disease to clinical applications, which could potentially provide curative therapy for this devastating condition.

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Conflict of interest:

DB and DDK have received research/grant support from Genzyme Corporation in the past. BT is an employee of Genzyme Corporation.

REFERENCES


Table 1: Comparison of AAV-LSPhGAA administration in DKO and GAA-KO mice

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**FIGURE LEGENDS**

**Figure 1: Enhanced efficacy following liver-targeted gene therapy plus clenbuterol treatment.** The GAA-KO mice were injected with AAV-LSPhGAA (2x10^{10} vector particles/mouse). Vector-treated mice were treated with clenbuterol (n=5) or untreated (n=5). GAA-KO mice were treated with clenbuterol (n=5) or untreated (n=5) to serve as controls. Mice were euthanized for tissue analysis 4 weeks after vector injection. (A) Rotarod latency, and (B) weight at indicated timepoints. (C) GAA enzyme levels and (B) glycogen content were evaluated in striated muscle and brain. (D) Glycogen content in striated muscle following treatment with either clenbuterol alone, or mock treatment. (E) Groups of GAA-KO mice were treated with clenbuterol or untreated for 4 weeks, without administering vector (n=5 per group). Glycogen content was evaluated in striated muscle. Mean +/- standard deviation are shown. Statistically significant alterations associated with clenbuterol treatment indicated (*=p<0.05; **=p<0.01). Statistically significant alterations associated with clenbuterol treatment indicated (*=p<0.05; **=p<0.01).

**Figure 2: Western blot analysis of CI-MPR expression in skeletal muscle and brain.** Western blot detection of CI-MPR and control proteins, β-actin or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in the tissues of DKO and GAA-KO mice is shown, with molecular weights indicated. Each lane represents an individual mouse. Equivalent quantities of tissue homogenate were loaded for each mouse. (A) Gastrocnemius. (B) Gastrocnemius (C) Cerebrum. (D) Cerebellum. (E) Signal for CI-MPR as quantified by densitometry of Western blots. Mean +/- standard deviation are shown. Statistically significant alterations associated with clenbuterol treatment indicated (*=p<0.05; **=p<0.01).

**Figure 3: Enhanced Rotarod performance and biochemical correction following long-term liver-targeted gene therapy plus β2-agonist treatment.** The GAA-KO mice were injected with AAV-LSPhGAA (4x10^{10} vector particles/mouse). Vector-treated mice were treated with clenbuterol (n=7 males and 4 females), albuterol (n=6 males and 4 females), or vector alone (n=5 males). Mock-treated GAA-KO mice (n=5 males) were controls. Mice were euthanized for tissue analysis 18 weeks after vector injection. Mean
+/− standard deviation are shown. Statistically significant alterations associated with clenbuterol treatment indicated (*=p<0.05; **=p<0.01). The following parameters were evaluated following clenbuterol treatment: (A) Rotarod latency, (B) GAA enzyme levels, and (C) glycogen content were evaluated in the target tissues (males only). Similarly, the following parameters were evaluated following albuterol treatment: (D) Rotarod latency, (E) GAA enzyme levels, and (F) glycogen content were evaluated in the target tissues (males only).

**Figure 4: Decreased glycogen accumulation following β-2-agonist administration.** Periodic-acid Schiff staining for glycogen in paraffin-embedded sections of the cerebellum (A-D), hippocampus (E-H), and quadriceps (I-L). Original magnification 400x. Two sections were examined for each group and representative images are shown. GAA-KO mice were untreated (A, E, I); or were treated with vector alone (B,F,J), vector plus albuterol (C,G,K), or vector plus clenbuterol (D,H,L). Glycogen accumulations in the brain of untreated mice are indicated (arrows).

**Figure 5: Impaired liver-targeted gene therapy in DKO mice did not respond to treatment with clenbuterol.** (A) The homozygous DKO mice (n=5) and GAA-KO mice (n=4) were injected with AAV-LSPhGAA (2x10^{10} vector particles/mouse) to evaluate the relative effect of MPR depletion upon GAA uptake. Mice were euthanized for tissue analysis 18 weeks after vector injection. GAA enzyme levels and (B) glycogen content were evaluated in the target tissues of mice from (A). (C) GAA activity in target tissues of DKO mice. DKO mice were treated with vector plus clenbuterol (n=5), vector alone (n=4), or mock-treated (n=7). The DKO mice were administered a higher dose of AAV-LSPhGAA (1x10^{11} vp), and euthanized for tissue analysis 4 weeks after vector injection. (D) Glycogen content was evaluated in the target tissues of DKO mice. (E) Cytochrome C oxidase (COX) was analyzed in the quadriceps of GAA-KO mice. GAA-KO mice were treated with vector (4x10^{10} vp), plus clenbuterol (n=5), vector plus albuterol (n=5), vector alone (n=4 DKO), and euthanized for tissue analysis 18 weeks after vector injection. Mean +/- standard deviation are shown. Statistically significant alterations associated with CI-MPR absence indicated (*=p<0.05; **=p<0.01).
Figure 6: Partial efficacy following liver-targeted gene therapy plus clenbuterol treatment in elderly GAA-KO mice. One year-old GAA-KO mice were injected with AAV-LSPhGAA (1x10^{11} vector particles/mouse). Vector-treated mice were treated with clenbuterol (n=6) or untreated (n=6). GAA-KO mice were treated with clenbuterol (n=7) or untreated (n=6) to serve as controls. (A) Wirehang and Rotarod latency at 18 weeks. (B) GAA enzyme levels and (C) glycogen content were evaluated in striated muscle. Mean +/- standard deviation are shown. Statistically significant alterations associated with clenbuterol treatment indicated (*=<0.05; **=<0.01).