Pompe disease: Shared and unshared features of lysosomal storage disorders

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Pompe disease, an inherited deficiency of lysosomal acid α-glucosidase (GAA), is a severe metabolic myopathy with a wide range of clinical manifestations. It is the first recognized lysosomal storage disorder and the first neuromuscular disorder for which a therapy (enzyme replacement) has been approved. As GAA is the only enzyme that hydrolyses glycogen to glucose in the acidic environment of the lysosome, its deficiency leads to glycogen accumulation within and concomitant enlargement of this organelle. Since the introduction of the therapy, the overall understanding of the disease has progressed significantly, but the pathophysiology of muscle damage is still not fully understood. The emerging complex picture of the pathological cascade involves disturbance of calcium homeostasis, mitochondrial abnormalities, dysfunctional autophagy, accumulation of toxic undegradable materials, and accelerated production of lipofuscin deposits that are unrelated to aging. The relationship of Pompe disease to other lysosomal storage disorders and potential therapeutic interventions for Pompe disease are discussed.

Lysosomal storage diseases (LSDs), a group of nearly 60 inherited disorders, are caused by deficiencies in lysosomal hydrolases or transmembrane proteins of late endosomes/lysosomes. LSDs are characterized by progressive accumulation of various undigested substrates and a dysregulation of intracellular trafficking pathways. A dysfunctional lysosomal system affects multiple cellular functions resulting in secondary abnormalities such as defective autophagy, accumulation of aberrant mitochondria, dysregulation of signaling pathways and calcium homeostasis, inflammation, and apoptosis. The complexity of the pathogenic cascade which became clear in the past decade, suggests that successful therapy most likely will require a combination of drugs to target specific aspects of pathogenesis rather than a "magic bullet" designed to address the primary defect (for a review see ref.[1]).

Pompe disease (Glycogen storage disease type II, a deficiency of lysosomal enzyme acid α-glucosidase) is a case in point. Acid α-glucosidase (GAA) is essential for the degradation of glycogen to glucose in lysosomes, and its deficiency is associated with a wide spectrum of clinical phenotypes. In the most severe infantile form, hypertrophic cardiomyopathy and muscle weakness lead to death within the first 2 years of life; in the less devastating childhood and adult onset forms, in which cardiac muscle is usually spared, slowly progressive skeletal muscle myopathy eventually leads to respiratory insufficiency, wheelchair-dependency, and a shortened life span.[2]

Enzyme replacement therapy (ERT) with recombinant human enzyme is available for Pompe patients. Two drugs, Myozyme and Lumizyme (both produced from the same CHO-cell line at different scales; Genzyme Corp., a Sanofi Company), were approved in 2006 and 2010 respectively. Clinical experience has

shown that the therapy reverses cardiac abnormalities but has limited effect in skeletal muscle. Because of the remarkable effect in cardiac muscle, the therapy significantly extends the life expectancy of infants, but leaves children with a myopathy often more severe than in milder later onset forms.[3] The limitations of ERT stimulated research on new therapeutic approaches; these include the development of a second-generation of recombinant human GAA with increased skeletal muscle targeting,[4,5] a combination of ERT with albuterol, a drug that enhances the expression of cation-independent mannose-6-phosphate receptor (CI-MPR) in skeletal muscle,[6] the use of pharmacological chaperones to increase the stability and half-life of the current drug,[7] and gene therapy.[8,9]

In search of new therapeutic targets we have systematically looked at the pathogenesis of muscle damage in Pompe disease using our GAA knockout mouse model (GAA KO), immortalized murine KO muscle cells (an *in vitro* model of the disease), as well as muscle cells and biopsies from Pompe patients. We have identified several secondary pathological changes, such as excessive lipofuscin accumulation, disturbance of mTORC1 signaling (our unpublished data), defective autophagy, mitochondria abnormalities, and dysregulation of calcium homeostasis in the diseased muscle cells.[3] In this commentary, we place particular emphasis on autophagic, mitochondrial, and calcium defects in Pompe disease, and we discuss these effects in the context of other LSDs to identify potential common and uncommon molecular targets.

Autophagy

Lysosomes are key players in the autophagic process, a major intracellular degradative pathway that involves sequestration of a portion of cytoplasm in double-membrane autophagosomes and delivery of the cytoplasmic materials to the lysosomes for break-down and recycling.[10] Many lysosomal storage diseases share a common autophagic defect, inefficient autophagosome–lysosome fusion, leading to the accumulation of polyubiquitinated protein aggregates, dysfunctional mitochondria, and cell death.[1]

Damaged lysosomes themselves may undergo selective autophagy. We have seen morphological evidence of this phenomenon, termed "lysophagy," in muscle fibers from biopsies of Pompe patients: immunostaining with lysosomal and autophagosomal markers showed the presence of lysosomes with compromised borders inside the autophagosomes.[11] Later studies by others demonstrated that injured lysosomes are ubiquitinated and recruited by autophagic proteins, which allows for the incorporation of lysosomes into autolysosomes for degradation; furthermore, autophagy of lysosomes is considered critical for the restoration of lysosomal degradation capacity.[12,13] It is unclear whether excessive lysophagy is a ubiquitous mechanism in LSDs.

The interconnection between the lysosomes and autophagosomes is further emphasized by a recent discovery of a coordinated lysosomal expression and regulation (CLEAR) gene network and a transcription factor EB (TFEB) that orchestrates the biogenesis and function of these 2 organelles.[14] We have demonstrated that a closely related transcription factor E3 (TFE3; a member of the same MiTF/TFE family) is another major regulator of lysosomal/autophagosomal biogenesis.[15] Both transcription factors stimulate lysosomal exocytosis, a Ca^{2+}-dependent process of attachment and fusion of lysosomes and autolysosomes (a product of lysosomal-autophagosomal fusion) with plasma membrane leading to a discharge of lysosomal content in the extracellular space. Activation of TFEB promoted cellular clearance of lysosomal storage materials in several LSDs,[16] thus, providing a conceptually new therapeutic avenue for this group of disorders. The activity of both TFEB and TFE3 is regulated by mTORC1-mediated phosphorylation on the lysosomal surface: when phosphorylated, these transcription factors are inactive and localized in the cytosol; inhibition of phosphorylation (for example, by nutrient deprivation or incubation with the mTORC1 inhibitor Torin-1) promotes their translocation to the nucleus where they stimulate the expression of multiple lysosomal and autophagic genes.[15,17-19] Recent study identified another signaling pathway involved in TFEB nuclear translocation and activation – calcineurin-mediated TFEB dephosphorylation.[20]

Of note, the mechanism of the effect of 2 drugs, genistein and 2-hydroxypropyl-β-cyclodextrin (HPβCD), used for therapy of mucopolysaccharidoses and Niemann-Pick type C disease (NPC), was shown to be TFEB-mediated lysosomal exocytosis.[21,22] HPβCD treatment also promoted TFEB-mediated clearance of the ceroid lipopigment in fibroblasts derived from patients with late infantile neuronal ceroid lipofuscinosis (LINCL).[23]

Defective autophagic flux, which is defined as the rate of lysosomal processing of autophagosomes, is particularly striking in Pompe muscle leading to massive autophagic buildup that disrupts muscle architecture and presents an obstacle for enzyme replacement therapy.[3] We have shown that overexpression of TFEB or TFE3 in Pompe muscle alleviated autophagic buildup and cleared cells of excess glycogen.[15,24] Consistent with the role of these transcription factors in stimulating lysosomal biogenesis,[14,25] a striking reduction in the number of large lysosomes in treated Pompe muscle cells was associated with the increase, rather than a decrease in the number of small "healthy" lysosomes as indicated by the level of LAMP1 (Lysosomal Associated Membrane Protein 1).[24] The fate of the discharged lysosomal content following TFEB- or TFE3- mediated lysosomal exocytosis remains unclear. This question is relevant not only to Pompe disease, but also to other lysosomal and neurodegenerative disorders, in which stimulation of lysosomal exocytosis is considered as a therapeutic approach. We have not addressed the question directly, but from our experience, overexpression of TFEB for 45 days in muscle of the GAA knockout mice did not result in any appreciable abnormalities; there were no signs of toxicity or gross alterations of the muscle architecture, and no increase in the number of apoptotic cells or signs of caspase-3 activation.[24,26] It is still, however, not clear whether TFEB- or TFE3-mediated cellular clearance in Pompe

muscle would lead to improved muscle strength and functional performance. Recent evidence indicates that neuropathology, in particular changes in respiratory motoneurons and limb muscle neuromuscular junctions, greatly contribute to muscle weakness in Pompe disease; these studies suggest that therapies targeting muscle alone may not be fully effective in Pompe disease.[27-30]

Although promising, activation of the TFEB/CLEAR network in Pompe disease as well as in other LSDs is unlikely to become a monotherapy, but rather a short-term "rescue" measure to promote cellular clearance so that other therapies, such as enzyme replacement therapy (in the case of Pompe disease), may provide a greater benefit.

Mitochondria/Mitophagy

Considering the role of autophagy in selective elimination of damaged mitochondria, a process known as mitophagy, it is not surprising that many LSDs exhibit signs of mitochondrial dysfunction, which include morphological changes, decreased mitochondrial membrane potential ($\Delta\Psi_m$), diminished ATP production, increased generation of reactive oxygen species (ROS), oxidative stress, and apoptosis (for a review see ref.[31]). A loss of $\Delta\Psi_m$ leads to the accumulation of PINK1, a serine-threonine protein kinase, on the outer mitochondrial membrane (OMM); PINK1 signals mitochondrial dysfunction to PARK2, a cytosolic E3 ubiquitin ligase, which ubiquitinates OMM proteins, thus tagging damaged mitochondria for autophagic degradation.[32]

Indeed, defective autophagic turnover of dysfunctional mitochondria has been implicated in several LSDs: mucolipidosis II, III, and IV, neuronal ceroid lipofuscinosis or Batten disease, Gaucher disease, multiple sulfatase deficiency, and mucopolysaccharidoses.[31,33] However, the mechanism of failed PARK2-mediated clearance of damaged mitochondria is not the same in these disorders. For example, in multiple sulfatase deficiency (MSD) this defect is attributed to a significant reduction in PARK2 levels leading to

accumulation of morphologically aberrant mitochondria with reduced $\Delta\Psi_m$ and ATP production, and apoptosis,[34] whereas in type II Gaucher disease, the ability of the dysfunctional mitochondria to recruit PARK2 is compromised, although the level of PARK2 remains unchanged.[35] In contrast, the level of PARK2 in Pompe disease is increased in KO myotubes and to a greater extent in GAA KO muscle. Furthermore, the levels of both PINK1 and PARK2 are increased in the mitochondrial fraction from GAA KO muscle, indicating that the translocation of PARK2 to damaged mitochondria and subsequent ubiquitination of mitochondrial proteins are not impaired. However, when the KO myotubes were infected with a virus expressing mCherry–GFP tag attached to the OMM localization signal of the FIS1 protein (residues 101 to 152), a significant number of damaged mitochondria remained in the cytosol, suggesting their inefficient clearance through mitophagy due to incomplete autophagic flux.

Specific therapy to address the mitochondrial component in LSDs has not been developed; however, correction of the autophagic defect is likely to improve the mitochondrial status. In addition, several drugs, which indirectly target mitochondria through calcium signaling, have shown beneficial effect in LSDs.

Calcium Homeostasis

Disturbed Ca^{2+} signaling is another characteristic feature in many lysosomal storage diseases, but the location of altered Ca^{2+} stores and the underlying mechanisms vary significantly and appear to be distinct for each disorder (for a review see ref.[36]).

Lysosomal calcium

Recent studies have demonstrated that acidic compartments store calcium that can be mobilized and released through 2-pore channels (TPCs) by engaging the Ca^{2+} mobilizing second messenger, nicotinamide adenine dinucleotide phosphate (NAADP) (for a review see ref.[37]) (Of note, the view of TPCs as molecular targets of NAADP has been challenged).

Lysosomal calcium defect is observed in the neurodegenerative lysosomal disorder NPC1. Lysosomal accumulation of sphingosine in NPC1 significantly diminishes lysosomal calcium stores leading to the reduced NAADP-mediated Ca^{2+} release; this in turn negatively affects endosomal-lysosomal fusion and trafficking resulting in secondary accumulation of cholesterol, sphingomyelin, and glycosphingolipids.[38] Improved trafficking and pathology in NPC1 cells was achieved by 2 pharmacological agents, thapsigargin and curcumin, which raised cytosolic Ca^{2+} levels to compensate for the reduced Ca^{2+} release from the acidic compartment; furthermore, treatment with curcumin slowed the rate of disease progression and increased the lifespan of NPC1 mice. A combination of 3 therapies, each targeting a specific aspect of the pathogenesis [miglustat (an inhibitor of glycosphingolipid synthesis) for substrate reduction therapy, curcumin to compensate for the calcium defect, and ibuprofen to reduce CNS inflammation] provided even greater benefit in the NPC1 mice.[39]

In our Pompe models (myotubes and fibers) Ca^{2+} imaging using Ca^{2+} binding fluorescent dye showed intensely bright fluorescent spots in addition to a diffuse Ca^{2+} staining; these spots were reminiscent of enlarged lysosomes, typical of Pompe disease, suggesting a possibility of selective accumulation of Ca^{2+} in lysosomes. However, Ca^{2+} imaging of KO myotubes and fibers in which lysosomes were labeled with mCherry-LAMP1 (a lysosomal marker) did not reveal appreciable co-localization of the 2 stains, indicating extralysosomal Ca^{2+} location in Pompe cells.

Cytosolic and mitochondrial calcium

Increased cytosolic Ca^{2+} has been reported in Niemann-Pick A disease (a deficiency of the acid sphingomyelinase) and in 2 sphingolipid storage diseases, GM2 gangliosidosis (Sandhoff disease; a deficiency of hexosaminidase B) and Gaucher disease (lysosomal glucocerebrosidase deficiency). In both Niemann-Pick A and GM2 gangliosidosis the increase is attributed to the reduced Ca^{2+} uptake by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), but the underlying

mechanism is different: low levels of SERCA expression in Niemann-Pick A and a direct inhibition of SERCA activity by the excess sialic acid residue of the accumulated ganglioside in GM2 gangliosidosis. Increased cytosolic calcium in murine models of neuronal forms of Gaucher disease, the most common LSD, is caused by the direct interaction of the accumulated glucosylceramide (GlcCer) with the ER calcium channel, the ryanodine receptor, leading to the activation of the receptor.[36]

We, too, found an increase in cytosolic Ca^{2+} and Ca^{2+} flux in *vitro* in KO myotubes. A significant age-dependent increase in Ca^{2+} levels was also detected in GAA KO muscle fibers, particularly in the areas of autophagic buildup. These data are consistent with the findings by Ishigaki and colleagues showing the excess of Ca^{2+} accumulation in high-density areas on computerized tomographic (CT) images of severely affected muscles in children with Pompe disease; an elevated Ca^{2+} signal was particularly strong in electron dense globular bodies (most likely lipofuscin inclusions[3]) within the area of autophagic accumulation.[40] The surge in cytosolic Ca^{2+} levels in Pompe muscle cells leads to mitochondrial Ca^{2+} overload, decreased mitochondrial membrane potential, ROS generation, and AIF (apoptosis-inducing factor)-mediated apoptosis. These changes, already seen in KO myotubes, precede the development of massive autophagic buildup, lipofuscin accumulation, and a respiration compromised state of mitochondria which are so prominent in whole muscle fibers.

Altered mitochondrial calcium homeostasis has been documented in at least 2 other LSDs. Fragmented mitochondria with reduced Ca^{2+} buffering capacity and increased susceptibility to apoptosis (activation of caspase-8-dependent pathway) have been observed in fibroblasts from patients with mucolipidosis type II, III and IV and neuronal ceroid lipofuscinosis 2 (CLN2).[41] In GM1 gangliosidosis (deficiency of lysosomal β-galactosidase), GM1- ganglioside accumulates in the glycosphingolipid-enriched fractions of mitochondrial associated ER membranes, where the substrate interacts and alters the activity of the phosphorylated form of the

Ca^{2+} releasing inositol triphosphate receptor leading to mitochondrial Ca^{2+} overload, mitochondrial membrane permeabilization, and activation of the mitochondrial apoptotic pathway.[42]

We have shown that the increase in intracellular Ca^{2+} in Pompe models is linked to the up-regulation of the L-type Ca^{2+} channel isoforms—*Cacnb1* (calcium channel, voltage-dependent, β 1 subunit) and *Cacng1* (calcium channel, voltage-dependent, gamma subunit 1). The increase in the CACNB1 was confirmed by western blot analysis of lysates from KO myotubes, human Pompe myotubes, and GAA KO fibers. Most importantly, we have shown that a Ca^{2+} channel blocker—verapamil—a drug approved by the US Food and Drug Administration (FDA) to treat cardiovascular diseases, reduced Ca^{2+} levels and reversed the mitochondrial abnormalities in the KO cells. Therefore, this drug in conjunction with enzyme replacement therapy may have significant therapeutic potential for Pompe disease.

Interestingly, selected L-type calcium channel blockers, such as amlodipine, has been found to have beneficial effects in a cell model of juvenile neuronal ceroid lipofuscinosis (JNCL; Batten disease), a lysosomal storage disorder due to mutations in the *CLN3* gene. A significant elevation of intracellular Ca^{2+} in this disorder is caused by the increased level of the β 1 subunit of the G-protein complex (Gβ1), which negatively regulates the N-type voltage-gated calcium-mediated synaptic transmission in neurons.[43] Amlodipine (primarily blocks the L-type voltage Ca^{2+} channels and also has effects on N-type and P/Q type calcium channels) significantly decreased Ca^{2+} levels and reversed neuronal apoptosis in a primary neuronal model of Batten disease.[44]

Furthermore, a combination of L-type Ca^{2+} channel blocker (lacidipine) with the inhibitor of ER-associated degradation (Eeyarestatin I) has been used to improve the folding, trafficking, and lysosomal activity of the unstable mutant form of glucocerebrosidase (L444P, the most prevalent mutation in the neurological form of Gaucher disease) in fibroblasts derived from patients with the disease.[45] Therefore, by lowering cytosolic calcium levels,

Ca^{2+} channel blockers might serve as chaperones to enhance the activity of acid α-glucosidase in Pompe patients with mutations that cripple but do not abolish the enzyme activity.

A question which remains to be elucidated in Pompe disease is how excessive accumulation of lysosomal glycogen leads to enhanced cellular Ca^{2+} intake. Two possible scenarios will be discussed here: one based on mechanistic target of rapamycin (mTORC1) inhibition and the other on degradation of β adrenergic (β-AR) receptor.

mTORC1, a master regulator of protein synthesis, is a protein kinase complex that has the general role of advancing biosynthesis and cell growth and proliferation in response to cues of nutrient abundance. The specific relevance of mTORC1 to LSDs lies in its activation upon lysosomal recruitment in nutrient-rich conditions. Under these conditions, the increased concentration of amino acids in the lysosome initiates a signal to a multiprotein lysosome-based complex (v-ATPase in cooperation with the guanine nucleotide exchange factor Ragulator and Rag GTPases) which culminates in the mTORC1 translocation to the lysosomal surface where this kinase is directly activated by Rheb (Ras homolog enriched in brain).[46] Conversely, amino acid starvation de-activates v-ATPase causing mTORC1 lysosomal release and inactivation. Perhaps even more relevant to Pompe disease is the finding indicating that glucose, like amino acids, controls mTORC1 recruitment to the lysosomal surface and its activation.[47]

Reduced mTORC1 activity has been documented in fibroblasts from Pompe patients and in GAA KO muscle.[48] We, too, observe inhibition of mTORC1 activity in cultured murine KO myotubes and whole muscle from Pompe mice (unpublished data). The link between lysosomal glycogen over-accumulation and reduced mTORC1 activity is supported by the data showing that the deficiency of the putative lysosomal sugar transporter Spinster (Spin) leads to lysosomal accumulation of carbohydrates in the enlarged lysosomes and an mTORC1 dysfunction (lack of its reactivation after prolonged starvation).[49]

Inhibition of sugar efflux from the lysosomes in Spin knockdown cells is similar to the effect of GAA deficiency in Pompe disease as both lead to lysosomal carbohydrate accumulation (probably glycogen in both conditions, although it has not been shown for the Spin knockdown). These results suggest that excessive carbohydrate accumulation in lysosomes, caused by different mechanisms (inhibition of glucose efflux and inhibition of glycogen breakdown to glucose) can impair mTORC1 activity. The lack or deficiency of acid α-glucosidase in Pompe disease is expected to reduce lysosomal glycogen breakdown to available glucose and thus induce local glucose starvation, which may contribute to energy deficit in skeletal muscle from Pompe mice.[50] Apart from possible effect of glucose starvation on mTORC1 activity in Pompe muscle, suppression of mTORC1 may result from lysosomal membrane damage and permeabilization leading to amino acid leakage and diminished available surface for mTORC1 binding; as mentioned above, a subset of lysosomes with compromised borders are often seen in muscle fibers from biopsies of Pompe patients. Inhibition of mTORC1 activates the MAPK pathway leading to nuclear translocation and activation of CREB (cAMP response element –binding protein), which may in turn induce CACNB1 transcription by binding to the conserved sites in its promoter.

The second scenario connecting excess lysosomal glycogen accumulation and upsurge of intracellular calcium also revolves around the lysosomal membrane permeabilization. Lysosomes are critical for the trafficking and degradation of the G-protein-coupled receptors, including β-AR receptor. Lysosomal dysfunction is expected to reduce lysosomal degradation of β-AR with its concomitant increase and activation of cAMP-dependent protein kinase A and L-type Ca^{2+} channels as was shown in a cardiac myocytes model.[51]

A larger view of the secondary abnormalities in LSDs which emerges from the discussion in this commentary could go something like this: imagine a pyramid with the autophagic defect, the most commonly shared piece among LSDs, at the apex, the less commonly shared mitochondrial abnormalities in the middle, and the most unique calcium aberrations at the base. This does not surprise us.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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