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Review

Lysosomal proteolysis in skeletal muscle

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Abstract

Lysosomal proteases are abundantly expressed in fetal muscles, but poorly represented in the adult skeletal muscles. The lysosomal proteolytic system is nonetheless stimulated in adult muscles in a variety of pathological conditions. Furthermore, recent investigations describe autophagosomes in muscle fibers in vitro and in vivo, and report myopathies with excessive autophagy. This review presents our current knowledge about the lysosomal proteolytic system and summarizes the evidences pertaining to the role of lysosomes and autophagosomes in muscle physiology and pathology.

Keywords: Skeletal muscle; Lysosome; Cathepsin; Autophagosome; Myopathy

Contents

Abbreviations: Atg, autophagy; DMRV, distal myopathy with rimmed vacuoles; E64, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; EDL, extensor digitorum longus; EE, early endosome; ER, endoplasmic reticulum; GABARAP, γ-aminobutyric acid receptor-associated protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; GFP, green fluorescent protein; Hsc73, heat-shock cognate protein of 73 kDa; IL6, interleukin-6; IκB, inhibor of the nuclear factor κB; KFERQ, Lys-Phe-Glu-Arg-Gln; LAMP, lysosome associated membrane protein; LIMP, lysosome integral membrane protein; LC3, microtubule-associated protein light chain 3; LE, late endosome; M6PR, mannose-6-phosphate receptor; PAS, pre-autophagosomal structure; PCR, polymerase chain reaction; $PI3K_{III}$, phosphoinositide 3-kinase of class III; RER, rough endoplasmic reticulum; TGN, trans-Golgi network; TNF-α, tumor necrosis factor-α; V-type H^+ ATPase, vacuolar-type ATP-proton pump; XLMTM, X-linked myotubular myopathy; XMEA, X-linked myopathies with excessive autophagy

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1. Introduction

Lysosomes are a major component of the degradative machinery in mammalian cells. Lysosomes are defined as membrane-bounded vesicles containing high concentrations of various acid hydrolases and they typically present an acidic lumen (pH 4–5) and a high density (Kirschke & Barrett, 1987). Lysosomal hydrolases contain proteases, glycosidases, lipases, nucleases and phosphatases. Lysosomes therefore act as intracellular compartments dedicated to the degradation of a variety of macromolecules. Should they escape from lysosomes, acid hydrolases can be devastating for cellular or extracellular constituents. Therefore, accurate synthesis, processing and sorting of lysosomal hydrolases to endosomes/lysosomes, not only determine the capacity for lysosomal proteolysis, but are also vital for cellular homeostasis.

The lumen of lysosomes topographically corresponds to the extracellular milieu. Lysosomal hydrolases are therefore implicated in the degradation of extracellular constituents, which may reach lysosomes by endocytosis, pinocytosis or phagocytosis. Endocytosis and secretion pathways also deliver cell membranes and vesicles to endosomes/lysosomes, and hence lysosomes play a central role in the turnover of membrane lipids and trans-membrane proteins. Lysosomes are further implicated in the turnover of cytoplasmic soluble constituents, and of cellular organelles including mitochondria, peroxisomes, and even nuclei (Roberts et al., 2003). In contrast to the other proteolytic systems (proteasomes, calpains) involved in the degradation of intracellular proteins, lysosomal hydrolases are physically isolated from cytoplasmic constituents by the lysosomal membrane. Various mechanisms of autophagy are then essential to bring cytoplasmic substrates inside lysosomes. Delivery of substrates, together with lysosomal hydrolytic capacity, will specify the role of lysosomes in the overall intracellular proteolysis.

The purpose of this review is to present a comprehensive summary of lysosomal protein degradation and to outline recent advances supporting important functions for lysosomes in the physiology and pathology of skeletal muscle.

2. Targeting nascent cathepsins to lysosomes

Cathepsins L, B, D and H are the major lysosomal proteases and they primarily determine the proteolytic capacity of lysosomes. Cathepsins are synthesized as pre-pro-enzymes (Hasilik, 1992) (Fig. 1). The pre-peptide drives the cathepsin precursor in the lumen of the rough endoplasmic reticulum and is hydrolysed early during translation. The pro-peptide enables a proper folding of the nascent cathepsin, inhibits its proteolytic activity and preserves its three-dimensional structure at neutral pH. Pro-cathepsins are also co-translationally glycosylated. Phosphorylation of mannose residues in the Golgi network is a key step for the trafficking of pro-cathepsins to endosomes/lysosomes (Braulke, 1996). Mannose-6-phosphate enables the specific binding of pro-cathepsins to mannose-6-phosphate receptors (M6PR) in the trans-Golgi network. M6PR with their bound ligand are subsequently translocated to late endosomes, where the mildly acidic pH (6.0–6.5) dissociates pro-cathepsins from M6PR. Proteolytic processing of pro-cathepsins into active proteases occurs in late endosomes or in lysosomes. A drop in pH weakens the interaction between the pro-peptide and the active site cleft, and enables degradation of pro-peptides (Turk, Turk, & Turk, 2001).

Endoplasmic reticulum (ER) and Golgi elements are the strategic cell centers for synthesis and trafficking of lysosomal enzymes. They are distributed throughout the whole myofiber of all muscle types (Ralston, Lu, & Ploug, 1999). ER elements, with typical protein translocation, glycosylation, folding, and export functions are located in perinuclear regions and also extend over the Z-line in myofibrils (Kaisto & Metsikko, 2003; Rahkila, Vaananen, Saraste, & Metsikko, 1997). In skeletal muscle, Golgi complexes important for mannose phosphorylation concentrate around the myonuclei at the surface of fibers, and also between the myofibrillar cores (Ralston, Ploug, Kalhovde, & Lomo, 2001). This distribution is fiber-type dependent: Golgi complexes accumulate as a perinuclear belt at the surface of slow-twitch fibers, but they show a more even

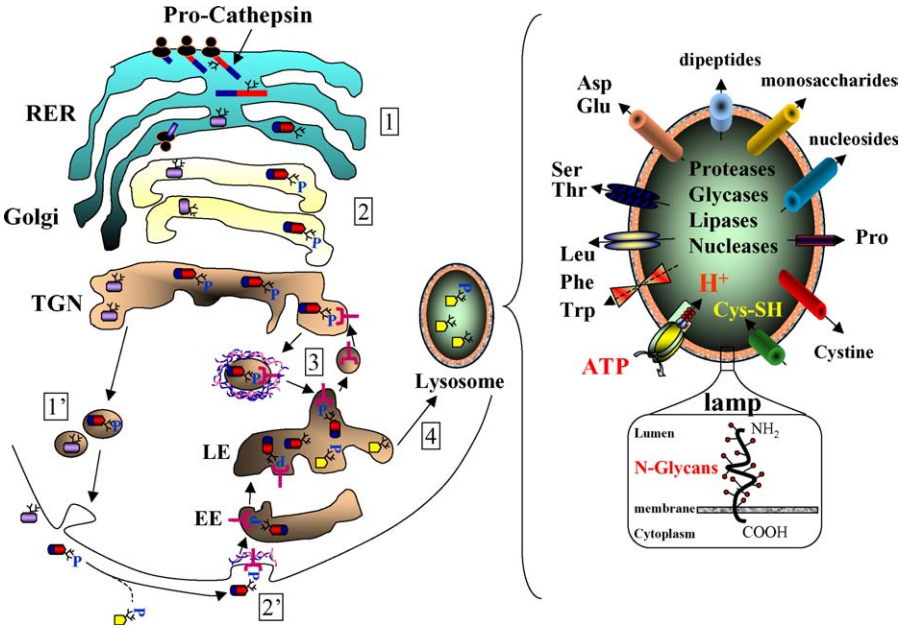

Fig. 1. Schematic representation of the processing and transport of nascent pro-cathepsins to lysosomes. (1) Translation in the rough endoplasmic reticulum (RER) and folding of inactive pro-cathepsins; (2) phosphorylation of selected mannose residues; (3) binding of pro-cathepsins to M6PR in the trans-Golgi network (TGN), and translocation to late endosomes (LE); (4) proteolytic processing of pro-cathepsins into active proteases (yellow) and transport to lysosomes. If newly synthesized pro-cathepsins fell to bind to M6PR in the trans-Golgi network, (1′) they follow the secretion pathway; (2′) M6PR localized at the plasma membrane may transport pro-cathepsins to lysosomes via early endosomes (EE) and receptor-mediated endocytosis. The right panel is a schematic representation of the organization of lysosomal structures.

distribution between the myofibrillar core and surface in fast-twitch fibers (Ralston et al., 1999). Using fluid-phase markers in isolated myofibers, Kaisto, Rahkila, Marjomaki, Parton, and Metsikko (1999) showed that late endosomes and lysosomes localize predominantly around myonuclei beneath the sarcolemma, and sparsely in the interior of the myofibers.

3. Organization of lysosomal structures

3.1. Lysosomal membrane proteins

Lysosomal hydrolysis depends on accurate synthesis and sorting of hydrolases, and also on characteristic functions of lysosomal membrane proteins, such as resistance to degradation by lysosomal hydrolases, generation of a lumenal acidic pH, and selective transport of degradation products to the cytoplasm (Fig. 1, right panel). Major protein components of the lysosomal membrane are lysosome associated (LAMP) and lysosome integral (LIMP) membrane proteins. LAMP and LIMP present a large lumenal domain decorated by a number of *N*-glycans and *O*-glycans (Fukuda, 1991). Highly abundant, they form a nearly continuous coat on the inner surface of the lysosomal membrane, and thus serve as a degradation-resistant barrier (Eskelinen, Tanaka, & Saftig, 2003). The lumen of lysosomes is maintained at a acidic pH (4.0–5.5) due to the presence of vacuolar-type ATP-proton pumps (V-type H^+ ATPase) in the lysosomal membrane (Forgac, 1999). V-type H^+ ATPases couple ATP hydrolysis to the electrogenic translocation of protons from the cytoplasm to the lysosomal lumen. The acidic pH favors the digestive activities of lysosomal hydrolases, and partly unfolds protein-substrates. In addition, the electrochemical proton gradient established by V-type H^+ ATPases acts as a driving-force for lysosomal membrane transporters (Lloyd, 1996). Therefore, the digestive function of lysosomes, which depends on its acidification, is an ATP-dependent process and requires cellular energy metabolism.

Transport systems are required for the efflux of degradation end-products from the lysosomes to the cytoplasm, where they are reused by cellular metabolism. The lysosomal membrane contains transporters for mono- and oligo-saccharides (Saint-Pol, Codogno, & Moore, 1999; Tietze, 1992), amino acids (Lloyd, 1996), oligopeptides (Zhou, Thamotharan, Gangopadhyay, Serdikoff, & Adibi, 2000), nucleosides and phosphate (Pisoni, 1991). Among lysosomal transporters, the cysteine-transporter is unique, as it imports cysteine from cytoplasm to lysosomes. In fact cystine-export (by cystinosin) and cysteine-import function together to keep a high cysteine/cystine ratio inside lysosomes. This is required to maintain the activity of cysteine hydrolases, and to reduce some disulfide bridges of protein-substrates.

The importance of lysosomal membrane proteins in muscle physiology is illustrated (see below) by the existence of several myopathies caused by a deficiency in LAMP (Nishino et al., 2000), in lysosomal acidification (Oyama, Murakami, & Ihara, 1998), or in cystinosin (Charnas et al., 1994; Vester, Schubert, Offner, & Brodehl, 2000).

3.2. Lysosomal proteases

Cellular and extra-cellular protein-substrates are properly folded at physiologic neutral pH. When delivered to lysosome for degradation, the lysosomal acidic pH weakens intermolecular charge–charge interactions and promotes their unfolding. A high ratio of cysteine/cystine concentration inside lysosomes further provides reducing power to break some disulfide bonds within protein-substrates. Misfolded proteins then become more susceptible to proteolysis by lysosomal proteases.

Protein hydrolysis in lysosomes is performed by an array of proteases encompassing endopeptidases and exopeptidases. The formers cleave inner peptide bonds of proteins, while the latters hydrolyse C- or N-terminal ends. Two classes of endopeptidases (aspartic and cysteine) are found in lysosomes (Turk et al., 2001). Lysosomal cysteine endopeptidases are active in the acidic cysteine-rich milieu found in lysosomes, but several (cathepsins S, K, B) remain active at neutral pH. Most lysosomal cysteine proteases are only endopeptidases (cathepsins F, K, L, S, V, legumain), but cathepsin B is also a carboxydipeptidase and cathepsin H, an aminopeptidase. Cathepsin D is the major aspartic endopeptidase identified in lysosomes, is only active below pH 6 and does not require a reducing environment. The cathepsins B, L, H and D function as ubiquitous lysosomal peptidases and are expressed in many tissues (Turk et al., 2001). The other lysosomal endopeptidases

exhibit a more limited distribution and tissue-specific function (Driessen et al., 1999; Gelb, Shi, Chapman, & Desnick, 1996).

As endopeptidase activities proceed, new C- and N-terminal residues are generated and they become substrates for exopeptidases. The major lysosomal carboxypeptidases are cathepsin A, carboxypeptidase B (cathepsin X), prolylcarboxypeptidase B and cathepsin B. The major lysosomal aminopeptidases include cathepsin H, cathepsin C and tripeptidyl peptidase I, which remove one, two and three amino acids from the N-terminus, respectively (Mason, 1996). Endopeptidases are at least 10-fold more concentrated than exopeptidases, and they probably initiate lysosomal proteolysis.

High hydrolase concentrations (1 mM for cathepsin B, L or D) inside lysosomes may favor protein–protein interactions and generate a lysosomal matrix (Zhu & Conner, 1994). Such a lysosomal matrix may enable lysosomes to retain their enzyme content when they fuse with other vesicles (endosomes, secretory vesicles). The lysosomal matrix may also be important to protect lysosomal hydrolases from degradation (Galjart et al., 1991), and to guide substrates to hydrolases inside the lysosome.

3.3. Hydrolysis of myofibrillar proteins by cathepsins

In vitro, the lysosomal endopeptidases were all shown to degrade purified myofibrillar proteins (Dufour, Ouali, Obled, Deval, & Valin, 1989; Katunuma, Kominami, Noda, & Isogai, 1983). The specificity of the hydrolysis varies from one cathepsin to another. Cathepsin H degrades mainly troponin T (Katunuma et al., 1983). Cathepsin B hydrolyses myosin heavy chain (Schwartz & Bird, 1977), troponin T, and more slowly troponin I and tropomyosin (Noda, Isogai, Hayashi, & Katunuma, 1981). Cathepsin L degrades most myofibrillar proteins with the exception of troponin C and tropomyosin (Matsukura, Okitani, Nishimuro, & Kato, 1981). Because the tremendous concentration and diversity of lysosomal hydrolases cannot be mimicked in vitro, the disruptive potential of cathepsins can be expected to be even more massive in vivo.

Assessing the role of lysosomal proteolysis is a difficult task, because it requires a massive inhibition of all types of lysosomal proteases (Tournu et al., 2001). Cathepsin knockout studies have not provided clear information about the role of cathepsins in intracellular proteolysis. For example, cathepsin D knockout (Saftig et al., 1995), or combined deficiency of cathepsins B and L (Felbor et al., 2002) are lethal shortly after birth. Moreover, cathepsin inhibitors were not found to reduce 3-methyl-histidine release (an index of myofibrillar proteolysis) by perfused muscles (Lowell, Ruderman, & Goodman, 1986). In fact, a total and selective inhibition of lysosomal proteolysis is often difficult to achieve, even with cultured cells. Thus, E64 (an inhibitor of cysteine proteases), leupeptin (an inhibitor of cysteine and serine proteases), and pepstatin-A (an inhibitor of aspartic proteases) are often used, but they are not readily membrane-permeable and variably inhibit lysosomal cathepsins in vivo (Tournu et al., 2001; Wilcox & Mason, 1992). In addition, neutralization of lysosomal acidity (by weak-base amines or by inhibitors of V-type H^+ ATPase) poorly affects cathepsins (S, B) active at neutral pH.

The role of lysosomal proteolytic pathway in muscle physiology and pathology is underlined by recent investigations on cathepsin expression, and on delivery of substrates to lysosomes in skeletal muscles.

4. Expression of cathepsins in skeletal muscles

4.1. Cathepsins in skeletal muscles

The lysosomal cathepsins B, H, L and D, although ubiquitously expressed, reveal tissue-specific distribution (Bando, Kominami, & Katunuma, 1986; Kominami, Tsukahara, Bando, & Katunuma, 1985). High levels of expression of cathepsins are found in tissues presenting high rates of protein turnover (kidney, spleen, liver, placenta), while low concentrations of cathepsins prevail in slowly turning-over skeletal muscles.

Despite low levels of cathepsins in adult skeletal muscles, many studies demonstrate the presence of these proteases in this tissue. Cathepsins were characterized in lysosomes isolated from adult skeletal muscles (Bechet, Deval, Robelin, Ferrara, & Obled, 1996; Belkhou et al., 1994; Kirschke, Wood, Roisen, & Bird, 1983), and they present enzymatic properties similar to those of cathepsins purified from classical target

tissues (Deval, Bechet, Obled, & Ferrara, 1990; Dufour et al., 1987). Muscle cathepsins were identified in various species, including human, rat, mouse, bovine, pig, chicken or fish. Similar enzymatic properties were observed for different muscles, independently of their metabolic and contractile type. Slow-twitch oxidative muscles, however, typically exhibit higher levels of cathepsins than fast-twitch glycolytic muscles. Many non-proteolytic lysosomal hydrolases were also identified in skeletal muscle. They include *N*-acetyl-β-glucosaminidase, β-D-glucuronidase, α- and β-galactosidase, α-*N*-acetyl-galactosaminidase, α- and β-glucosidase, α- and β-mannosidase, α-L-fucosidase and acid phosphatase (Belkhou et al., 1994; Johnson, Hong, & Knights, 1986; Sano et al., 1988).

To circumvent the uncertainty that cathepsins identified in whole extracts from skeletal muscle may originate from non-muscle cells (e.g. fibroblasts, endothelial cells, nerves, or macrophages), cathepsins and other lysosomal hydrolases were characterized in isolated muscle cells. The existence of a lysosomal apparatus with a full complement of acid hydrolases and cathepsins was thus established in muscle cell lines (Kirschke et al., 1983; Tassa, Roux, Attaix, & Bechet, 2003), as well as in primary cultures of fetal muscle cells (Bechet, Listrat, Deval, Ferrara, & Quirke, 1990; Bechet et al., 1991) and of adult human muscle satellite cells (unpublished data).

4.2. Microscopic localization of cathepsins in adult skeletal muscle

The presence of cathepsins and of other lysosomal hydrolases within muscle fibers was further demonstrated using histochemical and immunohistochemical investigations.

Immunohistochemical location of cathepsins was realized in different types of skeletal muscles (Stauber et al., 1985; Taylor, Almond, & Etherington, 1987). Cathepsins B and L immunoreactivities are found inside myofibers. In myofibers, immunoreactive cathepsins B and L are distributed mainly in perinuclear regions beneath the sarcolemma, but are also found diffusely throughout the sarcoplasm. The distribution of cathepsin D was localized by immunohistochemistry in adult human skeletal muscle (Whitaker, Bertorini, & Mendell, 1983) and was found preferentially beneath the sarcolemma in normal muscles.

Antibodies may recognize mature but also inactive pro-forms of hydrolases. Specific synthetic substrates were used to localize active hydrolases by cytochemistry. These studies confirmed that active cathepsin B is located in discrete vesicles in all muscle types, and that the vesicles are more frequently observed at the periphery of the myofiber beneath the sarcolemma (Stauber & Ong, 1981, 1982). Other cytochemical studies similarly located another lysosomal hydrolase, acid phosphatase, in dense bodies along the length of the fiber beneath the sarcolemma, and between the myofilaments (Trout, Stauber, & Schottelius, 1979).

Cytochemistry performed at the electron microscopy level further pointed to the existence of two types of lysosomes containing active cathepsins: one in the perinuclear region in the vicinity of Golgi stacks; and another in the interior of the myofibril contiguous to the sarcoplasmic reticulum (Bird, Spanier, & Schwartz, 1978).

4.3. Cathepsin expression during muscle development

Differentiation of skeletal muscle involves proliferation of myoblasts, withdrawal of myoblasts from the cell cycle, fusion into multinucleated myotubes and the coordinated induction of muscle-specific gene products. Muscle fibers are then produced by further fusion with additional myoblasts and hypertrophy of the myotubes. Different categories (embryonic, fetal and adult) of myoblasts predominate at different stages of muscle development, and each generation of myoblasts produces muscle fibers with distinct properties (Stockdale, 1992). Although the distinct myoblast lineages appear committed to generate fiber diversity, extrinsic influences, such as innervation and hormones together with spatial and temporal cues, all operate to generate and maintain the complex patterns of fiber type in skeletal muscle (Hughes & Blau, 1992). Satellite cells (or adult myoblasts) develop by mid-fetal life and are the only myoblasts present at birth and in adult muscles. These stem cells play a critical role in the regeneration of adult skeletal myofibers.

4.3.1. Myoblast-myotube differentiation

The initial steps of muscle differentiation, fusion of myoblasts into myotubes, can be reproduced in cell culture. Several groups established that isolated muscle

cells do express lysosomal cathepsins and that the specific activities of cathepsins increase during the fusion of fetal myoblasts (Bechet et al., 1991) and of adult satellite cells (Kirschke et al., 1983). The implication of proteolytic enzymes in myoblast-myotube differentiation has been suspected for a long time, and metalloproteinases (Couch & Strittmatter, 1983), cathepsins (Colella, Roisen, & Bird, 1986), proteasomes (Ebisui et al., 1995) and calpains (Dourdin et al., 1997) were successively reported to play a determining role.

Weintraub and coworkers employed retroviral gene traps to systematically identify the genes regulated during satellite muscle cell differentiation (Gogos et al., 1996). Integration of the vector adjacent to an actively transcribed gene places it under the control of the endogenous transcription unit and facilitates its identification. Using this technology, they isolated a myoblast cell line with a unique phenotype consisting of deficiency of myoblast fusion and decreased post-mitotic survival. This phenotype was due to the insertion of the gene-trap upstream of the leader-1b of the cathepsin B gene (Ferrara et al., 1990; Rhaissi, Bechet, & Ferrara, 1993). This phenotype was reproducible in parental myoblasts with antisense cathepsin B expression (Gogos et al., 1996). These observations clearly implicate cathepsin B in the proliferation and differentiation of myoblasts. Latter investigations revealed that cathepsin B is not only increased during myoblast differentiation, but also is secreted by differentiating mouse satellite myoblasts (Jane et al., 2002) or fetal myoblasts (Bechet et al., unpublished), which suggests a role for pericellular/secreted cathepsin B in myoblast fusion.

4.3.2. Skeletal muscle development

Because many of the processes implicated in myogenesis in vivo cannot be mimicked in cell culture, cathepsin expression was further investigated in skeletal muscles at different stages of development. A significant decrease in muscle cathepsin activities, protein and mRNA levels were found between 85 and 200 days of fetal calf development (Bechet et al., 1996). This period of fetal muscle development corresponds to the proliferation and fusion of fetal myoblasts, the generation of secondary myotubes, the formation and alignment of myofibers, and to their organization into bundles. All these phenomena involve a massive mobilization of membrane proteins and reorganization of cytoskeletal components which may require high levels of proteolytic enzymes, including lysosomal endopeptidases. Other investigations also report a decrease in cathepsin expression during the post-natal period (Goldspink & Lewis, 1985; Saunderson & Leslie, 1989).

Collectively, these observations indicate that (i) myogenic cells are committed to express high levels of lysosomal cathepsins, but that extrinsic influences play a primary function during muscle development to hinder expression of these proteinases. Potential candidates for repression of cathepsin expression include hormones, innervation, stretch or interaction with the extracellular matrix. (ii) Cathepsins in skeletal muscle are under developmental control during fetal and/or post-natal growth, which accounts for their low level of expression in adult myofibers. Noteworthy is that skeletal muscles maintain a small proportion of satellite myoblasts. These stem cells synthesize and secrete cathepsins at significant levels which may be important for their role in adult muscle regeneration.

4.4. Expression of muscle cathepsins during pathological conditions

Pronounced muscle wasting is observed in a variety of animal models of human diseases, such as fasting, diabetes, cancer, trauma, sepsis or disuse atrophy, and also occurs as a consequence of perturbations in circulating levels of various hormones. A coordinate stimulation of the lysosomal process with either the ubiquitin-dependent proteasome pathway (Baracos, DeVivo, Hoyle, & Goldberg, 1995; Wing & Goldberg, 1993), with Ca^{2+}-dependent calpains (Combaret et al., 1996), or with both (Mansoor et al., 1996; Taillandier et al., 1996; Voisin et al., 1996) prevails in different models of muscle wasting. However, amongst all endopeptidases implicated in intracellular proteolysis, cathepsin L has recently been recognized as a general marker of muscle atrophy.

4.4.1. Expression of muscle cathepsin L

Deval et al. (2001) used a rat model of infection, reproducing the sustained and reversible catabolic state observed in humans (Voisin et al., 1996), to identify the major differentially expressed transcripts during sepsis. Differential display reverse transcription-PCR lead to the identification of cathepsin L mRNA as over-

expressed in several muscles of septic rats (Deval et al., 2001). Compared to other muscle lysosomal and non-lysosomal endopeptidases, cathepsin L is induced early in catabolic states, correlates with increased protein levels for the mature and pro-enzyme and with enhanced protein breakdown.

An increased level of cathepsin L mRNA also characterizes other models of muscle wasting. Thus, cathepsin L mRNA is up-regulated in skeletal muscle of adult rats treated with dexamethasone (Deval et al., 2001) and appears as the earliest mediator of muscle proteolysis induced by this glucocorticoid analogue (Komamura et al., 2003). Increased cathepsin L mRNA levels are observed in rat soleus muscle following disuse atrophy (Taillandier et al., 1996), and impaired cathepsin L gene expression has recently been associated with type 2 diabetes (Huang et al., 2003). Cathepsin L mRNA is also up-regulated in skeletal muscle of tumor-bearing rats (Deval et al., 2001). Moreover, this increase in muscle cathepsin L mRNA is partly prevented when tumor-bearing animals are treated with pentoxifylline, which inhibits the production of tumor necrosis factor-α (TNF-α). TNF-α is implicated in the signaling of muscle wasting (Buck & Chojkier, 1996), and stimulates the production of interleukin-6 (IL6) which may mediate some of its effects. Transgenic mice overexpressing IL6 undergo muscle atrophy, which is indeed associated with increased muscle cathepsin L activity and mRNA (Tsujinaka et al., 1995).

Recently, transcriptome analyses of mRNA contents further pointed to the systematic induction of cathepsin L mRNA in atrophying muscles. Thus, cathepsin L was found to be overexpressed in muscles from fasted mice (Jagoe, Lecker, Gomes, & Goldberg, 2002), in muscle from rats bearing Yoshida hepatoma, from rats with streptozotocin-induced diabetes mellitus, and from rats with uremia induced by subtotal nephrectomy (Lecker et al., 2004).

4.4.2. *Expression of other muscle cathepsins*

Glucocorticoid-induced muscle wasting (Dardevet et al., 1995) and disuse atrophy of skeletal muscle (Taillandier et al., 1996) were also associated with increased levels of cathepsins B and D mRNAs. Increased cathepsins B and D activities (Bosutti et al., 2002) and cathepsin D mRNA (Mansoor et al., 1996) were reported in muscle biopsies from patients with head trauma. Muscle mRNA levels of cathepsin B were also increased in patients with lung cancer (Jagoe, Redfern, Roberts, Gibson, & Goodship, 2002), and in IL6-transgenic mice muscle (Tsujinaka et al., 1996).

However, increased cathepsins B and D gene expressions are not systematically observed in all models of muscle wasting, and when they are, they appear less pronounced than those of cathepsin L (see for example, Temparis et al., 1994). Variations in cathepsin mRNA levels also are not systematically correlated with similar modifications in lysosomal cathepsin activities (Combaret et al., 2003; Tournu et al., 1998; Tsujinaka et al., 1995). This presumably reflects the multiplicity of the control points during the post-translational sorting of newly synthesized cathepsins to endosomes/lysosomes.

4.5. *Expression of cathepsins in dystrophic muscles*

Marked increases in cathepsin B activities were observed in muscles of dystrophic mice (Sanada, Yasogawa, & Katunuma, 1978; Sano et al., 1988), dystrophic chicken (Noda, Isogai, Katunuma, Tarumoto, & Ohzeki, 1981), and of patients with Duchenne muscular dystrophy (Katunuma et al., 1978). X-chromosome-linked recessive muscular dystrophy (mdx) in mice is also associated with increased activities of cathepsins H and L in muscles (Sano et al., 1988). At early stages of myopathy and before the infiltration of macrophages, some myofibers exhibit increased cathepsin L reaction in subsarcolemmal, perinuclear, and intermyofibrillar regions (Kominami, Kunio, & Katunuma, 1987). At late stages of myopathy, immunoreactive cathepsins (B, L, H) are found in phagocytes infiltrating between and into myofibrils. Intramyofibrillar increase of cathepsins B and L before infiltration of macrophages is also observed in patients with Duchenne muscular dystrophy (Kominami et al., 1987). In Duchenne patients the increase in cathepsin D is prominent in small regenerating fibers and is visualized at the ultrastructural level in lysosome-like organelles (Whitaker et al., 1983).

Distal myopathy with rimmed vacuoles (DMRV) is caused by the partial loss of function of the rate-limiting enzyme in sialic acid biosynthetic pathway (Noguchi et al., 2004). Unlike muscular dystrophy, DMRV is not associated with macrophage infiltration. Activation of the intramyofibrillar lysosomal system also occurs in

patients with DMRV, with increased cathepsins B and H located in vacuoles in subsarcolemmal perinuclear and intermyofibrillar regions (Ii et al., 1986). Therefore, activation of the lysosomal system with increased expression of cathepsins appears as an early response to myofibrillar damage in various types of myopathies.

5. Delivery of substrates to lysosomes

Schematically, lysosomal-dependent degradation of cytoplasmic constituents (autophagy) involves the initial sequestration of protein substrates into the vacuolar system and their subsequent hydrolysis by lysosomal hydrolases. Different pathways (Fig. 2) may be used to deliver intracellular protein substrates to lysosomes: microautophagy, crinophagy, chaperone-mediated autophagy or macroautophagy (Blommaart, Luiken, & Meijer, 1997).

5.1. Microautophagy and crinophagy

During microautophagy, portions of cytoplasm are transferred into the lysosome by direct invagination of the lysosomal membrane and subsequent budding of vesicles into the lysosomal lumen. Microautophagosomes are thus formed inside the lysosome and are rapidly degraded. In vitro reconstitution of microautophagy with yeast vacuoles lead to the observation that the formation of invaginations is the rate-limiting step of the process (Kunz, Schwarz, & Mayer, 2004). During crinophagy, mature secretory vesicles fuse with endosomes or lysosomes. Crinophagy may control the secretion rate of export proteins, including peptide hormones. Neither microautophagy nor crinophagy have been described in skeletal muscle cells, and therefore their importance in muscle proteolysis remains to be established.

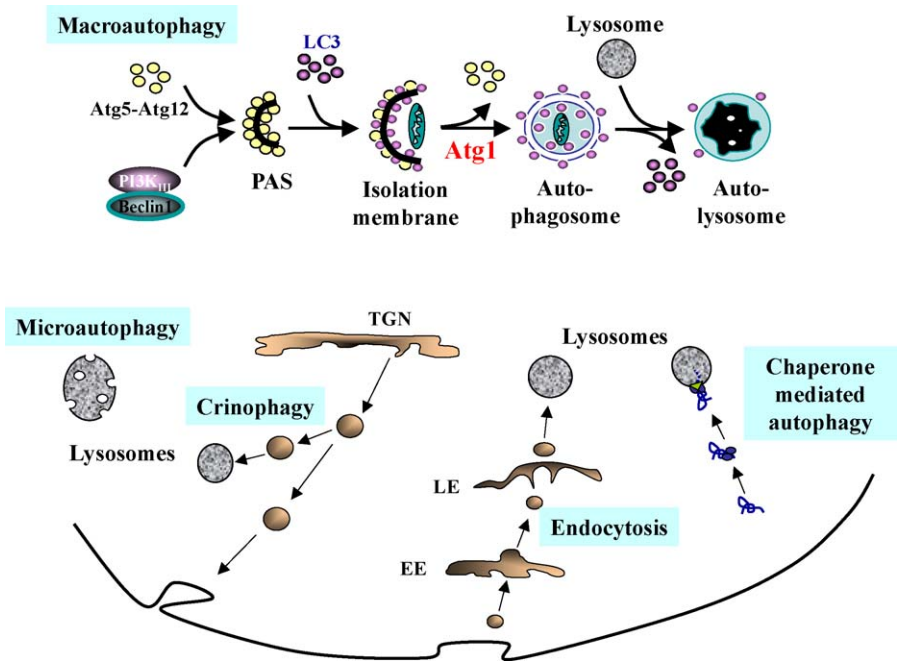

Fig. 2. Schematic representation of the major pathways delivering cellular substrates to lysosomes. Cytosolic proteins may enter the lysosome by microautophagy (invagination of the lysosomal membrane), or by direct transfer through the lysosomal membrane when they contain a KFERQ motif (chaperone-mediated autophagy). Crinophagy targets secreted proteins to lysosomes, while membrane lipids and proteins may reach lysosomes by endocytosis. Macroautophagy engulfs whole portions of cytoplasm together with various organelles (see text for details). In this process, PI3K$_{III}$ complex and Atg12-Atg5 conjugate are probably required for the generation of pre-autophagosomal structure, and lipid-conjugated LC3 is associated with the elongation of isolation membranes. Atg1 complex likely functions for the completion of the autophagosomes, before they fuse with lysosomes.

5.2. Chaperone-mediated autophagy

A selective degradation of cytoplasmic proteins by lysosomes can be achieved in mammalian cells through chaperone-mediated autophagy. The substrates for this selective pathway of lysosomal proteolysis are cytoplasmic proteins bearing sequences related to the pentapeptide Lys-Phe-Glu-Arg-Gln (KFERQ). Among recognized substrates are glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, aldolase, phosphoglucomutase), a transcription factor (c-fos), an inhibitor of nuclear factor (IκB), as well as the proteasome (Cuervo & Dice, 1998). The model for this selective transport to lysosomes involves three major steps: (i) the KFERQ-like sequence is recognized by the heat-shock cognate protein of 73 kDa (hsc73); (ii) the hsc73-substrate protein complex translocates to the lysosomal membrane and binds to its receptor, which was identified as the LAMP2a protein (Cuervo & Dice, 1996); (iii) the protein-substrate is internalized through the lysosomal membrane assisted by an intra-lysosomal isoform of hsc73 (Agarraberes & Dice, 2001).

LAMP proteins include LAMP1 and three alternative spliced products of the same gene, LAMP2a, 2b and 2c. The physiological importance of LAMP2 was supported by the finding that LAMP2 deficiency is the primary defect in human Danon disease (Nishino et al., 2000), the clinical characteristics of which is myopathy and cardiomyopathy with massive accumulation of autophagic vacuoles (Danon et al., 1981). The LAMP2a cytoplasmic tail serves as a receptor for hsc73-substrate protein complex (Cuervo & Dice, 1996), while the LAMP2 lumenal domain acts as a degradation-resistant barrier for the lysosomal membrane (Braulke, 1996). How the defect in muscle lysosomal function relates to these two functions of LAMP2a remains to be determined.

5.3. Macroautophagy

During the macroautophagic process, cytoplasmic constituents, including organelles, are sequestered by a pre-autophagosomal structure (PAS) to generate a double membrane-bound vesicle termed the autophagosome (Mortimore, Poso, & Lardeux, 1989). The autophagosome then fuses with a lysosome to become an autolysosome, where sequestered components are degraded. Time-course studies of macroautophagy indicated that this process is very dynamic. Vacuole formation is attained within 20 min under catabolic conditions, while vacuoles regress exponentially with a half-life of 8 min under anabolic conditions (Mortimore, Miotto, Venerando, & Kadowaki, 1996). In mammalians cells (Ogier-Denis & Codogno, 2003), including differentiated muscle cells (Mordier, Deval, Bechet, Tassa, & Ferrara, 2000; Tassa et al., 2003), lysosomal macroautophagy represents the major proteolytic pathway stimulated in response to nutrient limitation.

5.3.1. Molecular mechanisms of macroautophagy

Genetic screenings in yeast have identified 16 *ATG* genes required for autophagy, and many Atg products have related homologous proteins in mammals (Ohsumi, 2001). Moreover, Atg encoding mRNAs are broadly distributed in human and mouse tissues, and are often abundant in skeletal muscle (Mizushima, Sugita, Yoshimori, & Ohsumi, 1998; Yan et al., 1998). Expression studies revealed that post-translational modifications of Atg proteins are important for macroautophagic sequestration (Mizushima, Ohsumi, & Yoshimori, 2002). These post-translational modifications encompass two ubiquitin-like modification systems, which were also characterized in mammals: (i) protein conjugation of Atg12 to Atg5 (Mizushima et al., 1998), and (ii) conjugation of the microtubule-associated protein light chain 3 (LC3; mammalian Atg8) to membrane phospholipid(s) (Kabeya et al., 2000). Macroautophagic sequestration also involves two other multi-protein components. The first complex contains a protein kinase Atg1 (Kamada et al., 2000), whose potential mammalian homologue is Ulk1 (Yan et al., 1998). The second complex contains class III phosphoinositide 3-kinase ($PI3K_{III}$, or phosphatidylinositol 3-kinase), and Beclin1 (mammalian Atg6) (Kihara, Kabeya, Ohsumi, & Yoshimori, 2001; Kihara, Noda, Ishihara, & Ohsumi, 2001).

The present model for macroautophagic sequestration implicates the following steps: (i) the $PI3K_{III}$ activity is required probably at an early step of sequestration, and possibly for the generation of Atg12-Atg5 associated pre-autophagosomal structure (PAS) (Mizushima et al., 2001; Suzuki et al., 2001); (ii) Atg12-Atg5 conjugate is required for the elongation of the cup-shaped isolation membranes; (iii) Atg12-Atg5 conjugate is

important for the recruitment of lipid-conjugated LC3 to isolation membranes (Mizushima et al., 2001); (iv) Atg12-Atg5 conjugate detaches from the isolation membrane before autophagosome formation is completed; (v) Atg1 complex likely functions at a late step of autophagosome formation (Mizushima et al., 2002), and may be important for the completion of the autophagosome (Suzuki et al., 2001).

Strikingly, most Atg12 and Atg5 pre-exist as conjugated to each other in mammalian cells (Mizushima et al., 2001). In contrast, LC3/Atg8 is substantially induced under starvation conditions, and its lipidation is stimulated by nutrient limitation in various cells (Kabeya et al., 2000). Lipidated-LC3 remains bound to the membrane even after autophagosome formation is completed, and now serves as a marker protein for autophagosomes (Kabeya et al., 2000; Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004). Mammalian cells express two additional LC3/Atg8 homologues, Golgi-associated ATPase enhancer of 16 kDa (GATE-16) and γ-aminobutyric acid receptor-associated protein (GABARAP). Although this remains controversial, the three Atg8 homologues may have redundant functions (Tanida, Ueno, & Kominami, 2004).

5.3.2. Macroautophagy in skeletal myofibers

Cultured myotubes were used to investigate whether macroautophagy occurs in differentiated muscle cells. These studies revealed that this process is the major proteolytic pathway implicated in the amino acid-dependent regulation of proteolysis in myotubes (Mordier et al., 2000). The analysis of the transduction pathways through which amino acid limitation mediates macroautophagy provided further evidence that the stimulation of phosphatidylinositol 3-kinase activity of $PI3K_{III}$-Beclin1/Atg6 complex plays a major role (Tassa et al., 2003). The product of $PI3K_{III}$ (phosphatidylinositol 3-phosphate) is specifically dephosphorylated by the phosphatase myotubularin, whose deficiency is the primary cause of X-linked myotubular myopathy (XLMTM). In this myopathy, myotubularin deficiency strongly alters muscle maintenance (Buj-Bello et al., 2002), but the relative implication of macroautophagy or of other vesicular trafficking pathways remains to be established.

To identify the tissues where macroautophagy occurs in vivo, Mizushima et al. (2004) generated transgenic mice expressing LC3 fused to green fluorescent protein (GFP-LC3). This study emphasized that macroautophagy is regulated differentially in distinct organs, some tissues (thymus, lens) exhibiting constitutive and starvation-insensitive macroautophagy. Strikingly, skeletal muscles revealed a very clear induction of macroautophagy in response to starvation. This response is muscle-type specific, as macroautophagy appears rapidly and intensively in the fast-twitch extensor digitorum longus (EDL) muscle, and is moderate and slow to develop in the slow-twitch soleus muscle. Fluorescent microscopy further indicated that the subcellular distribution of autophagosomes differs among muscles: GFP-LC3 fluorescence is located between myofibrils and in the perinuclear region in EDL, but is limited to the periphery of soleus muscle fibers.

This study with GFP-LC3 mice further revealed that the size of the autophagosomes strongly differs among mouse tissues. Remarkably, skeletal muscle generates only small autophagosomes, even in starved mice, whereas hepatocytes produce large autophagosomes (Mizushima et al., 2004). This observation may explain why little attention has been previously paid to macroautophagy in skeletal muscle.

5.4. Impaired autophagy in skeletal muscle in vivo: myopathies

Direct evidences for impaired lysosomal functions in skeletal muscle have been obtained in various myopathies.

5.4.1. Chloroquine myopathy

Chloroquine is an anti-malaria drug and hydroxychloroquine is commonly prescribed for treatment of inflammatory arthritis. Chronic intoxication with chloroquine generates myopathies that may be associated with peripheral neuropathy and cardiac myotoxicity (Oyama et al., 1998). Muscle biopsies consistently reveal muscle fiber atrophy, with accumulation of rimmed-vacuoles containing acid phosphatase, ubiquitin and deposits of β-amyloid protein precursor or tau protein.

Chloroquine myopathy is directly related to perturbations of lysosomal function, as this alkylamine neutralizes lysosomal acidity and thereby inhibits some lysosomal hydrolases (Seglen, 1983). Recent immunohistochemical studies (Suzuki et al., 2002) show

the co-localization of LC3 with autophagosomes in chloroquine myopathy and in another myopathy with rimmed vacuole (DMRV), suggesting increased muscle macroautophagy in these diseases.

5.4.2. Pompe disease

Lysosomal storage diseases are inborn metabolic disorders characterized by the accumulation of non-metabolized material inside lysosomes. Skeletal muscle is primarily affected in one of the most frequently occurring lysosomal storage disease: Pompe disease (or "lysosomal glycogen storage disease type II"). Pompe disease is caused by a deficiency of lysosomal 1–4 α-glucosidase (or acid maltase), which results in the accumulation of glycogen inside lysosomes. Hepatocytes, like muscle cells, contain high levels of cytoplasmic glycogen that also accumulate in hepatic lysosomes. However, patients suffer from muscular insufficiency rather than liver malfunction in Pompe disease. Even in early stages of the disease, lysosome volume substantially increases between myofibers. Ultimately swollen lysosomes interrupt the myofibers and diminish the force generating capacity of the contractile machinery (Hesselink, Wagenmakers, Drost, & Van der Vusses, 2003).

5.4.3. Danon disease

Danon disease is an X-linked "lysosomal glycogen storage disease with normal acid maltase". Its clinical characteristics are myopathy, cardiomyopathy and variable mental retardation (Danon et al., 1981). Histochemical and electron microscopic features of patients with Danon disease mimic those of Pompe disease but with normal acid maltase activity. A deficiency of lysosomal membrane lamp2 is the primary cause of human Danon disease (Nishino et al., 2000). The phenotypic alterations in lamp2 knockout mice go beyond this pathology, as they encompass autophagic lesions in non-muscular tissues (Tanaka et al., 2000). The pathological hallmark of Danon disease is the accumulation of autophagic vacuoles containing cytoplasmic debris or sarcoplasmic membranes. They are most prominent in skeletal muscles and ultimately lead to myofiber splitting and degeneration.

5.4.4. Myopathies with excessive autophagy

X-linked myopathy with excessive autophagy (XMEA) is characterized clinically by progressive muscle weakness in lower limbs and histopathologically by vacuolation of myofibers (Villard et al., 2000). The vacuoles membranes in XMEA immunostain for dystrophin and laminin, suggesting invagination of the sarcolemma. XMEA vacuoles contain lysosomal enzymes and cellular debris, and appear to exocytose the debris. XMEA is associated with intense deposition of the complement C5b-9 membrane attack complex over the muscle fiber surface, suggesting complement-mediated damage.

Vacuolar membranes with sarcolemmal features similar to vacuoles in Danon disease and XMEA were also observed in other forms of myopathies, including infantile autophagic vacuolar myopathy (Yamamoto et al., 2001), and adult-onset autophagic vacuolar myopathy (Kaneda et al., 2003). The protein deficiencies accounting for myopathies with excessive autophagy and for autophagic vacuolar myopathies remain to be specified. The identification of the primary cause of these myopathies will undoubtly provide critical information for our understanding of macroautophagy in skeletal muscle.

6. Concluding remarks

The lysosomal cathepsins are strongly expressed in fetal muscles, are implicated in myoblast proliferation and differentiation, and likely contribute to the regenerative potential of adult satellite cells. Lysosomal cathepsins are under developmental control and are expressed at low levels in adult myofibers. Adult muscles nevertheless reveal the presence of lysosomal vesicles containing immunoreactive and enzymatically active cathepsins inside myofibers, in perinuclear regions beneath the sarcolemma and also between the myofilaments. Moreover, recent differential display technology and transcriptome analyses point to the overexpression of markers of lysosomal proteolysis in various models of muscle atrophy.

Immunofluorescent localization of GFP-LC3 in skeletal muscles further demonstrates the occurrence of autophagosomes in the perinuclear region and between myofibrils. Noteworthy is that skeletal muscle only generates small autophagomes, which certainly accounts for previous difficulties in identifying these structures in this tissue. A pathological hallmark of several myopathies is the accumulation of vacuoles inside

myofibers. The etiology of many of these myopathies remains unknown. However, chloroquine intoxication, Pompe and Danon diseases indicate that myopathies can result from mis-regulation of lysosomal acidity, deficiencies of lysosomal membrane protective protein or of lysosomal hydrolase. Strikingly, these deficiencies will target lysosomes in every tissue, but nevertheless they preferentially generate myopathies. These observations suggest that the functions of lysosomes and autophagosomes may have been underestimated in the adult skeletal muscle.

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