Differential localization of autolyzed calpains 1 and 2 in slow and fast skeletal muscles in the early phase of atrophy

Marianne Vermaelen,1,2,3,4 Pascal Sirvent,1,2,3,4 Fabrice Raynaud,5 Catherine Astier,6 Jacques Mercier,1,2 Alain Lacampagne,3,4 and Olivier Cazorla3,4

1Institut National de la Santé et de la Recherche Médicale (INSERM), ERI 25; 2Université Montpellier1, UFR de Médecine, EA 701; 3INSERM, U637; 4Université Montpellier1, UFR de Médecine; 5Centre National de la Recherche Scientifique, UMR 5539, Université de Montpellier2; and 6UPRES-EA 3799 “Appr`eche bio-psycho-sociale du dopage,” Université de Montpellier1, Faculté des Sciences du Sport, Montpellier, France

Submitted 21 July 2006; accepted in final form 15 December 2006

SKELETAL MUSCLE ATROPHY OCCURS in many unrelated conditions, including fasting, chronic diseases like diabetes or chronic obstructive pulmonary disease, and reduced mechanical loading as in immobilization. An increased rate of proteolysis has been identified as a major step in muscle atrophy, while the rate of protein synthesis is not always reduced (25). Although the etiology may differ, the loss of muscle proteins has been attributed in all cases mainly to the increased activity of the ubiquitin-proteasome proteolytic pathway (29). However, lysosomal cathepsins, uncharacterized serine proteases, and calpains are also involved in skeletal muscle atrophy (2, 32, 45, 50).

Calpains are Ca2+-dependent cysteine proteases that constitute a large and diverse family. Skeletal muscle fibers contain calpain 1, calpain 2, and a muscle-specific calpain known as calpain 3 or p94. The precise roles and normal regulation of the calpains in skeletal muscle are currently unclear, although they are likely to be involved in cytoskeleton organization, the cell cycle, and apoptosis (for a review, see Ref. 16). In addition, several studies have suggested that calpains are involved in the differentiation, atrophy, and regeneration of muscle, and it is evident that they have a major involvement in certain types of muscular dystrophy. It has been demonstrated that 1) calpains are elevated in atrophic conditions such as spinal cord transection (18), sepsis (50), and glucocorticoid treatment (20), but increased mRNA and/or protein levels do not necessarily reflect increased calpain activity; 2) the use of calpain inhibitors can reduce muscle proteolysis and atrophy (14, 48), but the inhibitors used to counteract atrophy in many studies are not specific to calpain and may also inhibit other proteases such as cathepsins or other cysteine proteases; and 3) calpain activity, as assessed by spectrofluorimetric assays with fluorescent substrates, is increased during disuse, fasting, and remodeling induced by chronic low-frequency stimulation (43, 45, 48). Regarding this last point, however, it should be kept in mind that in vitro activity does not necessarily reflect in vivo activity, since, in physiological conditions, regulations take place in cells that cannot occur in proteic extracts. Moreover, when calpain activity is quantified in whole tissue homogenates, the specific involvement of each calpain isoform cannot be distinguished.

As recently emphasized by Bartoli and Richard (2), the relative contribution of each calpain in the development of muscle atrophy is difficult to assess. The various calpains may play different roles, since their activation signals may differ and they may have distinct localizations in the cell. For example, a recent study reported that calpain 1 is mainly diffusible, whereas calpain 3 is mainly tightly bound within myofibers (35). Moreover, it is well documented that micromolar and millimolar Ca2+ concentrations ([Ca2+]i) are required to activate calpain 1 and calpain 2 in vitro, respectively (8). The in vivo regulation of calpain activity is complex and poorly understood, but it is assumed to involve several processes such as Ca2+ oscillations, calpastatin-inhibiting activity, phosphorylation, translocation to membrane, and autolysis. The respective contributions of these processes have not yet been elucidated (for a review, see Ref. 16). Although mechanisms of calpain activation are multifactorial, removal of the NH2 terminus region by autolysis is considered a sign of...
calpain 1 and 2 activation. Indeed, calpain 1 and calpain 2 are heterodimers constituted by an 80-kDa catalytic subunit and a 28-kDa regulatory subunit, and autolysis of the catalytic subunit increases calpain sensitivity to Ca\(^{2+}\) in vitro (44). Thus calpain autolysis measurements are used as signs of calpain activation (34, 40, 47).

The aim of this study was to clarify the involvement of calpains in skeletal muscle atrophy. To do so, we specifically examined 1) whether calpains 1 and 2 were more autolyzed after a short period of atrophy induced by plaster cast immobilization; 2) whether these two calpains and their autolyzed products, if any, had different patterns of localization in the myofibers; and 3) whether two different muscles, one slow-type muscle known to be quickly atrophied and one fast-type muscle known to be less sensitive to disuse, showed the same pattern of autolysis in the myofibers.

**MATERIALS AND METHODS**

**Animals and immobilization protocol.** Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC), and the protocol was approved by the local Ethic Committee. Sixteen male Wistar rats (6 mo old) were maintained in a controlled room (22°C) with a 12:12-h light-dark cycle and were given free access to a standard laboratory diet and water. They were randomly assigned to either the control (n = 8) or the immobilized (n = 8) group. All the rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), and the right hindlimbs of eight of them were then immobilized with a plaster cast as described previously (4). The right hindlimbs were fixed at the hip, ankle, and knee with the cast such that the soleus and plantaris muscles were in a shortened position. Great care was taken to ensure that the cast did not induce edema or ischemia. Immobilization lasted for 5 days. Food intake was checked to ensure that it was similar between groups. After 5 days, the rats were killed by cervical dislocation, and the soleus and plantaris muscles were removed, weighed, and quickly frozen in liquid nitrogen to ensure that it was similar between groups. After 5 days, the rats were killed by cervical dislocation, and the soleus and plantaris muscles were removed, weighed, and quickly frozen in liquid nitrogen for biochemical analysis or in isopentane cooled with liquid nitrogen to −80°C until analysis.

**Immunohistochemistry.** Ten-micrometer-thick cryostat sections were cut from the midbelly of the isopentane-frozen plantaris and soleus muscles. The sections were first stained with hematoxylin-eosin to verify that they were orthogonal to the muscle’s longitudinal axis. Unfixed sections were incubated on slides with anti-dystrophin antibody [H4, which was produced and characterized as previously described (39)] and either anti-myosin heavy chain (anti-MyHC I) (8H8) or anti-MyHC II (Sigma-Aldrich, Saint Quentin Fallavier, France; dilution 1/400) antibody for 1 h at room temperature. After a wash with PBS solution, the sections were then incubated for 1 h at room temperature with secondary antibodies [Cy3-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Chemicon International; dilution 1/2,000, and 1/6,000, respectively)]. Finally, the sections were washed with PBS and fixed 10 min with 3% paraformaldehyde. The slides were then mounted with Mowiol (Calbiochem) and observed under a fluorescence microscope (Nikon Optiphot-2). Cross-sectional areas (CSA) for slow and fast fibers (anti-MyHC I- and anti-MyHC II-labeled fibers, respectively) were determined with ImageJ software. For each fiber type, the CSA of 50–150 fibers were measured per muscle (n = 12). Each fiber was considered independently, and the means were the result of all fiber CSA in each group for soleus and plantaris muscle.

**Extraction of soluble and particulate fractions.** The soluble and particulate fractions were separated as described previously by our group (49). Each muscle was homogenized separately in a protein extraction buffer [20 mM Tris, pH 7.4, 5 mM EGTA, 1 mM DTT, 0.5 mM iodoacetamide, 10 μg/ml E-64, and a protease inhibitor cocktail: 1 tablet/10 ml (Roche, Mannheim, Germany)]. The homogenates were centrifuged at 1,700 g for 2 min at 4°C, and the supernatant was stored as the soluble fraction. Pellets corresponding to particulate fractions were suspended in the extraction buffer without DTT and completed with 250 mM NaCl and 1% SDS and centrifuged at 15,000 g for 2 min at 4°C. The supernatant (solubilized particulate fraction) was saved, and the insoluble pellet was discarded. Both fractions were aliquoted and stored at −80°C. Protein concentrations were determined by bicinchoninic acid assay (BCA kit, Pierce) using bovine serum albumin as the standard. The control of the particulate and soluble protein separation was performed by Coomassie blue staining of myosin (see Figs. 3D and 4D).

**Total protein extraction.** About 30 mg of soleus and plantaris muscles were homogenized at 4°C in a lysis buffer containing 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM EDTA, pH 7, 1 mM Na3VO4, 1 mM NaF, 0.1% SDS, 0.5 mM PMSF, 40 μM leupeptin, and E64. The homogenates were then centrifuged at 13,000 rpm for 3 min at 4°C. The supernatants were stored at −80°C. Total protein concentrations were determined by BCA.

**Lactate dehydrogenase isoenzyme activity.** The soluble proteins (1 μg) were separated on 1% agarose gels at 90 V for 30 min (Bio-Rad Sub-Cell system). An electrophoretic marker (LDH Isotrol, Sigma-Aldrich) containing lactate dehydrogenase (LDH) isoenzymes was used as a positive control. LDH isoenzyme activities were visualized by nitroblue tetrazolium reduction to formazan (Sigma Procedure 105). Gels were fixed in 5% acetic acid and were scanned with a Kodak image station. Bands were quantified with Kodak ID3.6 software.

**Citrate synthase activity.** Citrate synthase (CS) activity was assayed by a spectrophotometric method according to Srere (42) using acetyl coenzyme A (15 μM) and oxaloacetic acid (0.5 mM) as substrates. In the presence of coenzyme A, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; 100 μM) was turned into C6O4S₂⁻, which was recorded at 412 nm over 3 min at 25°C. Activity was expressed in micromoles per minute per microgram protein.

**MyHC isofrom analysis.** MyHC analysis was performed as described previously (46) with some modifications. Proteins from the particulate fraction (0.2 μg) were solubilized in a sample buffer (5% 2-β-mercaptoethanol, 100 mM Tris, pH 6.8, 5% glycerol, 4% SDS, and bromophenol blue), boiled for 3 min, and then loaded on gels. Electrophoresis was performed using the Mini Protein III system (Bio-Rad, Marnes-la-Coquette, France). The separating gel solution contained 30% glycerol, 8% acrylamide-bis (49:1), 0.2 M Tris, 0.1 M glycine, and 0.4% SDS. The stacking gel was composed of 30% glycerol, 4% acrylamide-bis (49:1), 70 mM Tris, 4 mM EDTA, and 0.4% SDS. Two lanes were loaded with protein extract from a control diaphragm muscle known to contain the four adult MyHC isoforms. Gels were run at constant voltage (72 V) for 31 h, silver stained, and scanned with the Kodak image station. Bands were quantified with Kodak ID3.6 software.

**Western blots.** The protein extracts were solubilized in a sample buffer (100 mM Tris, pH 6.8, 5% 2-β-mercaptoethanol, 5% glycerol, 4% SDS, and bromophenol blue). Calpains 1 and 2 were separated with 6% SDS-PAGE from the soluble and particulate fractions and transferred to polyvinylidene difluoride (PVDF) membranes that were incubated with anti-calpain 1 (Affinity BioReagent, OPA1-08803; dilution 1/5,000) and anti-calpain 2 [previously described (37); dilution 1/100] antibodies. Ubiquitin was separated with 2–12% SDS-PAGE using nitroblue tetrazolium reduction to formazan (Sigma Procedure 105). Gels were fixed in 5% acetic acid and were scanned with a Kodak image station. Bands were quantified with Kodak ID3.6 software.
anti-filamin antibody [previously described (28); dilution 1/400]. The 14-kDa actin product and cleaved caspase 3 were separated with 15% SDS-PAGE from the total fraction and transferred to nitrocellulose membranes that were incubated with anti-cleaved caspase 3 (Asp175) (Cell signaling, 9664; dilution 1/5,000) and anti-actin COOH-terminal (Sigma-Aldrich, A2066, dilution: 1/400) antibodies. All membranes were transferred using a semidry transfer protocol (1.5 mA/cm² of membrane for 70 min) and blocked with 5% nonfat milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with the corresponding antibody and then washed three times for 10 min and incubated with anti-rabbit or anti-mouse horseradish peroxidase secondary antibody. The membranes were again washed in TBS-Tween three times for 10 min. Specific bands were revealed with enhanced chemiluminescence reagent (West Pico, Pierce), and the signal was recorded with the Kodak image station. Analysis was performed with Kodak ID3.6 software.

Proteasome activity. Muscles were homogenized in a 0.1 M NaKH₂PO₄ ice-cold buffer containing 2 mM EDTA [1/20 (wt/vol)], stirred for 15 min, and sonicated on ice four times for 10 s. The homogenates were then centrifuged at 15,000 g for 10 min at 4°C. The supernatants were removed, and the pellets were resuspended at a 1/20 dilution (wt/vol) and processed as described above. Both supernatants were then combined. The chymotrypsin-like enzyme activity of 20S proteasome was fluorometrically measured (excitation wavelength = 380 nm, and emission wavelength = 460 nm; SFM25 fluorimeter, Kontron Instruments) with 10 μl of protein extract in 980 μl of 60 mM imidazole buffer (pH 7.4) (11). The reaction was started by the addition of 100 μM fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Bachem, I-1395).

Statistical analysis. All data were presented as means ± SE. Statistical differences were determined by unpaired Student t-tests of the control and immobilized groups, and one-way ANOVA and post hoc Neuman-Keuls tests for the MyHC isoforms and LDH isoenzymes of the control and immobilized groups; P < 0.05 was considered significant.

RESULTS

Muscle atrophy. Atrophy was assessed by the muscle mass-to-body weight ratio and the CSA of muscle fibers according to fiber type. The muscle mass-to-body weight ratio decreased significantly by 14% in soleus and 9% in the plantaris after 5 days of plaster casting (Fig. 1A). Moreover, immunohistochemistry analysis showed that type I fibers were more atrophied than type II fibers in both muscles. Indeed, CSA decreased by 35% for type I fibers and by 16% for type II fibers in soleus muscle and by 21% for type I fibers and by 16% for type II fibers in plantaris muscle (Fig. 1, B and C).

Muscle phenotype. Muscle phenotype was evaluated by MyHC isoform distribution and CS and LDH activities. The contractile phenotype was not changed in the soleus muscle (Fig. 2A). In plantaris muscle, the MyHC IIb proportion was significantly decreased (38 and 24% in the control and immobilized groups, respectively; P < 0.05) with a slight but not significant increase in the proportion of the IIa, IIx, and I isoforms. Concerning the metabolic phenotype, no changes in CS activity (Fig. 2B) or LDH isoenzyme expression (Fig. 2C) were observed between the control and immobilized groups in either muscle.

Calpain 1 and 2 autolysis in the soleus and plantaris muscles. In the soleus muscle, hindlimb immobilization resulted in increased calpain 1 autolysis in the particulate fraction (24% of
autolyzed calpain 1 vs. 7% for control; \( P < 0.05 \), while total calpain 1 content remained unchanged (Fig. 3A); hence, the 80-kDa form was decreased and the autolyzed form increased. In the soluble fraction, no significant difference in calpain autolysis was observed (Fig. 3B).

Conversely, calpain 2 autolysis was increased only in the soluble fraction with immobilization (54% of autolyzed calpain 2 vs. 38% for control; \( P < 0.05 \)), and the total calpain 2 content increased (Fig. 3B). Hence, the autolyzed form increased without any significant change in the 80-kDa form. In the particulate fraction, no calpain 2 autolysis was observed in either the control or the immobilized group (Fig. 3A).

In the plantaris muscle, the total content of calpains 1 and 2 remained unchanged, and no difference was seen for the autolyzed forms in either fraction after 5 days of immobilization (Fig. 4, A and B).

As previously described, filamin is a calpain substrate (36), and its degradation can be used as a marker of calpain activation (30). We thus assessed filamin degradation in our extracts. Filamin degradation products were significantly increased in the soleus muscle after immobilization (Fig. 3C), whereas no difference was found in the plantaris muscle (Fig. 4C).

In control vs. 1.22 ± 0.16 in immobilized plantaris muscles; \( P < 0.05 \), whereas no difference was found in the soluble fraction (Fig. 5A). The chymotrypsin-like activity of the proteasome was not different after immobilization in either muscle type (Fig. 5C). Cleaved caspase 3 was increased after immobilization only in the soleus muscle, whereas no difference was observed in the plantaris muscle (Fig. 5D). This activation was confirmed by Western blots of the 14-kDa actin fragment, which was previously described as a specific caspase 3 cleavage (10). Similar to cleaved caspase 3, the 14-kDa actin fragment was increased after immobilization in the soleus muscle, whereas no increase was observed in the plantaris muscle (Fig. 5E).

DISCUSSION

Calpain activation has been proposed to be involved in the process of skeletal muscle atrophy. However, the respective contributions of calpains 1 and 2 have not been well documented. In this study, we investigated autolyzed calpain 1 and 2 products according to their localization in the myofibers of slow and fast skeletal muscles after 5 days of hindlimb immobilization. Interestingly, we observed that this short period of disuse induced different responses depending on muscle phenotype and calpain localization.

Effects of 5 days of immobilization on muscle remodeling. Consistent with previous reports (25, 32, 49), 5 days of immobilization were sufficient to induce significant muscle atrophy,
showing that our experimental model works. The muscle mass-to-body weight ratio decreased slightly more in immobilized slow muscle than in immobilized fast muscle. It has been well documented that slow muscles are much more sensitive to disuse than fast muscles (1). We found that soleus and plantaris muscles from contralateral legs of immobilized animals were hypertrophied (data not shown), since these muscles undergo a mechanical overload due to disuse of immobilized leg. For this reason, we used a control group to assess the effects of disuse on calpain autolysis.

The short-term immobilization did not change the contractile phenotype in the soleus muscle, whereas a small but significant decrease in MyHC IIb occurred in the plantaris muscle. This relative decrease was compensated by a slight but not significant increase in the percentage of the IIa, IIx, and I isoforms. These changes can be explained by the fact that type IIb fibers seem to be more sensitive to atrophy than type IIA and IIX fibers in fast-type muscle, which has already been demonstrated for the plantaris muscle in different atrophic conditions (13, 24). Moreover, we can hypothesize that the synthesis rate of MyHC IIb was decreased compared with that of the other isoforms, which would also explain the decreased MyHC IIb percentage in the plantaris muscle. The absence of a phenotypic change in the soleus muscle can be explained by the short period of disuse. Indeed, it has been found that the MyHC isoform shift in the soleus muscle generally occurs after 8–15 days of inactivity (17). Five days of immobilization induced no change in the metabolic phenotype of either muscle. This is consistent with the findings of Desplanches et al. (9), who found no modifications in CS or LDH activities after 1 wk of hindlimb suspension while CS activity was decreased after 5 wk. These data suggest that metabolic remodeling does not occur in the early stages of muscle atrophy and instead begins later, when disuse is extended.

Effects of 5 days of immobilization on calpain autolysis. To prevent or reverse muscle atrophy, it is necessary to understand the early events of muscle wasting. Several studies have suggested that calpains are involved in skeletal muscle remodeling and atrophy. In our study, we thus assessed autolyzed calpains during the early stages of muscle atrophy. The use of autolysis as an index of calpain activity is debatable, since unautolyzed calpains may have proteolytic activity (31). Nevertheless, autolysis is known to occur consistently when calpains are active, and autolysis has been shown to increase calpain Ca$^{2+}$ sensitivity in vitro (44). Thus autolysis is acknowledged as an indicator of calpain activity in the cell (6, 16) and allows the assessment of calpains 1 and 2 separately. We chose to separate the particulate and soluble fractions to obtain information about the potential targets of calpains during early stage atrophy. The soluble fraction is mainly constituted by cytosolic...
proteins, whereas the particulate fraction contains mainly myo-
fibrillar proteins and, to a lesser extent, membrane-bound
proteins. These experiments allowed us to determine the frac-
tion in which the autolyzed calpain products are found and thus
the types of targets that are potentially cleaved.

In control conditions, it has been shown that calpain 1 can
be localized on or near Z disks and more weakly on I and A
bands (27, 38). Moreover, calpains are known to cleave
many myofibrillar proteins such as titin, nebulin, filamin,
troponin, and C protein. The increased products of calpain 1
autolysis and filamin degradation in the particulate fraction of
the soleus after immobilization suggested that calpain 1 may be
involved in cytoskeletal remodeling in the early stages of
muscle wasting, allowing further protein breakdown by the
proteasome, as already postulated by different authors (16, 21,
23). Moreover, these results are consistent with those of a
previous report showing that calpain activity measured with
colorimetric assay was increased in the particulate fraction of
the soleus muscle after short periods of unloading (12).

Compared with the particulate fraction, the soluble fraction
contains few structural/cytoskeletal proteins but many proteins
playing a role in cellular signaling pathways. Thus the in-
creased calpain 2 autolysis in the soluble fraction of immobi-
lized soleus muscles suggested that calpain 2 was more active
in this fraction after immobilization, which may imply that
calpain 2 plays a role in the signaling pathways that induce
muscle atrophy. Indeed, the limited cleavage sites on substrate
proteins suggest that calpains have a signaling or regulatory
function rather than a digestive function, such as the proteasome
has. Calpains are known to activate some of the kinases and
phosphatases such as PKC and calcineurin (5, 20) and other
molecules such as RhoA and IκBα (19, 26), which are key
elements of signaling pathways in skeletal muscle and involved
in muscle mass control (7, 41). In the particulate fraction of
both our groups, it might be suggested that calpain 2 was not
activated or was less activated. However, we cannot rule out
the possibility that the 80-kDa form was active without autol-
ysis in this fraction, since myofibrillar proteins such as nebulin
have been shown to be calpain 2 substrates (21).

Although our study provides some indications about calpain
targets during atrophy, the protocol did not allow us to deter-
mine where autolysis takes place. Indeed, it was recently
demonstrated that calpain 1 changes its localization when
\([Ca^{2+}]\) increases and autolysis occurs (35). This latter study
showed that, in vitro in extensor digitorum longus (EDL)
fibers, calpain 1 is mainly diffusible in control condition but
rapidly binds when \([Ca^{2+}]\) increases and then autolyses. The
authors suggested that this regulation could occur in vivo in

Fig. 4. Effects of 5 days of immobilization on autolyzed and unautolyzed calpains, ac-
cording to the fraction, and on filamin deg-
situations where [Ca\(^{2+}\)] is increased, such as eccentric exercise and Duchenne muscular dystrophy (35). Even though the level of [Ca\(^{2+}\)] in our model of disuse is unknown, Fraysse et al. (15) showed that 3 days of hindlimb suspension were sufficient to significantly decrease the resting calcium level in the soleus muscle. This may also have been the case in our model, which would be consistent with the lack of evidence for calpain relocalization from soluble to particulate fractions after immobilization, given the lack of difference in the total amount of calpain in each fraction between the control and immobilized conditions.

Moreover, Murphy et al. (35) showed that calpain 1 in EDL muscle is mostly present in the diffusible fraction. If its localization is similar in the soleus muscle, this would suggest that the increased autolyzed form of calpain 1 found in the immobilized soleus particulate fraction represents only a small proportion of total calpain 1. This point should be clarified by determining the relative proportions of calpains 1 and 2 in the bound and diffusible pools, using the same muscles and conditions as in the present study.

Interestingly, our data showed that the increase in autolyzed calpain 1 in the particulate fraction was accompanied by a reduction in the 80-kDa form, while total calpain 1 content did not change, after 5 days of immobilization. Conversely, the increase in calpain 2 autolysis in the soluble fraction was not accompanied by a change in 80-kDa form content. Thus total calpain 2 content increased after immobilization in the soleus soluble fraction, and it can be assumed that calpain 2 was upregulated during this short period of disuse, whereas calpain 1 was not. Therefore, calpains 1 and 2 may be separately and differently regulated, as previously suggested (2, 16).

No change in calpain autolysis was seen in the plantaris muscle in this study. This could imply that our short disuse period was not sufficient to increase calpain autolysis. Indeed, it has been well established that fast muscles are less sensitive to disuse-induced atrophy than the slow postural muscles (1), and our data are consistent with a recent study suggesting that calpain activation during atrophy is increased earlier in the slow type soleus muscle (after 12 h of hindlimb suspension) than in the fast type gastrocnemius muscle. In this muscle, calpain activation was not noted after 72 h of hindlimb suspension and began after 9 days of suspension (12).

We found that the plantaris muscle showed significant atrophy without an increase in autolyzed calpains after 5 days of immobilization, which suggests that other processes were involved. We thus assessed other proteolytic pathways known to play key roles in skeletal muscle atrophy. We found that the ubiquitin-proteasome pathway was activated in this early stage of immobilization.

Fig. 5. Effects of 5 days of immobilization on the ubiquitin-proteasome pathway and caspase 3 activation in the soleus and plantaris muscles. Western blot analysis of ubiquitinated proteins was performed in the soluble (A) and particulate (B) fractions in the soleus and plantaris muscles of the control (open bars) and 5-day immobilized (solid bars) groups. *P < 0.05. C: chymotrypsin-like activity of the proteasome was assessed by spectrophotometric method. Data are mean values ± SE. Activation of caspase 3 was investigated by detecting the cleaved caspase 3 (17–19 kDa) by Western blotting (D) and the 14-kDa actin fragment produced by caspase 3 cleavage (E). Note that cleaved (active) caspase 3 and 14-kDa actin fragment increased in the soleus but not in the plantaris after immobilization.
of wasting. As expected, we noted that protein ubiquitination was increased in the particulate fraction of the soleus and plantaris muscles, whereas ubiquitination was not changed in the soluble fraction of either muscle. These results are consistent with those of other studies suggesting that the ubiquitin-proteasome system is involved early on in skeletal muscle atrophy (3, 25) and that myofibrillar proteins are preferentially degraded during this atrophy (33). We also found no difference in the chymotrypsin-like activity of the proteasome between the control and immobilized groups for either muscle. Once again, this result is consistent with those of other studies suggesting that proteasome activity is increased after longer periods of disuse (22). These results indicate that the ubiquitin-proteasome pathway is involved in both muscles.

Caspase 3 has been shown to be an initial step in muscle atrophy development resulting from catabolic conditions, and its activation may result in accelerated muscle proteolysis by yielding proteins that are then degraded by the ubiquitin-proteasome pathway (10, 51). We therefore further assessed caspase 3 activation by cleaved caspase 3 and 14-kDa actin fragment Western blots. We hypothesized that its activation would occur preferentially in the plantaris muscle, which would explain the increased atrophy and ubiquitination in this muscle. We did not confirm this hypothesis, since we found no difference in the cleaved/active caspase 3 and 14-kDa actin fragment Western blots in this muscle, although this activation was evident in the soleus muscle.

Taken together, the simultaneous involvement of calpains, caspase 3, and the ubiquitin-proteasome pathway is consistent with a greater atrophy found in the soleus muscle compared with the plantaris muscle, in which only the ubiquitin-proteasome pathway is involved.

In summary, our results showed that 1) calpains are autolyzed in the early stage of skeletal muscle atrophy; 2) this autolysis is specific to the particulate fraction for calpain 1 and to the soluble fraction for calpain 2, indicating specific micro localization of calpain autolysis regulation; 3) calpain 2 autolysis is associated with an increased amount of calpain 2 content, whereas calpain 1 autolysis occurs without any modification in the total amount; 4) calpain autolysis is only seen in the slow soleus muscle, while the fast plantaris muscle is not affected; and 5) calpain autolysis and caspase 3 activation found in the soleus muscle could explain a more atrophied condition of this muscle compared with the plantaris muscle. These new data improve our knowledge about the regulation of the ubiquitous calpain system in the early stages of skeletal muscle atrophy and provide some indications about the respective targets of calpains 1 and 2 in this process. They point out that the regulation of these proteases is highly complex. A better understanding of these mechanisms is required to develop efficient strategies to counteract skeletal muscle atrophy.

ACKNOWLEDGMENTS

We thank Gérald Hugon for help with immunohistochemistry and Damien Freyssenet, Didier Moukoko, Karen Lambert, Karim Hnia, and Dominique Mornet for advice.

GRANTS

M. Vermaelen is a recipient of a grant from the Association Française contre les Myopathies.

REFERENCES


