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Review

Ca^{2+}-dependent proteolysis in muscle wasting

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Abstract

Skeletal muscle wasting is a prominent feature of cachexia, a complex systemic syndrome that frequently complicates chronic diseases such as inflammatory and autoimmune disorders, cancer and AIDS. Muscle wasting may also develop as a manifestation of primary or neurogenic muscular disorders. It is now generally accepted that muscle depletion mainly arises from increased protein catabolism. The ubiquitin–proteasome system is believed to be the major proteolytic machinery in charge of such protein breakdown, yet there is evidence suggesting that Ca^{2+}-dependent system, lysosomes and, in some conditions at least, even caspases are involved as well.

The role of Ca^{2+}-dependent proteolysis in skeletal muscle wasting is reviewed in the present paper. This system relies on the activity of calpains, a family of Ca^{2+}-dependent cysteine proteases, whose regulation is complex and not completely elucidated. Modulations of Ca^{2+}-dependent proteolysis have been associated with muscle protein depletion in various pathological contexts and particularly with muscle dystrophies. Calpains can only perform a limited proteolysis of their substrates, however they may play a critical role in initiating the breakdown of myofibrillar protein, by releasing molecules that become suitable for further degradation by proteasomes. Some evidence would also support a role for lysosomes and caspases in muscle wasting. Thus it cannot be excluded that different intracellular proteolytic systems may coordinately concur in shifting muscle protein turnover towards excess catabolism.

Many different signals have been proposed as potentially involved in triggering the enhanced protein breakdown that underlies muscle wasting. How they are transduced to initiate the hypercatabolic response and to activate the proteolytic pathways remains largely unknown, however.

Keywords: Muscle wasting; Calpains; Cachexia; Proteolysis; Protein turnover

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Contents

1. Protein degradation in the skeletal muscle

Atrophy of the skeletal muscle may be the result of local factors or of systemic effects. On one hand, it may depend on molecular alterations that directly affect this tissue or reflect disturbances in its nervous supply, such as occurring in muscular dystrophies, neurological myopathies, disuse and denervation. On the other hand, atrophy of the skeletal muscle (not a synonym for cachexia) may occur as a significant component of an organismal wasting syndrome (cachexia), such as observed in AIDS, cancer, chronic obstructive pulmonary disease, or autoimmune disorders. The wasting syndrome complicates the management of patients, impairs recovery, and decreases the effectiveness of therapeutic regimens. Skeletal muscle depletion significantly concur to reduce life quality and expectancy because both the contractile and the protein reserve function of the tissue are compromised.

The skeletal muscle protein mass depends on the balance between synthesis and degradation. Although modulations of both sides of turnover eventually converge to produce a new steady-state, physiological increases of muscle protein mass appear primarily associated with modulations of protein synthesis, which responds earlier than degradation to the inducing stimuli. By contrast, in many situations characterized by muscle atrophy enhanced protein breakdown seems primarily responsible for muscle protein depletion. Bulk cell protein degradation is a first order process described by a fractional rate constant, implying that, under a given set of regulations, the fraction of proteins degraded is not affected by the size of the protein pool. As a consequence, if the degradation rate constant persistently exceeds the level required to maintain a steady-state, protein loss will occur irrespectively of the protein synthesis rate (cf. Costelli & Baccino, 2003). Noteworthily, protein loss may affect the skeletal muscle compartment in itself (primary muscular or neurogenic disorders) or, in part at least, may reflect its role as main protein reserve of the organism (wasting syndromes). In some circumstances, the mobilization of muscle proteins may indeed serve the purpose of providing substrates for energy production and gluconeogenesis, synthesis of acute phase reactants or other anabolic processes. In other instances, however, muscle protein depletion appears basically purposeless, in part at least, and it is obviously so if the catabolic state persists when excess nutrients are supplied by parenteral feeding (Viganò & Bruera, 1996). Of potential interest, in this regard, is the report that muscle hypertrophy induced in mice by overexpression of the *c-ski* oncogene does not provide protection against wasting induced by the Lewis lung carcinoma (Carbó et al., 2004).

The view that in wasting syndromes muscle atrophy mainly depends on enhanced protein degradation is widely accepted at present, but the mechanisms by which factors such as classical hormones, cytokines, and nutritional status converge to generate this hypercatabolic response and to activate muscle proteolysis are largely undefined. Moreover, the contribution of the different intracellular proteolytic pathways to the enhanced breakdown rates is still debated.

Four major proteolytic systems exist in muscles. The lysosomal and the proteasomal systems can operate an exhaustive degradation of cell proteins, into amino acids or small peptides, while the Ca^{2+}-dependent and the caspase systems can only perform a limited proteolysis, due to their restricted specificity.

The endosome–lysosome system relies on the activity of acidic proteases, is relatively non selective and is mostly involved in the degradation of long-lived proteins (reviewed in Kadowaki & Kanazawa, 2003). Recently it has been reported that autophagy is

induced in the skeletal muscle by 24 h nutrient starvation (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004). Moreover, autophagins, a class of cysteine proteases putatively involved in the formation of autophagosomes, are particularly abundant in the skeletal muscle (Marino et al., 2003). The contribution of the lysosomal pathway to muscle protein hypercatabolism in wasting disorders is still unclear. Results obtained incubating muscles isolated from cachectic animals led to rule out a substantial role for lysosomal proteolysis in overall protein degradation (Baracos, DeVivo, Hoyle, & Goldberg, 1995; Llovera, Garcia-Martinez, Agell, Lopez-Soriano, & Argiles, 1995; Temparis et al., 1994). On the other hand, an elevation of total lysosomal protease activities has been observed in muscles and liver of tumor-bearing rats (Greenbaum & Sutherland, 1983; Lundholm, Ekman, Karlberg, Edstrom, & Schersten, 1980; Tessitore, Costelli, Bonetti, & Baccino, 1993). Furthermore, increases of cathepsin L mRNA in the skeletal muscle of septic or tumor-bearing rats and of cathepsin B mRNA in muscle biopsies from lung cancer patients have been reported (Deval et al., 2001; Jagoe, Redfern, Roberts, Gibson, & Goodship, 2002). In addition, leupeptin, an inhibitor of cysteine proteases, has revealed at least partially effective in preventing the onset of muscle wasting in experimental cancer cachexia and sepsis (Ruff & Secrist, 1984; Tessitore, Costelli, & Baccino, 1994).

The ubiquitin–proteasome system is believed to have a major part in muscle wasting-related protein breakdown. The expression of genes pertaining to this system and the amount of ubiquitin–protein conjugates are increased in atrophying muscles in conditions such as sepsis, denervation, AIDS, diabetes, and cancer (reviewed in Costelli & Baccino, 2003). Recently, the identification of muscle-specific components of the so-called SCF ubiquitin–ligase complexes has improved our knowledge on the regulation of the ubiquitin–proteasome system in the skeletal muscle (Bodine et al., 2001a; Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). This complex is involved in targeting proteins for proteasomal degradation. It is formed by two molecules, SKP-1 (S) and Cullin-1 (C), that may be associated with a large series of F-box subunits (F), responsible for substrate specificity (Kipreos & Pagano, 2000). A muscle-specific F-box protein, FBX32 (also known as MAFbx/atrogin-1), has been shown to be upregulated in different models of muscle atrophy (Lecker et al., 2004), likely as a consequence of reduced signaling through the insulin/IGF-1 anabolic pathways (Sandri et al., 2004; Stitt et al., 2004). The relevance of this protein to muscle wasting is also supported by the observation that knock-out mice are resistant to muscle wasting induced by denervation or unloading (Bodine et al., 2001b).

Caspases are a family of cysteine proteases mostly known for their role in the execution of the apoptotic process. The possibility that muscle protein depletion may also be a consequence of apoptotic events has been suggested by the observation that muscle atrophy occurring in heart failure or in Duchenne muscular dystrophy is associated with a reduction in the number of myonuclei (Adams, Gielen, Hambrecht, & Schuler, 2001; Sandri, 2002). Moreover, recombinant caspase-3 has been recently shown to cleave actomyosin complexes and caspase-3 inhibitors can prevent the accumulation of actin fragments in the skeletal muscles of diabetic or uremic rats, suggesting that activation of caspase-3 may be an initial step in enhanced muscle protein degradation (Du et al., 2004).

Finally, the role of the calpain system in the degradation of skeletal muscle protein and in muscle wasting is the main object of the present review and is discussed in the next sections. The reader should be warned that some important issues concerning the calpain system, such as most molecular details or the calpain action on transcription factors, have been largely or totally omitted. This choice was intentional in view of the scope of the present discussion. To fill the gaps, the reader is referred to the various available reviews (particularly: Goll, Thompson, Li, Wei, & Cong, 2003).

2. The Ca^{2+}-dependent proteolytic system

Ca^{2+}-dependent proteolysis relies on the activity of cysteine proteases known as calpains (calcium-activated neutral protease, E.C. 3.4.22.17), firstly reported by Guroff (1964). Although most studies have been focused on two particular isoforms, the μ- and m-calpain, respectively activated in vitro by μM and mM Ca^{2+} concentrations, at least 15 different calpains have been identified in mammals to date. Not all the calpain family members have been fully characterized yet, and some of them seem to lack residues necessary for the proteolytic activity (reviewed in Suzuki, Hata,

Kawabata, & Sorimachi, 2004). Calpains are generally expressed ubiquitously, but a few of them are mainly detectable in particular tissues, such as the muscle-specific calpain 3, also known as p94 calpain (Huang & Wang, 2001). The μ- and m-calpains, that share a 50–60% homology, exist as heterodimers formed by two subunits of about 80 and 30 kDa, respectively. The former contains the catalytic domain, the latter has regulatory functions (Carafoli & Molinari, 1998). Four domains have been identified in both μ- and m-calpain: (I) the N-terminal domain, (II) a domain containing a sequence characteristic of cysteine proteases, (III) a connecting domain, and (IV) a Ca^{2+}-binding domain (Ohno et al., 1984). The structure of m-calpain has been defined more precisely by crystallographic analysis, showing that it contains three different Ca^{2+}-binding sites, two calmodulin-like domains, and the catalytic domain (Hosfield, Elce, Davies, & Jia, 1999). It has been proposed that dissociation of the two subunits allows the catalytically active 80 kDa molecule to exert its functions (Pal, Elce, & Jia, 2001; Yoshizawa, Sorimachi, Tomioka, Ishiura, & Suzuki, 1995).

Calpains are normally present in the cytosolic compartment in an inactive state. When the intracellular Ca^{2+} concentration rises, inactive calpain molecules translocate to the plasmamembrane where they are activated by Ca^{2+} and phospholipids, resulting in dissociation of the dimers and release of the 80 kDa subunit (Suzuki, Sorimachi, Yoshizawa, Kinbara, & Ishiura, 1995). Ca^{2+}-binding has been suggested to induce conformational changes that abolish structural constraints on the catalytic domain and allow assembly of the active site (Alexa, Bozoky, Farkas, Tompa, & Friedrich, 2004; Strobl et al., 2000). However, there is wide consensus on the observation that purified calpains show low affinity for Ca^{2+}, the concentrations needed for half-maximal activity being in the range 2–75 μM for μ-calpain and 0.2–1 mM for m-calpain, too high to occur in living cells. It has been suggested that inactive calpains have to enhance their affinity for Ca^{2+} in order to become active. Factors such as phosphatidylinositides, a putative calpain activator protein, or the proximity to Ca^{2+} channels have been suggested to contribute to lower their Ca^{2+} requirements (Arthur & Crawford, 1996; Melloni, Michetti, Salamino, & Pontremoli, 1998). According to another view, calpains are regulatory enzymes, only a small fraction of which is activated at any given time point so that their actual Ca^{2+} requirement would be significantly lower than that needed for half-maximal activation (Friederich, 2004). In fact, calpains possess several Ca^{2+}-binding sites that concur differently to the transition from the inactive to the active conformation. Two of them have been proposed to play a major role, while saturation of the other sites is required for maximal activation. In addition, Ca^{2+} ions bind to calpains in a cooperative manner and lack of cooperativity significantly increases the Ca^{2+} requirement (Moldoveanu, Jia, & Davies, 2004). Besides Ca^{2+}, other factors contribute to regulate calpain such as phosphorylation or partial autolytic cleavage of domain I (reviewed in Averna, De Tullio, Salamino, Melloni, & Pontremoli, 1999; Carafoli & Molinari, 1998; Melloni, Minafra, Salamino, & Pontremoli, 2000). Phosphorylation, in particular, can result in enhanced or reduced activity depending on the kinase involved (Glading, Chang, Lauffenburger, & Wells, 2000; Shiraha, Glading, Chou, Jia, & Wells, 2002). Last but not least, the activity of the system is regulated by calpastatin, a physiological calpain-specific inhibitor. Other regulatory factors, such as the pro-apoptotic factor Gas2, may play a role as well (reviewed in Carafoli & Molinari, 1998; Suzuki et al., 2004).

The physiological significance of the Ca^{2+}-dependent proteolytic system still is largely unknown. As already noted, calpains only effect a limited proteolysis on their substrates, resulting in irreversible modifications that lead to activity changes or to degradation by other proteolytic systems (cf. Saido, Sorimachi, & Suzuki, 1994; Williams et al., 1999). A number of proteins, among which protein kinase C, Cdk5, Ca^{2+}/calmodulin-dependent protein kinase IV, and calcineurin have been proposed as in vivo calpain substrates (reviewed in Nixon, 2003). Mice knocked out for the gene encoding the μ-calpain 80 kDa subunit have been developed, while the lack of m-calpain 80 or 30 kDa subunits results in death of the embryos (Arthur, Elce, Hegadorn, Williams, & Greer, 2000), suggesting that the m-isoform is necessary for embryonal development. Calpains have been involved in processes such as cell proliferation, differentiation, migration, apoptotic death, and gene expression (Carafoli & Molinari, 1998; Nixon, 2003; Suzuki et al., 2004). It has been observed that both intracellular Ca^{2+} and calpain activity increase during ageing (Averna et al., 2001; Blalock, Porter, & Landfield, 1999; Romero, Salas,

& Hernandez, 2002). The enhanced activity may be independent of changes in calpain protein levels and rather associated with age-related modifications of substrates (Manya et al., 2002; Saito et al., 2003). Increased calpain activity has been suggested to contribute to age-related processes such as cataract formation, reduction of the major transmembrane anion-transport protein in erythrocytes, and enhanced spectrin breakdown in fibroblasts (Azuma & Shearer, 1992; Peterson & Goldman, 1986; Schwarz-Benmeir, Glaser, Barnoy, & Kosower, 1991).

3. Physiological role of calpains in skeletal muscles

Six different calpains are expressed as mRNA in the mammalian skeletal muscle, yet only m- and μ-calpains and, with difficulties due to its rapid turnover, the p94 isoform can be detected at the protein level (Sorimachi & Suzuki, 2001; Spencer, Croall, & Tidball, 1995). Several studies have stressed the role played by calpains in myogenesis. As an example, the cleavage of cytoskeletal proteins by m-calpain is likely involved in cell fusion during myogenesis (reviewed by Cottin et al., 2000). In addition, m-calpain expression has been found to increase during the recruitment of satellite cells into the cell cycle, while their entry into the S phase can be prevented by inhibiting calpain activity (Raynaud, Carnac, Marcilhac, & Benyamin, 2004). Overexpression of μ-calpain in myoblasts results in decreased levels of proteins such as caveolin, vinculin and myogenin, suggesting that this isoform is involved as well in the regulation of the myogenic process (Moyen et al., 2004). Moreover, C_2C_{12} myoblasts overexpressing calpastatin are unable to migrate in order to achieve the alignment that precedes fusion into myotubes (Dedieu et al., 2004). Whether these observations can also apply to the regenerative processes that might occur in muscles in the course of wasting syndromes is an open question.

An involvement of Ca^{2+}-dependent proteolysis in bulk muscle protein degradation was suggested by the observation that exposing incubated muscles to elevated extracellular calcium concentrations or to the calcium ionophore A23187 resulted in increased protein degradation rates (Baracos, Greenberg, & Goldberg, 1986; Furuno & Goldberg, 1986). At present, this involvement is supported by a number of observations. Nevertheless, to assess whether and to what extent calpain is active in vivo is not an easy task because of various methodological difficulties. Studies performed on isolated muscle preparations have shown that the release of free tyrosine, assumed as an indicator of overall protein degradation rates, can be partially blocked by inhibitors of the Ca^{2+}-dependent proteolytic system (Taillandier et al., 1996; Voisin et al., 1996). These results must be considered with caution, however, since most of the inhibitors used are not specific and may also block lysosomal proteases and/or the proteasome (reviewed in Wang & Yuen, 1997). Other studies have reported increased calpain protein or mRNA levels in the skeletal muscle (Llovera et al., 1995; Medina, Wing, & Goldberg, 1995), yet these data provide no information about the degree of activation of the system. Besides, since less than 10% of total calpain is normally activated in the skeletal muscle (see Goll et al., 2003), an increased expression of calpain per se is unlikely to make a significant difference in activity (this limitation does not necessarily apply to cells or tissues engineered to overexpress calpains or calpastatin wherein the changes in the amount of enzymes or inhibitor can be quite higher). In fact, as discussed above, the regulation of calpain activity mainly relies on factors such as Ca^{2+} concentration, enzyme phosphorylation or calpastatin levels.

The enzyme activity of calpains has been evaluated from the cleavage of exogenous substrates in vitro (Arthur, Booker, &, Belcastro, 1999; Belcastro, 1993; Reid & Belcastro, 2000) or of endogenous substrates in vivo (Carafoli & Molinari, 1998; Nath et al., 1996; Salamino, De Tullio, Mengotti, Melloni, & Pontremoli, 1994), including calpain itself (Spencer, Lu, & Tidball, 1997; Spencer et al., 1995). Unfortunately, even these approaches suffer from limitations. On one hand, the cleavage of exogenous substrates is generally measured at high Ca^{2+} concentrations, not representative of those occurring in vivo. On the other, specific cleavage of endogenous substrates is a sign of calpain activity, but not of actual activation nor of its degree. Interventions at the gene level to suppress calpain expression or to enhance calpastatin levels or activity have provided more direct information. Thus expression of a dominant-negative m-calpain or of the calpastatin inhibitory domain in rat L8 myoblasts resulted in significant reduction of protein degradation (Huang

& Forsberg, 1998; Purintrapiban, Wang, & Forsberg, 2003). Calpastatin-overexpressing mice have been recently generated, providing a model system in which calpains are specifically inhibited in vivo (Spencer & Mellgren, 2002; Tidball & Spencer, 2002). These mice show a marked muscle hypertrophy (Otani et al., 2004), suggesting that calpains somehow concur to the physiological maintenance of the skeletal muscle mass.

The complex molecular architecture of the striated muscle fibers poses remarkable constraints on the activity of the proteolytic systems on myofibrillar proteins. How the degradative enzymes can come into contact with protein substrates that are mostly organized in highly ordered supramolecular structures is not a secondary issue. In addition, as noted above, calpains can only affect a limited proteolysis of their substrates and, differently from the lysosomal and the proteasomal systems, basically exert a regulatory rather than a digestive function. In fact, evidence has been accumulating that calpains are necessary to initiate the degradation of myofibrillar proteins, while they are not involved at all in the bulk degradation of sarcoplasmic or sarcolemmal proteins (see Goll et al., 2003).

A two-step model has thus been proposed to account for the degradation of myofibrillar proteins and its enhancement in wasting situations (Goll et al., 2003; Hasselgren, Wray, & Mammen, 2002). In the first step, calpains are involved to sever thick and thin filaments from myofibrils, thereby permitting the subsequent step in which suitable protein substrates are allowed to gain access to the degradative machinery. Calpains have indeed been shown to exert their action on several sarcomeric proteins such as nebulin, titin and desmin, which are involved in the correct assembly of myofilaments and Z-disks or in the attachment of Z-disks to the sarcolemma. Cleavage of calpain substrates leads to dismantlement of the Z-disk, a structure that anchors and organizes myofilaments, mechanically links actin from one sarcomere to the next one along the myofibril and cross-links actin filaments by means of α-actinin bridges (Squire, 1997). The anchorage of actin to the Z-disk likely depends on the actin-capping protein CapZ, that contributes to prevent actin filament depolymerization (Maun, Speicher, DiNubile, & Southwick, 1996), while myosin filaments are anchored to the Z-disk by titin (Labeit, Kolmerer, & Linke, 1997). In this regard, the observed distribution of calpains and calpastatin in close proximity of the Z-disk is likely to have a precise functional significance (Kumamoto et al., 1992). For the subsequent breakdown of the myofibrillar proteins, once released from the sarcomeric structures, the main candidate is the ubiquitin–proteasome system. This is generally also held responsible for the enhanced protein breakdown in different situations of muscle wasting. Interestingly, even the 20 S proteasomes have been reported to have a sarcomeric distribution in striated muscle cells (Foucrier et al., 2001).

Therefore, the two-step model purports that Ca^{2+}-dependent proteolysis is responsible for the release of myofibrillar proteins, producing substrates suitable for degradation by the proteasome. A two-fold limitation is thus overcome, namely, that calpains do not afford extensive proteolysis and that proteasomes do not degrade whole myofibrils (reviewed in Goll et al., 2003; Hasselgren & Fisher, 2001). As already noted, caspases might also play a role in releasing protein substrates from myofibrils (Du et al., 2004). Whether and to what extent lysosomal mechanisms may also concur to the degradation of sarcomeric proteins is not clear at present.

4. Ca^{2+}-dependent proteolysis in muscle wasting

In general, calpains are believed to play a major role in enhancing muscle protein degradation only when cytosolic Ca^{2+} homeostasis is perturbed (Anderson et al., 1998; Tidball & Spencer, 2000). Several reports have shown that muscle wasting in muscular dystrophies, inclusion body myositis, denervation atrophy, burn injury and sepsis indeed is associated with increased intracellular Ca^{2+} levels (Christensen, Shtifman, Allen, Lopez, & Querfurth, 2004; Fischer et al., 2001; Joffe, Savage, & Isaacs, 1981; Tidball & Spencer, 2000; Turinsky, Gonnerman, & Loose, 1981). Moreover, the enhanced proteolysis in experimental models of muscle wasting such as sepsis and hindlimb immobilization has been suppressed with drugs that restore normal intracellular Ca^{2+} concentrations (Wagatsuma, Fujimoto, & Yamada, 2002; Williams et al., 1999). An important group of muscular dystrophies are due to loss-of-function mutations of dystrophin, a molecule that bridges cytoskeletal proteins with the extracellular matrix. Dystrophin defects are thought to result in weakening of the sarcolemma and leakage of extracellular Ca^{2+}

into the myofiber. Consistently, a significant intracellular Ca^{2+} elevation occurs in muscles of the *mdx* mouse, the most widely used experimental model of Duchenne type dystrophy (Hopf, Turner, Denetclaw, Reddy, & Steinhardt, 1996). The involvement of calpains in its pathogenesis has been further suggested by the effectiveness of leupeptin in suppressing the development of the dystrophic phenotype in *mdx* mice (Badalamente & Stracher, 2000). Although the latter inhibitor is by no means specific for calpain, this view has received strong support from the observation that muscle wasting in *mdx* mice is prevented by overexpression of calpastatin (Spencer & Mellgren, 2002). Moreover, also proinflammatory cytokines, which are frequently elevated in pathological states characterized by muscle wasting (Bick et al., 1997; Furukawa & Mattson, 1998; Grounds & Torrisi, 2004), can cause increases of intracellular Ca^{2+} in skeletal muscles, but again the extent of these increases per se can hardly account for an enhancement in calpain activity. To get round this discrepancy, Goll et al. (2003) have recently made the suggestion that elevations of intracellular Ca^{2+} could alter calpain activity through processes distinct from the classical direct regulation, though still unknown.

A different mechanism operates in limb girdle muscular dystrophy (LGMD) type 2A. This disease is caused by inactivating mutations of p94 calpain (Richard et al., 1995), an isoform that is protected from autoproteolysis by binding to connectin, and that neither is susceptible to inhibition by calpastatin, nor binds the 30 kDa regulatory subunit (Sorimachi, Ishiura, & Suzuki, 1993; Sorimachi et al., 1995). Binding of this calpain to muscle proteins has been proposed to play a protective role against their breakdown by ubiquitous calpains; such a hypothesis relies on the observation that p94-bound connectin is spared from degradation (Kinbara, Sorimachi, Ishiura, & Suzuki, 1998; Spencer et al., 2002). The capability to cleave m- and μ-calpains as well as calpastatin would further qualify p94 calpain for a regulatory role on Ca^{2+}-dependent proteolysis, although the existence of a cross-talk among these molecules has not yet been demonstrated (Ono et al., 2004).

Muscular dystrophies apart, alterations or even loss of the Z-disks and increased release of myofibrillar proteins in skeletal muscles have been reported in situations such as sepsis, starvation, cancer, and glucocorticoid administration (Acharyya et al., 2004; Dahlmann, Rutschmann, & Reinauer, 1986; Williams et al., 1999). In all cases, muscle depletion mainly depends on enhanced protein degradation and is associated with increased proteasome activity or higher expression of proteasome subunits (reviewed in Costelli & Baccino, 2003). In addition, various reports have shown that the calpain system is affected as well, at least in cancer and sepsis (see Goll et al., 2003; Hasselgren & Fisher, 2001). In the latter situation, calpain activation is accounted for by reduced calpastatin activity (Wei et al., 2005). Of special interest is the observation that treatment of septic rats with dantrolene, an inhibitor of the mobilization of Ca^{2+} from sarcoplasmic reticulum stores, prevents the release of myofibrillar proteins in skeletal muscles, implying that this release and, likewise, the disruption of the Z-disks are Ca^{2+}-dependent processes (Williams et al., 1999; see also Goll et al., 2003; Hasselgren & Fisher, 2001). Noteworthily, inhibition of calpain activity in muscle preparations isolated from animals with various models of muscle atrophy has often failed to suppress protein hypercatabolism (Baracos et al., 1995; Furuno, Goodman, & Goldberg, 1990; Llovera et al., 1995; Temparis et al., 1994). By themselves, however, calpains do not contribute significantly to the release of free amino acids (or equivalent degradation end products) when this is the parameter used to measure overall protein breakdown.

Increased m-calpain expression has been detected in the skeletal muscles of rats bearing the AH-130 sarcoma (Temparis et al., 1994). More recently, rats transplanted with the Yoshida AH-130 ascites hepatoma were found to have a significantly reduced expression of the p94 calpain isoform (Busquets et al., 2000), which, as discussed above, may act as a negative regulator of Ca^{2+}-dependent proteolysis. In addition, we have provided two kinds of independent evidence that muscle depletion in rats bearing the AH-130 hepatoma is associated with increased calpain activity. The first was the progressive decline after tumor transplantation in the level of two muscle proteins that are known calpain substrates, namely, calpastatin and the 130 kDa Ca^{2+}-ATPase, which is suggestive for a sustained activation of the calpain system (Costelli, De Tullio, Baccino, & Melloni, 2001). Secondly, the increased cleavage in vitro of specific fluorogenic substrates assayed in the absence of exogenous Ca^{2+} in the incubation medium (Ruiz-Vela, Gonzales de Buitrago, & Martinez, 1999)

also pointed to an activation of calpain (Costelli et al., 2002). Consistent with this possibility is our earlier report that muscle depletion in the AH-130 hosts can be suppressed by treatment with the cysteine protease inhibitor, leupeptin (Tessitore et al., 1994).

5. Perspectives and concluding remarks

Altogether, the data reviewed in the present discussion are largely consistent with the view that excess protein degradation in muscle wasting disorders may proceed according to the two-step model mentioned above and, more specifically, that Ca^{2+}-dependent proteolysis plays a significant role in its production (Fig. 1). In particular, this view is supported by results such as our own data on a model of cancer cachexia, the observation that mice overexpressing calpastatin in skeletal muscles exhibit a marked resistance to muscle wasting, the protection afforded by dantrolene from the wasting associated with sepsis, or the recent report that cultured myotubes exposed to the calcium ionophore A23187 develop an enhanced proteasome activity via, at least in part, a calpain-dependent pathway (Menconi, Wei, Yang, Wray, & Hasselgren, 2004; Tidball & Spencer, 2002; Williams et al., 1999). Whether and to what extent caspase activities and lysosomal proteolysis may also concur to the enhanced muscle protein catabolism, respectively in the initiating and in the degradative phase predicted by the above two-step model, is not known at present.

How precisely the relevant stimuli are transduced to the calpain system and its activity is regulated in the skeletal muscle, both physiologically and in pathological situations, still remains elusive. A number of factors are potentially involved, including phosphorylation/dephosphorylation, changes in calpain and/or calpastatin levels and in their ratio, altered Ca^{2+} requirements of these components, perturbed intracellular Ca^{2+} homeostasis. The latter is the factor most often taken into consideration, however tricky may be to assess its role in vivo. In this regard, it is worth noting that proinflammatory cytokines such as TNFα and IL-1 (reviewed in Argilés & Lopez-Soriano, 1998; Costelli

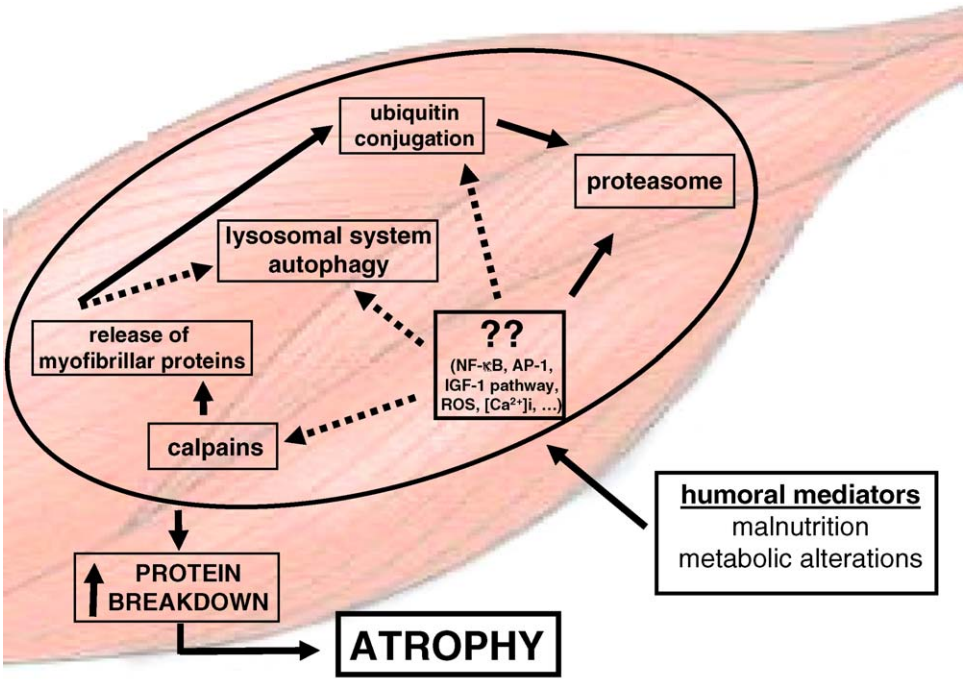

Fig. 1. Scheme of the interplay that may take place among the different proteolytic pathways in the development of muscle wasting. Extracellular signals evoke intracellular pathways, most of which still unknown (discontinuous arrows), leading to activation of the different proteolytic systems, in series or in parallel, ultimately resulting in enhanced protein breakdown and muscle atrophy.

& Baccino, 2000, 2003), which are believed to play a causative role in muscle wasting in many situations, have been shown to elicit increases of intracellular Ca^{2+} in the skeletal muscle. TNFα is an important mediator of cachexia in rats bearing the Yoshida ascites hepatoma: the elevation of calpain activity that occurs in their muscles is prevented by treatment with pentoxifylline, an inhibitor of TNFα synthesis (Costelli et al., 2002). As another piece of circumstantial evidence, TNFα-induced apoptosis in various cell types has been shown to be mediated by calpains, implying that the calpain system can be activated by this cytokine (Chang et al., 2004; Diaz & Bourguignon, 2000). Moreover, calpastatin can be cleaved by caspase-3 or caspase-1/interleukin-1 converting enzyme, which plays a role in activating proinflammatory cytokines, suggesting further possible links between cytokines and Ca^{2+}-dependent proteolysis (Barnoy & Kosower, 2003; Porn-Ares, Samali, & Orrenius, 1998). Treatment of HepG2 hepatoma cells with TNFα results in increased calpain activity and nuclear translocation of the transcription factor NF-κB; the latter effect is partially abrogated in HepG2 cells engineered to overproduce calpastatin, suggesting that to some extent it may be calpain-dependent (Han, Weinman, Boldogh, Walker, & Brasier, 1999). These observations may also be relevant to muscle wasting, since activation of NF-κB has been observed in muscle atrophy by disuse or sepsis (Hunter et al., 2002; Penner, Gang, Wray, Fischer, & Hasselgren, 2001) and has been involved in the increased expression of proteasome subunits mRNA (Whitehouse & Tidsale, 2003). Moreover, transgenic mice overexpressing IKKβ and with constitutive activation of this transcritpion factor develop severe muscle wasting (Cai et al., 2004).

The evidence that links the different proteolytic systems to muscle wasting still is largely circumstantial, in spite of the many recent progresses. A notable exception is the prevention of disuse-induced atrophy observed in transgenic mice overexpressing calpastatin (Tidball & Spencer, 2002) or in mice knocked out for the atrogenes *MuRF1* or *atrogin-1* (Bodine et al., 2001a). Much evidence indicates that the expression of calpains, lysosomal proteases and proteasome constituents or of enzymes involved in protein ubiquitylation are frequently up-regulated in muscle-wasting disorders, whereas endogenous inhibitors such as calpastatin are down-regulated. Generally speaking, the meaning of these regulations is not clear in terms of causal relationship. Often there may be some propensity to present them as controlling events, yet the possibility cannot be ruled out that they rather are secondary adaptive, perhaps permissive responses to a sustained hypercatabolic state. The available evidence may favor the concept of a concerted action (in series or in parallel) of the different proteolytic pathways to effect the hypercatabolic response in muscle wasting, even if their respective contribution may well vary depending on the kind of process and other concomitant factors. The key point is to clarify what really triggers the whole sequence(s), where the initial controlling events operate, and how the different signals may converge into the hypercatabolic response.

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