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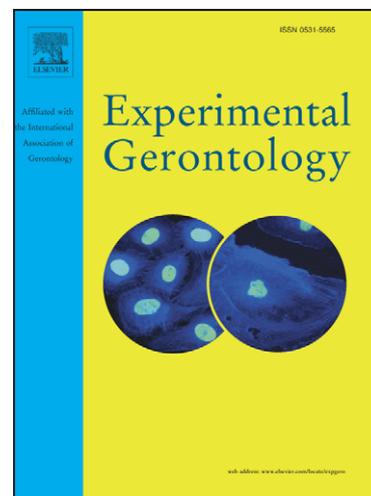
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Involvement of the calcium-dependent proteolytic system in skeletal muscle aging.

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Running title: Calpains and sarcopenia.

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Abstract

Aging is associated with a progressive and involuntary loss of muscle mass also known as sarcopenia. This condition represents a major public health concern with high socio-economics implications. Although sarcopenia is well documented, the aetiology of this condition still remains poorly understood. Calpains are ubiquitous proteases regulated in part by a specific inhibitor, calpastatin. They are well known to have major implications in muscle growth and differentiation. The aim of the present study was to determine if this proteolytic system could be involved in the phenotype associated with sarcopenia. Calpains and calpastatin levels, subcellular distributions and activities were compared between muscles from 3 and 24 months old rats. Altogether, the results we obtained showed an overall increase in calpain activities associated with muscle aging. These findings suggest that the calcium-dependent proteolytic system is indeed involved in sarcopenia.

Introduction

Aging is associated with a progressive and involuntary loss of muscle mass also known as sarcopenia. This condition is characterised by impaired muscle strength and function that affects 25% of person under the age of 70 years and 40% of those aged 80 years or more (Baumgartner et al., 1998). Sarcopenia has been reported even among healthy, physically active subjects (Hugues et al., 2002), and is becoming one of the major public health problems of our aging populations.

Because of the impairment of muscle mass and strength, sarcopenia has a very negative impact on old people's quality of life, with an increased incidence of falls and bone fractures (Szulc et al., 2005). Muscle is also the major reservoir of proteins in the body. After an injury or a stress, amino acids are released from this compartment to help tissue repair and maintain energetic balance (Griffiths et al., 2000). For old persons however, muscle atrophy leads to a very slow recover after a disease or trauma. All these situations require social and health care and consequently represent a massive cost for our society.

Although sarcopenia is now well documented, the aetiology of this condition still remains poorly understood and could well be a multifactorial disorder.

From an histological point of view, sarcopenia is characterised by a decrease in the number and the size of muscle fibres. Several different components have been proposed to contribute to this phenotype such as hormonal changes, malnutrition, changes in neuronal stimulation, lack of exercise or chronic inflammation (Carmeli and Reznick, 1994; Evans, 2004; Lamberts et al., 1997; Morley and Baumgartner, 2004; Visser et al., 2002). The reduction in the number of fibres occurring during muscle aging could be partly due to apoptosis activation (Dirks and Leeuwenburgh, 2005). If caspase-dependent pathways have been well characterised in apoptotic process, calpains could also play a role in cell death associated with sarcopenia

(Dirks and Leeuwenbergh, 2004). According to some authors an imbalance between the rates of protein synthesis and proteolysis could also account for the loss of muscle mass (Attaix et al., 2005; Yarasheski, 2003). For example, subunits of the proteasome as well as ubiquitin protein levels were found to increase in old rat muscle (Cai et al., 2004; Husom et al., 2004). Calpains belong to a large family of calcium dependent cystein proteases. 14 isoforms have been identified so far. They are ubiquitous or tissue-specific enzymes (Goll et al., 2003). The two best characterised isozymes are the ubiquitously expressed μ - and m-calpains. Both are heterodimeric proteins consisting of a distinct large catalytic subunit (80 kDa) and a common small regulatory subunit (30 kDa). Besides its regulation by calcium, calpain activity is tightly controlled by calpastatin, the specific endogenous inhibitor, binding to phospholipids, autoproteolysis and phosphorylation (Cottin et al., 2001; Glading et al., 2004; Saido et al., 1992). Although a great deal of informations has been gathered for the last decades in the calpain field, the physiological function of the enzymes still remains poorly understood. Calpains are however believed to be involved in a variety of cellular processes such as cytoskeletal remodelling, cell spreading and motility, signal transduction, cell cycle regulation, apoptosis and myogenesis (Aragon et al., 2002; Dedieu et al., 2004; Del Bello et al., 2006; Lebart and Benyamin, 2006; Mazeret et al., 2006; Raynaud et al., 2004). A main role played by calpains during muscle development and differentiation has been partly established in our laboratory (Cottin et al., 2000; Dedieu et al., 2004; Dulong et al., 2004; Leloup et al., 2006). Calpains are also involved in several diseases. Among them, some are directly linked to the disruption of a calpain gene like LGMD2A, associated with mutations in the calpain 3 gene (Richard et al., 1995), and others are a consequence of a loss of calcium homeostasis. For instance, calpain activity has been shown to be involved in cataract formation, Alzheimer's disease or muscular dystrophies (Biswas et al, 2004; Raynaud and Marcilhac, 2006; Tidball and Spencer, 2000).

Because calcium homeostasis is frequently altered during aging, it could explain why unregulated calpain activities are often associated with degenerative diseases. An increase in calpain activity has been indeed reported, as well as an elevation of the calcium influx in several aging tissues (Manya et al., 2002; Ouanounou et al., 1999; Romero et al., 2002; Sloane et al., 2003). The most striking example being the Alzheimer's disease where increased calpain activity has been correlated with impaired calcium homeostasis (Nixon, 2003).

Calcium plays a critical role in muscle function. Several studies have recently shown that an impaired calcium homeostasis could be involved in the loss of muscle mass occurring with aging (Delbono, 2002; Fraysse et al., 2006). Among other deleterious effects, high calcium level could trigger apoptosis via calpain activation and subsequent caspase 12-mediated pathway (Tan et al., 2006). Still, little information is available today concerning the link between calpains and sarcopenia.

In the present study, we thus characterised the effects of muscle aging on the calcium dependent proteolytic system. The levels of calpains and calpastatin were quantified, the specific activities were measured and all data accordingly show a significant tendency of calpain activities to raise with muscle aging.

Materials and methods

1-Materials

NBT (nitrobluetetrazolium) and BCIP (5-bromo-4-chloro-3-indoyl-1-phosphate) were purchased from Promega. Immobilon P transfer membrane was obtained from Millipore. The Coomassie Protein Assay Reagent was purchased from Interchim. The M-MLV reverse

transcriptase was from Invitrogen and random primers from Promega. The Hitrap Phenyl FF and HiTrap DEAE FF columns were obtained from GE Healthcare. Ac-DEVD-AMC and Ac-DMQD-CHO were purchased from Calbiochem. Anti-calpastatin and anti-calpain small subunit were from TEBU Santa Cruz Biotechnology. The m-calpain antibodies were raised in sheep as described by Cottin et al. (1994). The antibodies for fodrin were purchased from AbCys. Anti- μ -calpain antibodies, all secondary antibodies and other chemicals were from Sigma. Molecular weight markers were obtained from Biorad.

2-Animals

The study was conducted in accordance with the European Communities Council Directives (861609/EEC). All the experiments conformed to the Guidelines on the Handling and Training of Laboratory Animals. Male Wistar rats, purchased from Charles Rivers, were maintained in a room with a constant airflow system, controlled temperature (21-23°C) and a 12 h light/dark cycle. The rats were allowed ad libitum access to food and tap water. They were divided into two groups (6 animals per group) designated as young and old. Young rats were studied at 3 months of age and old rats at 24 months. The animals were killed by decapitation and all hindlimb muscles from both legs were rapidly removed, quick frozen in liquid nitrogen and stored at -80°C. Frozen muscle was then carefully collected from each individual in the same group and pooled for further experiments.

3-Electrophoresis and immunoblotting procedures

The samples were run on a 10% SDS-PAGE, as described by Laemmli (1970). The proteins were then transferred onto Immobilon P membrane. The membranes were blocked at room

temperature for 2 h using TBS (tris-buffered saline: 50 mM tris-buffer pH 8, 138 mM NaCl, 2.7 mM KCl) containing 5% (w/v) skim milk. The membranes were washed with TBS and incubated with the primary antibodies in TBS containing 1% skim milk for 2 h at room temperature. The following antibody dilutions were used: 1/1 000 for m-calpain, 1/5 000 for μ -calpain, 1/250 for calpastatin, 1/300 for calpain small subunit, 1/1 000 for fodrin. The membranes were washed with TBS and incubated with the secondary antibodies (alkaline phosphatase conjugated antibodies, 1/10 000) in TBS containing 1% skim milk for 1 h at room temperature. After washing with TBS, the NBT/BCIP were used as detection substrates.

4-Extraction of total RNA and reverse transcription

Total RNA was extracted using the Promega SV total RNA isolation system. The integrity of the RNA extracted was verified by electrophoresis. The purity and concentration of total RNA were estimated by measuring the 260nm/280nm absorbance ratio. Then, 300 ng RNA was reverse transcribed to cDNA using random primers and M-MLV reverse transcriptase. The obtained cDNA were conserved at -20°C and used in q-PCR.

5-Quantitative PCR

Quantitative PCR was performed using Roche LightCycler[®] FastStart DNA Master SYBR Green I kit according to manufacturer's instructions. The PCR reactions were performed in capillary tubes in a final volume of 20 μ l containing 1X LC-DNA Master SYBR Green I mix, 4mM MgCl₂, 0.5 μ M of each primer and 2 μ l cDNA. Forward and reverse primer sequences for μ - and m-calpain amplification are: agtcgtgccgcagcatggtga, acttgtccaggcaaaacttccg and gccatcaagtacctcaaccagg, gctccttggcagatgtctgtg, respectively.

After a denaturation step (10 min at 95°C), amplification was carried out for 40 cycles of 10s at 95°C, 5 s at 58°C, and 10 s at 72°C. At the end of the run, the specificity of the reaction was confirmed by analysing the melting curves. Crossing points were calculated using the second derivative maximum method in the LightCycler[®] software. Standard curves were done by plotting crossing points versus log concentration of serial dilutions of plasmids containing μ - and m-calpain cDNA. Transcript concentration of both isoforms was determined from the standard curve and compared between young and old samples.

6-Cell fractionation

Frozen muscle (100mg) was homogenized in PBS (137 mM NaCl, 2.68 mM KCl, 4 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.5) containing 1 mM NaN₃, 5 mM EGTA, 0.5 mM DTT and protease inhibitor cocktail (Roche). The homogenates were sonicated and centrifuged 1 h, 100 000 g at 4°C. Supernatants were kept and designated as the cytosolic fractions. Pellets were further homogenized in PBS containing 1 mM NaN₃, 5 mM EGTA, 1 mM DTT, protease inhibitor cocktail and 0.1 % Triton X-100, left on ice for 15 min and centrifuged 10 min, 1 000 g at 4°C. Supernatants were kept and designated as the particulate fractions.

7-Calpain activities

Calpain activities were assayed using fluorescein isothiocyanate-labeled casein (FITC-casein) as a substrate and by measuring the fluorescence of a 2.5% trichloroacetic acid supernatant (Wolfe et al 1989). Briefly, 50 μ L of sample were mixed with 50 μ L of FITC-casein (250 mM Tris-acetate pH 7.5, 250 mM KCl, 6.75 mg/mL casein-FITC, 85 mM DTT) and 25 μ L of CaCl₂ (25 mM) to start the reaction. As a reference, EGTA (10 mM) was added instead of

CaCl₂. After a 30 min incubation at 37°C, 125 µL of stop solution (5% TCA, 0.01% Triton X-100) was added and the samples were centrifuged 10 min, 3500 g at 4°C. The 200 µL supernatants were diluted in 800 µL phosphate buffer (0.3 M Na₂HPO₄) and fluorescence was measured. The results are expressed in fluorescence unit (FU)/mg of protein. One FU corresponds to the amount of enzyme able to increase the fluorescence intensity ($\lambda_{\text{exc}} = 495$ nm, $\lambda_{\text{em}} = 518$ nm) of the supernatant by 1 unit for 30 min at 37°C compared to the reference without calcium (EGTA).

8-Calpain purification

Frozen muscle (600 mg) was homogenized in buffer A (20 mM Tris-buffer pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM NaN₃, 1 mM DTT). Homogenates were sonicated and centrifuged 15 min, 5000 g at 4°C. Supernatants ionic strength was adjusted to 1 M NaCl and pH to 7.5. After filtration through a 0.45 µm filter and protein quantification, samples were applied to a 1 mL hydrophobic interaction column (HiTrap Phenyl FF) first equilibrated with buffer A containing 1 M NaCl. Elution was carried out with buffer A (0 M NaCl) containing 1% ethyleneglycol. Both calpains were recovered in the elution peak whereas calpastatin didn't bind to the column (Cottin et al., 1994). Calpain activities were measured and positive fractions were pooled and loaded onto a 1 mL anion exchange column (HiTrap DEAE FF) first equilibrated with buffer A (0 M NaCl). Elution was realised with a two step ionic strength method. First, buffer A containing 0.2 M NaCl gave a peak containing µ-calpain. Then, buffer A containing 0.35 M NaCl allowed to recover m-calpain.

9-In vitro digestion of α-fodrin

Frozen muscle (100 mg) from 3 months old rats was homogenised in 50 mM Tris-buffer pH 7.5. After a quick centrifugation (1000 g 5 min at 4°C), supernatant (100 µg of proteins) was incubated with µ-calpain purified from rabbit muscle (Cottin et al., 1994). CaCl₂ or EGTA (5 mM) was added to the reaction and tubes were left 10 min at room temperature. The reaction was stopped by addition of sample buffer (50mM Tris-HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, 30% glycerol, 2% β-mercaptoethanol).

10-Calpastatin activity

Frozen muscle (600 mg) was homogenized in buffer A (20 mM Tris-buffer pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM NaN₃). Homogenates were sonicated and centrifuged 15 min, 5000 g at 4°C. Supernatants were heated for 10 min at 90°C, cooled on ice for 5 min and centrifuged for 10 min 12 000 g at 4°C. Supernatants were then used in a calpain activity assay, using calpains previously purified from rabbit (Cottin et al., 1994).

The assays were carried out like described in paragraph 4. The 50 µl samples contained 10 µL of purified enzyme (2 µg) and 40 µL of heated supernatant (10 µg proteins). The results are expressed in percentage of calpain activity.

11-Caspase 3 activity

Frozen muscles (400 mg) were homogenized in ice-cold buffer (pH 7.5) containing 20 mM HEPES, 20 % glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.1 % Triton X 100 and 1 mM DTT. The homogenates were centrifuged at 1500 g (5 min., 4°C). Cytosolic proteins from the resulting supernatant were extracted by three successive centrifugations (6000 g, 5 min, 4°C). The last supernatant was stored at -80°C until caspase 3 activity measurement.

Caspase 3 activity was determined by measuring the hydrolysis of the fluorogenic substrate Ac-DEVD-AMC in the presence or absence of the specific inhibitor Ac-DMQD-CHO. To measure caspase 3 activity, 50 μ l containing 50 μ g of cytosolic proteins were incubated with 50 μ l of reaction buffer (100 mM PIPES, 0.2 mM EDTA, 20 % Glycerol, 0.1 mM DTT, 0.1 mM Ac-DEVD-AMC) containing or not 0.1 mM of the caspase 3 inhibitor IV, Ac-DMQD-CHO. Caspase 3 activity was determined by measuring the accumulation of the fluorogenic cleavage product (AMC) at 37°C during 1 h using a luminescence spectrometer FLX800 (Biotek). Fluorescence was recorded continuously at 380 nm excitation wavelength and 440 nm emission wavelength. The difference between the rates of substrate hydrolysis recorded with or without the caspase inhibitor in the reaction medium was calculated, and the final data were corrected by the amount of protein in the reaction.

12-Protein quantification

Protein concentrations were determined using a Coomassie Protein assay reagent with BSA as standard.

13-Densitometric analysis

The apparent densities of bands on the membranes were estimated after scanning with a video-densitometer (Gel-Doc 2000, Biorad). The linearity response and the reproducibility of densitometric analysis were controlled by duplicate calibration curves.

14-Statistical analysis

All data are expressed as means \pm S.E.M. and are representative of an average of at least three separate experiments. Each single experiment was conducted with frozen muscle pooled from 6 rats of each group of age. The statistical significance of the difference between multiple groups was determined using ANOVA followed by a Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Muscle aging and calpain subcellular distribution

Many cytoskeletal and membrane bound proteins belong to calpain substrates, and a membrane activation model has already been proposed by several authors (Suzuki and Sorimachi, 1998). In that context, changes in subcellular localisation of calpains could be linked to a change of activity during aging. Subcellular distribution of the enzymes and their inhibitor was compared after a 100 000 g centrifugation of muscle extracts from 3 or 24 months old rats followed by Western blot detection (Aragon et al., 2002). Results shown in Fig. 1A first indicated a major cytosolic representation of both ubiquitous calpains and an exclusive cytosolic localisation of calpastatin. After signal quantification, the lower proportion of μ - and m-calpains in the particulate fraction was depicted in Fig. 1B. However, no significant change in the subcellular distribution of the proteases could be noticed between young and old muscle extracts.

Muscle aging and calpain/calpastatin expression levels

In order to study changes in ubiquitous calpains and calpastatin expression level during aging, Western blots were carried out using muscle extracts from 3 and 24 months old rats. As shown in Fig. 2A and B, the calcium-dependent proteolytic system underwent significant changes during muscle aging. μ -calpain expression increased by 38% between 3 and 24 months. Calpastatin, the endogenous calpain inhibitor, showed a 28% decrease of its expression level between young and old specimen. m-calpain level didn't change with muscle aging. At last, the expression of the small subunit was quantified. Although its level increased by 16% between 3 and 24 months, this difference was not significant.

Specific mRNA levels for μ - and m-calpains were quantified by real time RT-PCR. As shown in Fig. 2C, μ -calpain transcripts increased 1.7 fold between 3 and 24 months. On the other side, m-calpain mRNA level was unchanged in both conditions. This data are in accordance with what we observed at the protein level, suggesting that muscle aging triggers an up-regulation of μ -calpain expression at the transcriptional level.

According to the present data, changes in calpain expression level varies between both isoforms. μ -calpain expression level would be more sensitive to aging than m-calpain in muscle. However, altogether, the results clearly indicated a change of the calpain/calpastatin balance with muscle aging.

Muscle aging and calpain activity

We next tested the calpain activity, from 3 and 24 months old rat muscles. Crude extracts were first submitted to an hydrophobic interaction chromatographic (HIC) column in order to discard calpastatin whose presence would interfere with the protease activity (Cottin et al., 1994). Both μ -and m-calpains were eluted in the same fractions as controlled by Western blot using specific antibodies (Fig. 3A). Positive fractions (10 to 13) were pooled and protein

amounts were measured. FITC-casein was then used as a substrate to measure the calcium dependent activity of the pool, expressed in fluorescence units (FU). Specific activities were calculated and expressed in fluorescence unit per mg of protein. As shown in Fig. 3B, calpain activity was 3.3 times higher in 24 months than in 3 months muscle extract (4957 versus 1477 FU/mg). This experiment allowed us to measure the overall calpain activity. We were able to confirm that in muscle, it increases during aging, like it has been shown previously in other tissues (Manya et al., 2002).

Showing that global calcium-dependent activity was indeed greater in old muscles, was a first step in our study. However, we next wanted to measure calpain activities individually. For that purpose, the pooled positive fractions collected from the HIC column were loaded onto an anion exchange chromatography column. Elution was performed using a two step procedure. μ -calpain was first collected by running a 0.2 M NaCl buffer and m-calpain elution was done by raising the ionic strength up to 0.35 M NaCl. These conditions have been previously used in our laboratory (Cottin et al., 1994). Fractions were collected and μ - and m-calpains were identified by Western blots (Fig. 4A). As previously described fractions positive for μ - and m-calpains (29 to 31 and 46 to 48, respectively) were pooled and calcium-dependent activity was measured for each pool. The protein content was then quantified and μ - and m-calpains specific activities were calculated. As depicted in Fig. 4B, enzymatic activity of both proteases significantly increased with age. μ -calpain specific activity was 3.1 times higher in old muscle extracts than in young ones (14745 versus 4627 FU/mg, Fig. 4B), while m-calpain activity increased by 2.3 times between 3 and 24 months (27 890 compared to 11 820 FU/mg, Fig. 4B).

Muscle aging and α -fodrin degradation

All the data reported above were based on in vitro activity assays using FITC-casein as a substrate. To confirm the increase of calpain activity with muscle aging in vivo, we compared the levels of α -fodrin in young and old rats muscle by Western blot using specific antibodies. Cleavage of this 240 kDa cytoskeletal protein has been used in different studies as an indication of calpain activity in vivo (Manya et al., 2002; Rice and Blough, 2006). As shown in Fig. 5 the level of full length α -fodrin decreased with muscle aging. After signal quantification, a 40 % reduction of the 240 kDa band was estimated between 3 and 24 months extracts ($P < 0.05$). In the mean time, we could also detect an increase of the 150 kDa degradation product. Although the intensity of the band was 20% higher in old samples than in young ones, this difference was not significant (Fig. 5B). According to some researchers, α -fodrin can also be cleaved by caspase 3 (Janicke et al., 1998). In order to clearly show that what we observed was linked to calpain activity, we carried out an in vitro digestion assay. Western-blot analysis of the digests clearly indicate a reduction of native fodrin in the presence of calcium (Fig. 5C). Altogether these data confirmed in vivo the rise in calpain activity we observed in FITC-casein proteolysis assays.

Muscle aging and calpastatin activity

As previously mentioned, calpain activity is tightly regulated in vivo by a specific endogenous inhibitor, calpastatin. It was thus very important for our study to next address the evolution of calpastatin activity during muscle aging. This protein being heat-stable (Goll et al., 2003), crude extracts were first heated at 90°C for 10 min in order to discard most other proteins including calpains (data not shown). The presence of calpastatin was then checked by Western blot using specific antibodies (Fig. 6A). The heated extracts from 3 or 24 months old rats muscle were used in a calpain assay using μ - or m-calpain purified from rabbit muscle

(Cottin et al., 1994). Fluorescence was measured and compared to a control without added calpastatin. Results were then expressed as a percentage of calpain activity inhibition. As shown in Fig. 6B, calpastatin dependent inhibition of m-calpain activity significantly decreased, from 71% in 3 months extracts to 48% in 24 months old muscle. Surprisingly, no apparent effect could be detected on μ -calpain activity between young and old muscle samples (Fig. 6B).

Although a discrepancy of calpastatin activity behaviour between both ubiquitous calpains was noticed, the present results confirmed a global tendency of calcium-dependent activity to increase with muscle aging.

Muscle aging and caspase-3 activity

Given the numerous cross-talks between calpains and caspases in the regulation of cell death (Harwood et al., 2005; Pineiro et al., 2007), we decided to study the behaviour of caspase-3 activity during muscle aging. In this experiment, cytosolic proteins were extracted from young and old muscles and incubated for one hour with a fluorogenic substrate (Ac-DEVD-AMC) in the presence or absence of the specific inhibitor (Ac-DMQD-CHO). As indicated in Fig. 7, no significant difference could be detected between 3 months and 24 months muscle extracts. The same result was obtained with various incubation times (data not shown).

It thus appears that caspase-3 activation is not associated with the increased calcium-dependent proteolysis observed in old rat muscle.

Discussion

All data reported here unambiguously showed a global increase of the calcium-dependent proteolytic activity during muscle aging. Western blot analysis and mRNA level

quantification indicated an up-regulation of μ -calpain expression (Fig. 2). This result corroborated studies realised in cardiac and skeletal muscles where calpain expression was shown to be up-regulated in the course of aging (Dardevet et al., 1995; Johnson and Hammer, 1993). Surprisingly m-calpain mRNA and protein levels remained unchanged between young and old muscle in our conditions. Concerning the endogenous inhibitor, we found a 28 % decrease of its protein level in old muscle compared to young. A decline of calpastatin amount associated with aging has already been observed by others in mice kidney, or human erythrocytes (Manya et al., 2002; Schwarz-Benmeir et al., 1994). This drop in intracellular calpastatin rate could be put together with the loss of muscle mass occurring during sarcopenia. Indeed, overexpression of a calpastatin transgene has been shown to trigger an increase in the number of muscle fibres and prevent atrophy after unloading (Tidball and Spencer, 2002).

An up or down-regulation of enzyme expression level doesn't necessarily reflect a modification of intracellular activity. It has been shown for instance that calpain mediated proteolysis could be modulated simply by translocation of the proteases to the membranes (Molinari et al., 1994). In that context we compared the enzymes subcellular localisation in young and old muscle. First, only a small proportion of calpains was localised in the particulate fraction, while calpastatin was exclusively found in the cytosol (Fig. 1). This result confirmed the possible relocation of the proteases away from the inhibitor and in the vicinity of their specific substrates upon activation (Gil-Parrado et al., 2003). However, we were unable to show any change in calpain and calpastatin distribution with muscle aging. Further experiments will be done using immunohistochemistry techniques to analyse more closely this aspect.

Altogether these data pointed to an imbalance of the calpastatin/calpains ratio in favour of an increased proteolysis in muscle during aging. Our activity assays confirmed this statement.

First, the overall calpain activity was found to be significantly higher in 24 months old muscle than in 3 months old (Fig. 3). Similar increases of calpain activity have been previously associated with aging (Nixon, 2003). Looking more closely at each individual activity (μ - or m-calpain) we found that both enzymes were significantly more active in old specimen than in young ones (Fig. 4). This result corroborated the Western blot data for μ -calpain. However, m-calpain specific activity was found to be higher in 24 months old muscle, even though its expression was identical in 3 and 24 months old muscle. The lack of correlation between calpain expression level and activity has been widely noticed and just confirmed the role of calpain activity regulators (Averna et al., 2007). In the present case, m-calpain activity could be up-regulated by phosphorylation through the ERK/MAPK pathway (Glading et al., 2004). Calpain dependent proteolysis in vivo results from the balance between the activities of the proteases and their inhibitor. We showed a significant decrease of calpastatin mediated inhibition of m-calpain with aging (Fig. 6). This result probably reflected the lower amount of inhibitor we found in old muscle extracts compared to young ones. Similar behaviour of calpastatin activity during aging has been reported earlier (Schwarz-Benmeir et al., 1994). On the contrary, the lack of effect observed on μ -calpain activity was unexpected. This age-related discrepancy between calpastatin behaviour on the two ubiquitous enzymes could be explained by the existence of a differential regulation by distinct phosphorylated forms of the inhibitor like postulated in the past (Salamino et al., 1994). Moreover it is important to highlight that in our study, we tested calpastatin activity on each purified enzyme (μ - and m-calpain), while most of the reports have been done using one single calpain isoform (Averna et al., 2007; Schwarz-Benmeir, 1994).

If we focus on m-calpain only, the amount of protease did not change with aging, but its higher activity in old muscle together with a decrease of calpastatin level and inhibition indicated an overall increase of m-calpain activity in 24 months old rat muscles. Similarly, we

could show that μ -calpain level and activity were higher in old muscle. This result combined with a decrease of the inhibitor resulted in a significant rise in the isoform's activity with aging. In conclusion, calpain-dependent proteolysis was up-regulated in the course of muscle aging.

Since the above-mentioned results were obtained from in vitro activity assays, we needed to confirm them in vivo. Alpha-fodrin, together with β -spectrin, is a major component of the membrane associated cytoskeleton of muscle fibres (Zhou et al., 1998). Full length protein (240 kDa) has been shown to be specifically cleaved by calpains into a 150 kDa breakdown product. This characteristic has been recently used as an indicator of calpain activity in muscle (Razeghi et al., 2007; Rice and Blough, 2006; Takamure et al., 2005; Xiao et al., 2003). In our Western blot experiments, we showed a significant decrease of native α -fodrin in 24 months old muscle together with an increase of the 150 kDa degradation product (Fig. 5). This result confirmed in vivo the up-regulation of calpain activity with muscle aging.

In conclusion, in the present study, we clearly showed an involvement of the calcium dependent proteolytic system in sarcopenia. The enhanced activity we observed could be explained by an increase of the resting cytosolic calcium levels in skeletal muscle of aged animals (Frayssé et al., 2006). However sarcopenia is a multifactorial disorder and other deregulations have been described in aged muscle. Reactive oxygen species (ROS) accumulation is one probable factor responsible for muscle loss during aging (Fulle et al., 2004). Sarcopenia has also been associated with apoptosis (Marzetti and Leeuwenburgh, 2006). Interestingly, apoptosis is tightly linked to ROS accumulation and calcium homeostasis in mitochondria and could involve calpain activity (Fulle et al., 2005 ; Dirks et al., 2006). Interestingly, we did not find any significant change in caspase3 activity between 3 and 24 months old extracts (Fig. 7). Similar observations have already been reported by others (Leeuwenburgh et al., 2005). Altogether, these data strongly suggest that, during sarcopenia,

calpains could induce apoptosis by a caspase3 independent mechanism. Further experiments are currently being done in our laboratory to identify what calpain regulated pathways could be affected during muscle aging.

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Legends

Fig. 1 : Comparison of calpains and calpastatin levels in young and old rat muscle in cytosolic and particulate fractions.

Subcellular fractions were prepared from rat muscles as described in Material and Methods section. 50 μ g of protein were run on a 10% SDS-PAGE. Immunodetection was carried out using specific antibodies for μ -calpain, m-calpain and calpastatin (A). Pictures shown are representative of at least 3 separate experiments. C: cytosolic fraction, P: particulate fraction. The level of μ -calpain and m-calpain was quantified and expressed as a percentage of the level obtained in the cytosolic fraction (B). Bars on the graph represent the standard deviation.

*Significantly different from the control ($P < 0.01$).

Fig. 2: Expression of μ -calpain, m-calpain, calpastatin and calpain small subunit (ssu) in muscle from young (3 months) and old rats (24 months).

Proteins were extracted and 50 μ g were subjected to SDS-PAGE. Immunodetection was carried out using specific antibodies for μ -calpain, m-calpain, calpastatin and ssu (A). The level of μ -calpain, m-calpain, calpastatin and ssu was quantified and expressed as a percentage of the level obtained in the young rats muscle (B). Total RNA were extracted and 300 ng were reverse transcribed using random primers. Standard curves were done by plotting crossing points versus log concentration of serial dilutions of plasmids containing μ - and m-calpain cDNA. Transcript concentration of μ - and m-calpain was determined from the standard curve. Results are expressed as a percentage of the level obtained in the young rats muscle (C). Pictures shown are representative of at least 3 separate experiments. Bars on the graph represent the standard deviation. *Significantly different from the control ($P < 0.01$), **significantly different from the control ($P < 0.05$).

Fig. 3: Quantification of calcium-dependent proteolytic activity in young and old rat muscles. Calpain activity was quantified after a HiTrap Phenyl FF column as described in Material and Method section. Fractions positive for both μ - and m-calpains were identified by Western blot (A). Elution profiles were identical in young and old samples. Specific activity levels are expressed as arbitrary units from the fluorescence of FITC-casein and values are the means \pm S.D. for at least 3 separate experiments (B). *Significantly different ($P < 0.05$).

Fig. 4: Quantification of μ -calpain and m-calpain activities in young and old rat muscle. Proteins were eluted from a HiTrap DEAE FF column as described in Material and Method section. Fractions were collected and tested for μ - and m-calpains by Western blot (A). Identical elution profiles were obtained in old and young samples. Specific activity levels are expressed as arbitrary units from the fluorescence of FITC-casein and values are the means \pm S.D. for at least 3 separate experiments (B). *Significantly different ($P < 0.01$).

Fig. 5: α -fodrin degradation in young and old rat muscle. Proteins were extracted and 50 μ g were subjected to SDS-PAGE. Immunodetection was carried out using specific antibodies for α -fodrin (A). The level of fodrin 240 kDa and 150 kDa fragments was quantified and expressed as a percentage of the level obtained in the young rat muscles. Bars on the graph represent the standard deviation (B). Protein extracts from 3 months old rat muscles were incubated with purified μ -calpain with 5 mM CaCl_2 or EGTA, as described in Material and Method section. Samples were subjected to SDS-PAGE and immunodetection was carried out using specific antibodies for α -fodrin (C). Pictures shown are representative of at least 3 separate experiments. *Significantly different from the control ($P < 0.05$)

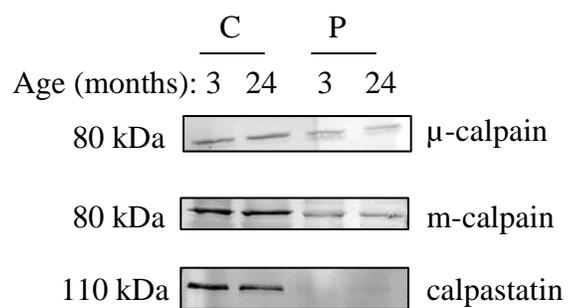
Fig. 6: Quantification of calpastatin activity in young and old rat muscle.

Calpastatin activity was quantified as described in Material and Methods section. Calpastatin level was controlled by Western blot in the heated samples (A). Specific activity levels are expressed as a percentage of calpain activity inhibition. Data shown are the means \pm S.D. for at least 3 separate experiments (B). *Significantly different ($P < 0.01$).

Fig. 7: Quantification of caspase 3 activity in young and old rat muscle.

Caspase 3 activity was quantified as described in Material and Methods section. Specific activity levels are expressed as arbitrary units from the fluorescence of Ac-DEVD-AMC and values are the means \pm S.D. for at least 3 separate experiments.

(A)



(B)

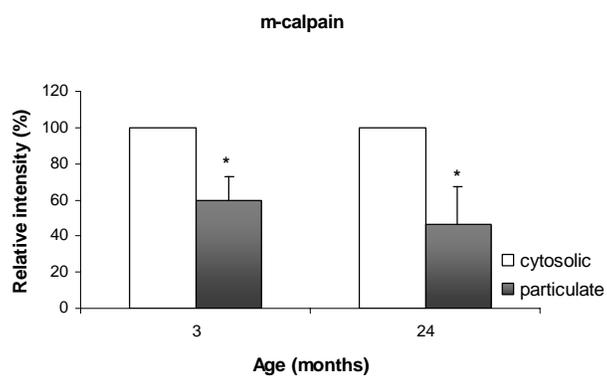
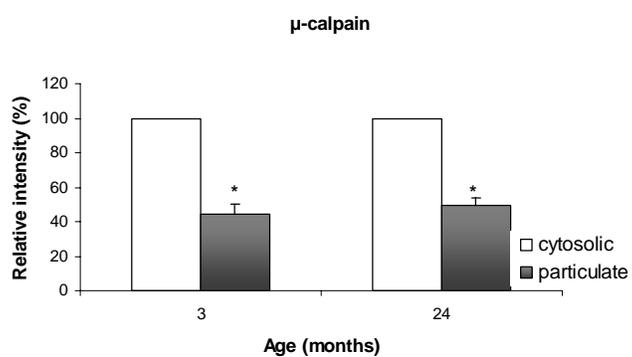
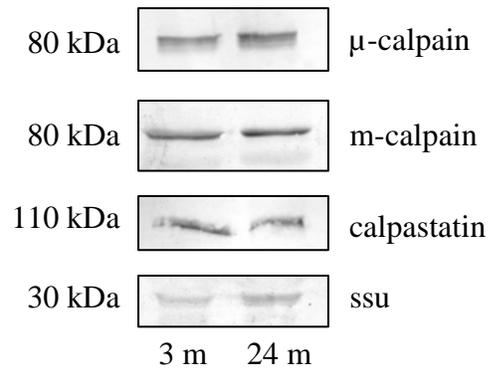
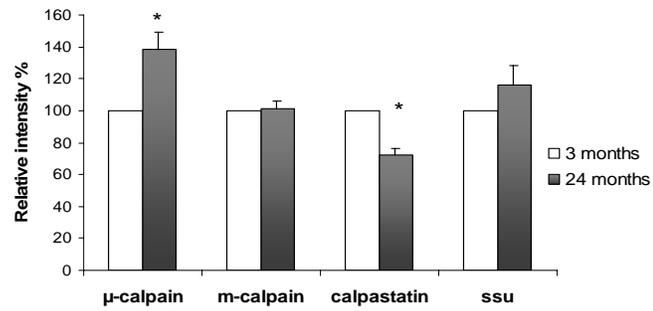


Figure 1

(A)



(B)



(C)

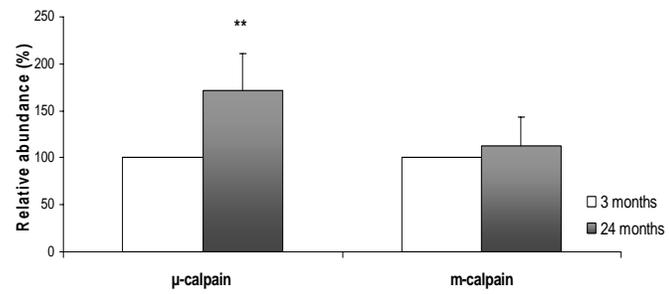
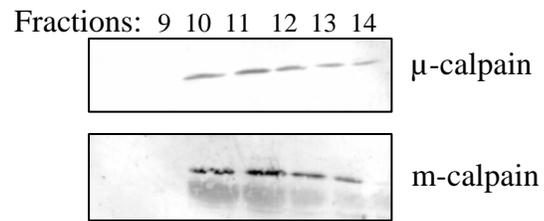


Figure 2

(A)



(B)

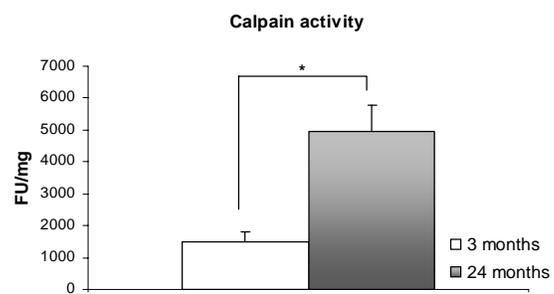
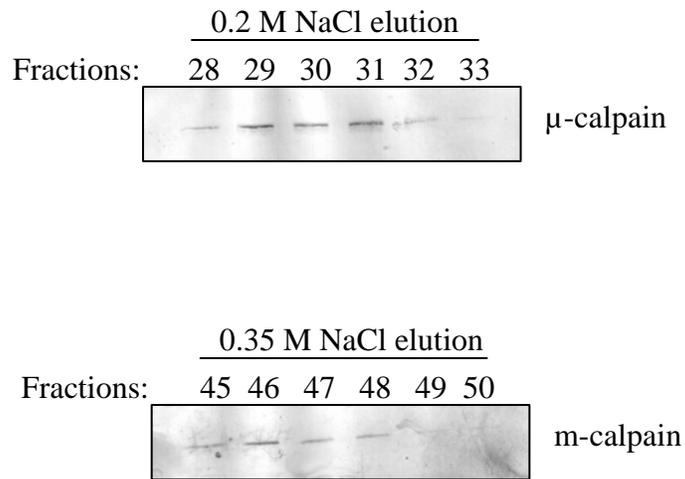


Figure 3

(A)



(B)

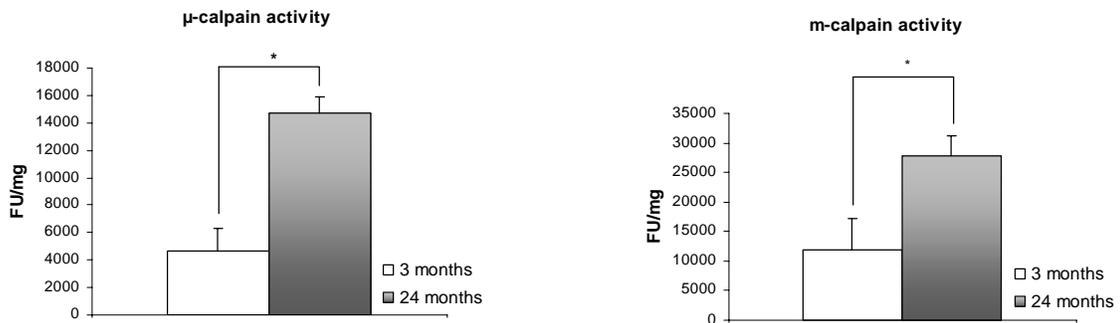
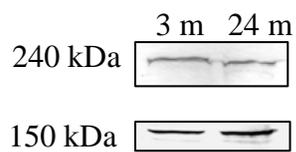
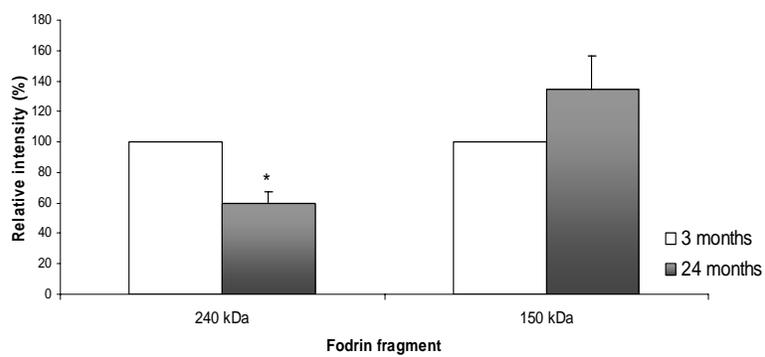


Figure 4

(A)



(B)



(C)

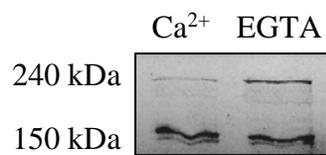
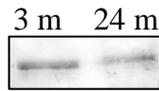


Figure 5

(A)



(B)

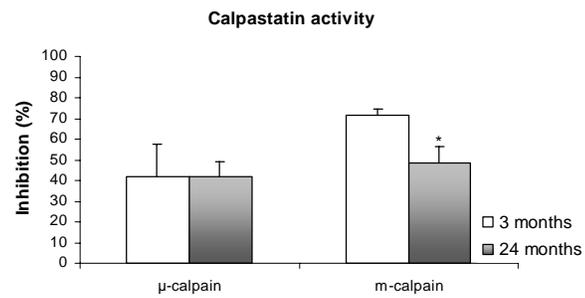


Figure 6

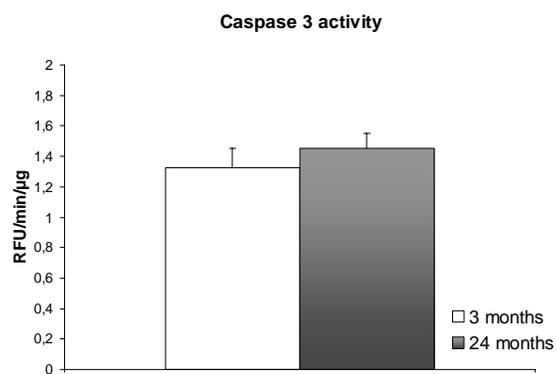


Figure 7