Immunological detection of m- and µ-calpains in the skeletal muscle of Marchigiana cattle

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Abstract

Calpains are Ca²⁺-dependent proteases able to cleave a large number of proteins involved in many biological functions. Particularly, in skeletal muscle they are involved in muscle tenderizing during post mortem storage. In this report we analyzed the presence and expression of µ- and m-calpains in two skeletal muscles of the Marchigiana cattle soon after slaughter, using immunocytochemical and immunohistochemical techniques, Western blotting analysis and Casein Zymography. Therefore, the presence and the activity of these proteases was investigated until 15th day post mortem during normal process of meat tenderizing. The results showed m- and µ-calpain immunosignals in the cytoplasm both along the Z disk/I band regions and in the form of intracellular stores. Moreover, the expression level of µ-calpain but not m-calpain decreased after 10 days of storage. Such a decrease in µ-calpain was accompanied by a gradual reduction of activity. On the contrary, m-calpain activity persisted until 15 days of post mortem storage. Such data indicate that expression and activity of both µ-calpain and m-calpain analyzed in the Marchigiana cattle persist longer than reported in literature for these proteases.

Introduction

Calpains are a large family of intracellular cysteine proteases. To date, 14 members have been identified, which are expressed in an ubiquitous or tissue-specific manner. In skeletal muscle, the calpain system consists of three proteases, ubiquitously expressed isoforms µ-calpain, m-calpain, and p94 (or calpain 3). The terms µ-calpain and m-calpain refer to the micromolar Ca²⁺-requiring (µ-calpain) and millimolar Ca²⁺-requiring (m-calpain) proteases, respectively. Both proteases are heterodimers, each one composed of a 80 kDa catalytic subunit and a 28 kDa regulatory subunit. The 28 kDa subunit is identical in the µ- and m-calpains. Both the 80 kDa and the 28 kDa subunits undergo auto-proteolysis from the N-terminus resulting in the conversion of the 80 kDa subunit into a 76 kDa form through a 78 kDa intermediate. In both µ- and m-calpains the 80 kDa subunit is divided into four domains based on the amino acid sequence: domain I or NH₂-terminal domain; domain II; domain III which contains two potential EF-hand Ca²⁺ binding sites. Associated with the calpain proteolytic enzyme family is the calpain-specific endogenous inhibitor, calpastatin. Calpastatin contains 4 inhibitory domains, each one capable of inhibiting calpain activity.

Key words: m-calpain, µ-calpain, skeletal muscle, Marchigiana cattle, immunohistochemistry, Electron Microscopy.

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Materials and Methods

Animals and tissue preparation

Samples of masseter and diaphragm muscles were collected from ten males 24-month-old Marchigiana cattle obtained from a local slaughterhouse (Campolattaro, Benevento, Italy). The animals were born and farmed in the skeletal muscle and breed examined.
the province of Benevento (Italy) and their meat was intended for human consumption. Sampling was made immediately after death. Cubic fragments of 1 cm of length underwent different treatments for immunohistochemistry and scanning electron microscopy (SEM) analysis. For immunohistochemistry, muscle samples were submerged in 2-methylbutane, extra pure (Acrros Organics, NJ, USA) for 5 seconds and then frozen in liquid nitrogen before being cut at the cryostat. For immunogold-labeling SEM analysis, specimens were submerged in PBS for 1 h at room temperature (RT). Additional muscle samples were stored at 4°C, collected and frozen (-80°C) at 0, 5, 10 and 15 days post-mortem for Western blotting analysis and Casein Zymography.

Immunohistochemistry

Frozen samples of masseter and diaphragm muscles were serially cut at a cryostat in transversal and longitudinal sections of 10 µm. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide for 20 min at RT, the sections were rinsed in 0.01 M phosphate buffered saline (PBS), pH 7.4, for 15 min. Primary antibodies were monoclonal antibodies raised in mouse against domain III/IV of m-calpain (C-268; Sigma, Sant Louis, MO, USA) and polyclonal antibodies raised in rabbit against domain IV of µ-calpain (C-5611; Sigma). Primary antibodies were diluted 1:50 and applied on the sections overnight in a moist dark chamber at 4°C. The other components of the immunological reaction were contained in the Envision Dako (K4006, DakoCytomation, Glostrup, Denmark) employed with mouse antibodies and Vectastain Elite ABC Kit (PK-6101; Vector Laboratories Inc., Burlingame, CA, USA) employed with rabbit antibodies. The final staining was performed using a solution of 3-3’-diaminobenzidine tetrahydrochloride (DAB; Sigma) of 10 mg in 15 mL 0.5M Tris buffer, pH 7.6, containing 0.03% hydrogen peroxide. The images of the immunostainings were acquired and photographed using the microscope Leica DMRAx2 (Leica, Wetzlar, Germany) equipped with a DC300F digital camera.

Negative controls were obtained substituting the primary antisera with PBS or normal serum in the specific step, or alternatively, by absorbing each primary antisera with an excess of the relative peptide (100 µg of peptide/mL of diluted antisera).

Immunogold-labeling SEM analysis

Samples were incubated for 2 h in a solution containing normal goat serum (900.077; Aurion, Wageningen, The Netherlands) diluted 1:10 in PBS, and then incubated with primary monoclonal antibodies raised in mouse against the domain III/IV of m-calpain (C-268; Sigma) and primary polyclonal antibodies raised in rabbit against the domain IV of µ-calpain (C-5611; Sigma), diluted 1:50 in PBS, overnight at 4°C. After washing in PBS, the samples were incubated with gold-conjugated goat anti-mouse IgG (806.022, Aurion) and goat anti rabbit IgG (106.011, Aurion) diluted 1:200 in PBS for 1 h at RT. The secondary antibody was conjugated with gold particles of different sizes (5 and 15 nm). After washings in PBS, samples were fixed in 2.5% glutaraldehyde in 0.1 M Cacodylate buffer, at pH 7.2, for 30 min. After washings with distilled water, samples were subjected to silver enhancement (500.055, Aurion). The silver enhancement process enables the use of antibodies conjugated with small (6 nm) gold particles allowing fast penetration and high labeling efficiency.41 Samples were then dehydrated through an ethanol series and dried to the critical point. The specimens, mounted on stubs, were examined under a LEO 435 VP scanning electron microscope at variable pressure (80-120 Pa) in the backscattered electron mode, which allows the detection of gold particles associated with cells even if they are located intracellularly.42 Since the samples were not coated by gold, only conjugated gold deriving from immunocytochemical reaction was observed by SEM and photographed.

Western blot analysis

Proteins from masseter and diaphragm muscle samples were extracted with Lysis buffer (220 mM D-Mannitol, 70 mM Saccharose, 1 mM EDTA, 20 mM Tris pH 7.4, containing protein inhibitors 2 mM PMSF, 1 mM pepstatin A, 2 mM trypsin inhibitor from chicken egg white). Muscle samples were homogenized with ultra-turrax T25 (IKA-Labortechnik, Staufen, Germany) for three times at 500 rpm, 800 rpm and 14,000 rpm for 10 min each. The supernatants were collected and underwent protein determination with the Bio-Rad dye protein assay (Bio-Rad laboratories, Hercules, CA) twenty-five µL of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT, pH 6.8) were added to 75 µL of the supernatant and 15 µL sample were loaded into each well of the gel. Before loading the samples, the gel was prerun for 15 min at 80 V (running buffer: 25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.3).

Electrophoresis was carried out at 80 V for 3 h at 4°C. Gels were then removed, rinsed with deionized H2O, and incubated with shaking at RT in 100 mL of incubation buffer (50 mM Tris, 4 mM CaCl, 10 mM monothioglycerol, pH 7.5) for 1 h; gels were rinsed twice. The calpain activity was stopped by washing the gel overnight with shaking, using 20 mM Tris, 10 mM EDTA, pH 7.0. Gels were stained for 6 h with colloidal Comassie Brilliant Blue G4 and destained overnight with deionized H2O. Signals were detected by UV transillumination with Chemidoc (Bio-Rad).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Turkey’s test. The analysis were carried out with the Statistica version 7.0 statistical package (Statsoft inc., Tulsa, OK, USA). Data are expressed as mean ± SEM.

Casein zymography method

To determine activity of p- and m-calpain the casein zymography method based on the protocol described by Raser, Posner, and Wang43 was used. One gram of each sample was homogenized with ultra-turrax T25 (13,500 rpm) in 6 mL of extraction buffer (50 mM Tris, 3 mM EDTA, 10 mM Monothioglycerol; one tablet per 50 mL of Protease Inhibitor Cocktail Tablet COMPLETE, RAS Roche Applied Science, Mannheim, Germany; pH 8.0) and centrifuged for 30 min at 4°C and 15,000g. Each sample was run using 12.5% casein precast gel (Bio-Rad Laboratories, Hercules, CA) twenty-five µL of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT, pH 6.8) were added to 75 µL of the supernatant and 15 µL sample were loaded into each well of the gel. Before loading the samples, the gel was prerun for 15 min at 80 V (running buffer: 25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.3).

Homogenized samples were boiled at 98°C for 10 min/each. The supernatants were collected and underwent protein determination with the Bio-Rad dye protein assay (Bio-Rad laboratories, Hercules, CA) twenty-five µL of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT, pH 6.8) were added to 75 µL of the supernatant and 15 µL sample were loaded into each well of the gel. Before loading the samples, the gel was prerun for 15 min at 80 V (running buffer: 25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.3).

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Results

Since the results obtained for both masseter and diaphragm muscles were similar, we will refer to them as the skeletal muscles of Marchigiana cattle.

Immunohistochemistry

In the longitudinal sections of the skeletal muscle of Marchigiana cattle, both m- and µ-calpain immunopositivity could be seen (Figure 1a,b). The positivity was present for both calpains along Z disk/I band regions. In the A-band area the positivity was absent (Figure 1a,b).

Immunogold-labeling SEM analysis

Both m- and µ-calpain immunoreactive gold particles were detected in the cytoplasm (Figure 2). In particular, immunopositivity was localized along the Z disk/I band regions (Figure 2a,b) and in the intracellular stores (Figure 2c,d).

Western blot analysis

The expression of m- (Figure 3a) and µ-calpains (Figure 3b) was detected at 0, 5, 10 and 15 days of storage at 4°C in the masseter (Figure 3a) and diaphragm (Figure 3b) muscles. Both m- and µ-calpains showed a molecular mass of about 80 kDa. A significant decrease in the level of expression for µ-calpain was detected at the 10th and the 15th day (Figure 3b); m-calpain showed a significant decrease at the 15th day (Figure 3b). Densitometric analysis of the immunoreactive bands was performed and β-actin (molecular mass of about 42 kDa), as an internal marker, was used to normalize the optical density.

Casein zimography

Four bands of enzymatic activity were identified on the gels. Two of these bands were located on the top of the gels, where µ-calpain normally is positioned; the other two migrated further into gels, where m-calpain runs.43,45 We assumed that the two bands located on top of the gels corresponded to native and autolyzed µ-calpain activities (Figure 4, thin arrow), and the other two bands represent native and autolyzed m-calpain activities (Figure 4, thick arrow). The autolyzed forms of both µ- and m-calpain migrated slightly faster than the native forms.

Controls

Negative controls did not show specific immunostaining. Moreover, the incubation of m- and µ-calpain antiserum preincubated with its homologous antigen showed no immunoreactivity. In Western blotting analysis, rat skeletal muscle, used as a positive control, showed a band of about 80 kDa when antibodies against m- and µ-calpains were employed (Figure 3a,b).

Discussion

In this study, we report on the presence, level of expression and enzymatic activity of m- and µ-calpain in the masseter and diaphragm muscle of Marchigiana cattle.
Immunohistochemistry and immunogold-labeling SEM analysis revealed the presence of calpains both along the Z disk/I band areas and in intracellular stores. These results are in agreement with the current literature. In fact, in normal skeletal muscle the majority of calpains are located on or next to the Z-disk with very few in correspondence to the I-band, as well as in vesicles and subcellular organelles. Particularly, during the first few hours post-mortem, m- and µ-calpains are localized in subcellular organelles in the inner sarcoplasm, and then spread along Z disk/I-band areas of myofibrils beginning the transformation of muscle into meat.

The presence of m-and µ-calpains in correspondence to the Z disk/I band areas of the diaphragm and masseter muscles of Marchigiana cattle suggests that in these muscles the proteolytic action of calpains occurs on those proteins that are involved in keeping miofilaments attached to the myofibril. In fact, calpains rapidly cleave titin and nebulin at the point where these two polypeptides enter the Z disk. Titin and nebulin cleavage, together with that of desmin and filamin, release α-actinin, the principal Z disk protein, from the myofibril. Calpains do not degrade actin and myosin, the two major proteins in skeletal muscle myofibrils, implying that calpains had a limited and very specific subsite specificity. Calpains also degrade M proteins, tropomyosin and troponin, albeit at slower rates than titin and nebulin. In general, calpains cleave proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides or amino acids. Because of the limited specificity of the calpains, further degradation of myofibril proteins in aminocids requires the participation of other proteases. It seems likely that the proteasome plays a major role in the degradation of the active site of calpains, myosin and the other myofibrillar protein fragments. The proteasome, on the other hand, cannot degrade intact myofibrils or cytoskeletal complexes, likely because the entrance to the central cavity of the proteasome containing the active site is only 19-13 Å in diameter and is much too narrow to allow entry of myofibrils that range from 10 to 100 µm in diameter. Therefore, the calpains begin the process of muscle transformation in meat that in turn, requires other proteases to be completed. Among these, the caspase system could be active post-mortem and contribute to tenderization throughout an interaction with the calpain system. In fact, caspases may contribute to decrease the calpastatin level in the muscle aging and this, in turn, could result in the activation of calpains and thus reducing toughness.

In the skeletal muscle of Marchigiana cattle the level of expression of m- and µ-calpains...
and their enzymatic activity was detected up to 15 days post-mortem. Our results indicate a decrease in the level of expression of μ-calpain after 10 days of storage, while m-calpain expression persisted up to 15 days of post-mortem storage. The trend of activity of both μ- and m-calpain overlap with their expression pattern. It is well known that post-mortem activation of m-calpain is due to the increasing concentration of Ca²⁺. The activity of μ- and m-calpain is synergistic: μ-calpain contributes to early post-mortem proteolysis, while m-calpain is partially activated and contributes to tenderization during prolonged ageing. In the bovine skeletal muscles (longissimus dorsi, semimembranosus, triceps brachii and psoas major), the proteolytic activity of μ-calpain decreases rapidly during post mortem storage and very little activity can be detected after 48 h post-mortem storage at 4°C, so that only 10 to 20% of m-calpain activity remained after 144 h post-mortem. The proteolytic activity of calpains depends on the Ca²⁺ concentration and pH of the muscle during the post-mortem storage.

Our results indicate a greater persistence of expression and enzymatic activity of m- and μ-calpains in Marchigiana cattle skeletal muscle. Such disagreement could be ascribed to the type of muscle chosen or to the breed considered for this study. Certainly, further investigations, involving, also, ultrastructural analysis, may help to study the role of calpains in meat tenderness in the Marchigiana cattle.

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