Activation of skeletal muscle calpain-3 by eccentric exercise in humans does not result in its translocation to the nucleus or cytosol
calpain-3 was full-length or autolyzed. Other groups proposed that when calpain-3 becomes activated, it may move from a sarcomeric location to the nucleus, where it could mediate control of apoptosis in healthy individuals by interaction with IκBα/NF-κB pathways (2). Whether such nuclear translocation of calpain-3 actually occurs, however, has not been investigated. It has also been suggested that calpain-3 translocates to the cytosol following downhill treadmill running in mice (21). At rest, only a very small proportion of calpain-3 is evident in the cytosol in adult rat muscle (16, 20), although it is not known what proportion of calpain-3 is cytosolic in human skeletal muscle. No data are available to show whether such cytosolic translocation occurs following calpain-3 activation in human skeletal muscle.

In the present study, to investigate the likely site of action of calpain-3 in human skeletal muscle, we investigated calpain-3 in skeletal muscle from healthy individuals following eccentric exercise, which is the only physiological stimulus known to activate calpain-3 in vivo. In undertaking the study, we hypothesized that only a small proportion of the total calpain-3 pool in skeletal muscle would be present in the nucleus or the cytosol. We further hypothesized that, following eccentric step exercise, full-length and autolyzed calpain-3 remains bound within the muscle fibers, predominantly at the myofibrillar lattice, and does not translocate to the nucleus or the cytosol.

METHODS

Subjects

The present study is an extension of a larger study that is described elsewhere (31). For this study, tissue was available from a subsample of the participants (11 subjects for whom full data sets were available; mean ± SD: 24 ± 3 yr old, 183 ± 9 cm height, 79 ± 8 kg mass) in the previous study. All subjects were male, healthy, and physically active; for 6 mo prior to the study none had participated in resistance training, nor had they experienced recent trauma to the knee joint. Subjects were informed of all test procedures and associated risks before giving written informed consent. All experimental protocols were approved by the Danish Ethical Committee of Aarhus (J. nr. 20040159) and the La Trobe University Human Ethics Committees and carried out in accordance with the Declarations of Helsinki. In an additional study, another group of four healthy individuals (3 men and 1 woman; mean ± SD: 24 ± 8 yr old, 175 ± 16 cm height, 71 ± 13 kg mass) was recruited; from these subjects, we obtained resting muscle biopsies, from which single fibers could be isolated from the fresh tissue and examined for protein diffusibility. This protocol was approved by the Victoria University and La Trobe University Human Ethics Committees.

Eccentric Exercise Protocol

The exercise protocol is described in detail elsewhere (31). Briefly, the subjects performed a single bout of 30 min of bench-stepping at a predetermined step height of 110% of the lower leg length. Stepping up with one leg requires muscles involved in knee extension to work concentrically, while stepping down with the same leg that had been working eccentrically while the subjects were supine. Samples were immediately frozen directly in liquid N₂, or, from a small sample, fibers were aligned, laid in Tissue-Tek, and frozen in liquid N₂-cooled isopentane and stored at −80°C until analyzed for calpains. Only muscle biopsies obtained from the eccentrically working leg were used for analyses in the present study.

Human Skeletal Muscle: Preparations of Exercised Muscle

Enriched cytosolic and nuclear preparations. To establish whether calpain-3 translocates to the cytosol or the nuclei following exercise-induced activation, enriched cytosolic and nuclear fractions were prepared as described in detail elsewhere (30). Total protein in the final samples was measured using the QuantIt protein assay (QuBit, Invitrogen, Sydney, Australia), with BSA used as the standard. Samples were diluted to the same concentration for the cytosolic and the nuclear preparations, added (2:1 vol/vol) to 3× solubilizing buffer (SB; 0.125 M Tris·HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromphenol blue, pH 6.8), and stored at −20°C until Western blot analysis. From each cytosolic preparation, ~5 μg of total protein were analyzed; from each nuclear preparation, ~30 μg of total protein were analyzed. For analysis of cytosolic data, the amount of calpain-3 in the cytosol was expressed relative to the total calpain-3 pool in skeletal muscle. This was based on the amount of diffusible calpain-3 determined using the mechanically skinned fibers described below (see Fig. 3E). For analysis of nuclear data, the amount of calpain-3 in the nuclei was also expressed relative to the total calpain-3 pool in human skeletal muscle (see Fig. 4D).

Whole human muscle preparation. To determine the level of calpain-3 autolysis in muscle samples with all constituents present (i.e., unfraccionated), 10-μm cryostat (model CM1950, Leica) sections were cut for each muscle sample. Depending on the approximate diameter of the muscle sample, four to six sections were placed immediately in 50–70 μl of cold Na⁺ homogenization solution [165 mM Na⁺, 1 mM free Mg²⁺ (10.3 mM total Mg²⁺), 90 mM HEPES, 50 mM EGTA, 8 mM ATP, and 10 mM creatine phosphate (pH 7.10), 295 ± 10 mosmol/kgH₂O], where the strong EGTA buffering kept the free [Ca²⁺] very low (<10 nM) at all times. Samples were kept on ice for 30–60 min, during which they were vortexed a number of times; then they were added (2:1 vol/vol) to 3× SB. Samples were stored at −20°C until Western blot analysis.

Rat skeletal muscle preparations. With approval of the La Trobe University Animal Ethics Committee, male Long-Evans hooded rats (~6–8 mo old) were killed by overdose with isoflurane (4% vol/vol). EDL muscle was rapidly excised and immediately homogenized at room temperature using three 8- to 10-s pulses of a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 10 volumes of Na⁺ homogenization solution (see above). Whole muscle homogenate was diluted 1:40 in Na⁺ homogenization solution and then to a concentration of 2.5 μg wet wt muscle/μl with 3× SB and stored at −20°C for Western blot analysis.

Human Skeletal Muscle: Preparations of Nonexercised (Quiescent) Muscle

Relative amount of diffusible (cytosolic) calpain-3. To determine the diffusibility of calpain-3 in human skeletal muscle, our novel approach used for rat skeletal muscle fibers (16, 20) was adapted to human skeletal muscle fibers, allowing the proportion of any truly cytosolic calpain-3 to be determined. This approach prevents any sample loss that typically occurs during high-speed centrifugation steps employed to obtain pure cytosolic fractions. Fibers were collected from resting muscle biopsies obtained from healthy active individuals who had not undertaken a specific eccentric exercise regimen in the preceding week (n = 4). Immediately after the biopsy procedure, a bundle of muscle fibers were placed under paraffin oil and set on an ice pack to maintain the temperature at ~10°C. Individual fibers were dissected under paraffin oil, and their surface membrane, or sarcolemma, was removed by mechanical dissection along half the length of the muscle fiber (see Fig. 3A). The resulting
“skinned” region of the fiber (F-sk) was tied with surgical thread and cut off and placed in a small volume (10 μl) of physiological buffer (diffuse (Diff) solution: Na\(^+\) homogenization solution except with 129 mM K\(^+\), to isoionically replace the same amount of Na\(^+\) (final Na\(^+\) concentration = 36 mM)) at room temperature for 30 min, during which time it was vortexed four to five times. After 30 min, the skinned fiber segment (F-sk) was removed from the Diff solution and placed in a different tube containing 1× SB [2:1 (vol/vol) intracellular physiological buffer-3× SB] (see Fig. 3); 3× SB was also added (1:2 vol/vol) to the Diff solution, which now contained only the proteins that had diffused out of the skinned fiber in the 30-min wash period (16, 20). A similar-length segment of the intact region of the same muscle fiber (F-in) was collected directly into 1× SB (see Fig. 3A). All preparations were stored at −80°C until side-by-side Western blot analysis.

Crude fractions for determination of percentage of calpain-3 in nuclei. To determine the amount of calpain-3 in the nuclei as a percentage of the total calpain-3 in skeletal muscle, crude fractionation experiments were performed using human (“Pre” samples from the human exercise trial) and rat skeletal muscle samples. It was not possible to use the enriched preparations described above (which allowed us to obtain purified fractions); although these samples were very pure, a proportion of the sample (hence, possibly of the total calpain-3 pool) was discarded along the way. Homogenization and centrifugation steps described in Fig. 1 were followed to separate muscle into crude cytosolic, myofibrillar, and nuclear fractions. Na\(^+\) homogenization solution was used in all homogenization and resuspension steps, with the free [Ca\(^{2+}\)] strongly buffered to very low levels at all times.

Western blotting. Total protein from preparations was separated using 10% SDS-polyacrylamide gels or 10% Criterion Stain Free gels (Bio-Rad, Hercules, CA) (17) and then transferred to nitrocellulose. Membranes were exposed to anti-calpain-3 antibody and, in some cases for verification that a cytosolic fraction was obtained, to anti-μ-calpain antibody; then goat anti-mouse horseradish peroxidase secondary antibody was added to the membranes. Characterization of the various fractions was performed by probing for particular proteins, including lamin A/C (nuclear), GAPDH (cytosolic), creatine kinase (cytosolic), tropomyosin (myofibrillar), myosin heavy chain (MHC) II, and/or sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1). Bands were visualized using West Femto chemiluminescent substrate (Thermo Scientific), and densitometry was performed using Quantity One software (Bio-Rad). The relative positions of molecular mass markers were visualized, and images were captured under white light prior to chemiluminescent imaging, which was done without moving the membrane; the separate images were superimposed, as shown in Fig. 4. Full-length calpain-3 is observed as a 94-kDa protein that autolyzes to ~60-, 58-, and 55-kDa proteins when activated (4, 10, 27–29). One method by which Western blot data were quantified was by expression of the density of the bands of the autolyzed products relative to the total density of all the bands for calpain-3 (i.e., autolyzed and unautolyzed) in that sample. This indicated the proportion of calpain-3 that was autolyzed in a particular sample, irrespective of any minor differences in protein loading. To examine the effect
of exercise, data were normalized to the Pre value for a given subject. For calpain-3 and µ-calpain, the amount of diffusible protein in a given fiber was determined when matched Diff and F-sk were run side-by-side on a gel, together with F-in (see Fig. 3, B–D). The Diff sample contained all the components that had been freely diffusible (i.e., cytosolic) in the skinned fiber segment, and the F-sk sample contained the remaining nondiffusible components. Hence, the percentage of diffusible calpain-3 in the fiber was derived simply from the total of all calpain-3 bands (i.e., autolyzed and full-length) in the Diff sample expressed relative to the grand sum of all such bands in the Diff sample and the matching F-sk sample. The percentage of diffusible µ-calpain was calculated in a similar way.

Chemicals and Antibodies

All chemicals were obtained from Sigma (Sydney, Australia) unless otherwise stated. Primary antibodies were as follows: mouse anti-calpain-3 antibody (1:200 dilution; monoclonal 12A2, Novocasttra, Newcastle, UK), which also detects m-calpain (~82 kDa) in rodent, but not human, skeletal muscle (Mollica, Murphy, and Lamb, unpublished observation); mouse anti-µ-calpain (1:1,000 dilution, 1 µg/ml; clone 15C10, Sigma); rabbit anti-lamin A/C (1:1,000 dilution; catalog no. 2032, Cell Signaling, Beverly, MA); goat anti-creatine kinase (1:200 dilution; catalog no. sc-15161, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-GAPDH (1:10,000 dilution; clone 6C5, catalog no. ab 8245, Abcam, Cambridge, UK); mouse anti-SERCA1 [1:200 dilution, clone CaF2-5D2, Developmental Studies Hybridoma Bank (DSHB), University of Iowa]; MHC II (1:200 dilution; no. A4.74, DSHB); and mouse anti-tropomyosin (1:1,000 dilution; no. CH1, DSHB). Secondary antibodies were goat anti-mouse horseradish peroxidase (1:20,000 dilution; Bio-Rad), goat anti-rabbit (1:60,000 dilution; catalog no. 31460, Thermo Scientific, Rockford, IL), and rabbit anti-goat (1:20,000 dilution; catalog no. 81-1620 Invitrogen, Carlsbad, CA).

Statistics

Values are means ± SE unless otherwise stated. Data were analyzed using one-way ANOVA with repeated measures and the New-
RESULTS

Muscle Damage

As described in detail elsewhere (31), because of a number of indicators of eccentric exercise-induced muscle damage, it was likely that muscle damage had occurred. Briefly, results for the eccentrically working leg comprised a $\sim 17\%$ muscle force drop during 1–2 days after eccentric exercise. Perceived muscle soreness in the eccentrically working leg increased by up to $\sim 40\%$ over that in the concentrically working leg on day 2 after exercise, and $\sim 100$-fold increases in plasma myoglobin and $\sim 10$- to 30-fold increases in plasma creatine kinase were observed on days 3 and 4 after eccentric exercise.

Calpain-3 Localization and Activation

Calpain-3 activation. The percentage of autolyzed (60-, 58-, and 55-kDa isoforms) calpain-3 in whole muscle samples was increased 3 and 24 h after exercise compared with before exercise (Fig. 2). The total amount of calpain-3 in the muscle did not significantly change between preexercise and 24 h after the eccentric exercise bout ($P > 0.05$, by 1-way ANOVA, $n = 11$ subjects); the total amount of calpain-3 at each time point was derived from the sum of the densities for all the calpain-3 bands (i.e., 94, 60, 58, and 55 kDa), normalized by the amount of sample loaded in the given lane, as indicated by the MHC signal in the stain-free gels (see METHODS).

Cytosolic calpain-3. To determine baseline values for the amount of calpain-3 in the cytosolic compartment as a proportion of the total calpain-3 pool in freshly obtained, nonexercised, human skeletal muscle, we used a technique developed by us on rat skeletal muscle (16, 20) and described here for the first time in human muscle fibers (Fig. 3). Importantly, when the truly diffusible components were separated into a physiologically based solution, all the constituents of single muscle fibers were analyzed (i.e., without the loss that typically occurs with centrifugation and fractionation; see METHODS). Comparison of the densities obtained for the intact fiber segments that were run side-by-side with the diffusible constituents and the matched skinned fibers demonstrated that all calpain-3 was accounted for (i.e., the sum of the calpain-3 in the Diff + F-sk samples was not different from the amount of calpain-3 in the matched F-in segment). We found that only $5 \pm 1\%$ of the total calpain-3 pool ($n = 10$ fibers from 4 individuals) was present in the nuclei, where it is predominantly autolyzed. A: Western blot of calpain-3 in crude nuclear, myofibrillar, and cytosolic fractions from a rat extensor digitorum longus muscle (lanes 5–7) and from 2 resting human subjects (subjects A and B; lanes 9–14). Fractions were prepared as described in Fig. 1 from muscle cryosections; the 3 fractions together contain all calpain-3 present in the muscle sample. Lane 8 shows an additional nuclear fraction from another rat. Samples of rat whole muscle homogenate (lanes 1–4), not fractionated in any way, were used to produce calibration curve. Calpain-3 was detected with 12A2 antibody, which in rat muscle also detects m-calpain (at $\sim 82$ kDa). Molecular mass markers are superimposed at right. Amount of autolyzed calpain-3 (% of total) detected in nuclear, myofibrillar, and cytosolic samples in lanes 5–14 is shown below lanes. B: verification of cytosolic (GAPDH), myofibrillar (tropomyosin), and SR proteins (SERCA1) present/absent in nuclear, cytosolic, and myofibrillar fractions (lanes 1, 2, and 3, respectively). Given the presence of SERCA1 in the nuclear fraction, enriched and purified SR vesicle preparations were analyzed for the presence/absence of calpain-3 (lanes 4–7). No 94- or 55-kDa calpain-3 bands were present in enriched or purified SR vesicles. RyR1, ryanodine receptor type 1. C: percentage of autolyzed calpain-3 in nuclear fractions from human and rat skeletal muscle. D: amount of calpain-3 in all forms in the nuclear fraction as percentage of total calpain-3 in matching nuclear, cytosolic, and myofibrillar fractions for that subject (e.g., for subject A, lane 9 compared with lanes 9, 11, and 12). Values are means ± SE.
as diffusible cytosolic protein when mechanically skinned fibers were exposed to the physiological solution for 30 min (Fig. 3). Sometimes calpain-3 autolysis was detected, and this was dependent on the signal for any given band being above threshold limits of detection. In the intact segments of muscle fibers that showed detectable autolysis (n = 8), this amounted to 5 ± 2% autolysis. Given the very small amount of full-length calpain-3 found as a diffusible constituent, autolysis typically could not be detected (only 1 of the 10 fibers showed detectable autolysis in the Diff sample), likely because the amount of autolysis, if any, was below detection thresholds. Importantly, however, where diffusible calpain-3 was detected, it was predominantly in its full-length, unaautolyzed state. These findings demonstrate that, in resting human skeletal muscle, 1) ~95% of the total calpain-3 is tightly bound at myofibrillar or membranous (e.g., nuclear) compartments within skeletal muscle and 2) diffusible calpain-3 is predominantly present as the full-length protein. In stark contrast to these findings and as expected for the freely diffusible μ-calpain (20), 95 ± 2% of the total μ-calpain pool was present in the Diff lane (Fig. 3, D and E).

Nuclear calpain-3. To ascertain the amount of calpain-3 in the nuclei of skeletal muscle in its full-length and/or autolyzed forms, human skeletal muscle was divided into crude cytosolic, crude myofibrillar, and crude nuclear fractions by centrifugation (Fig. 1). Importantly, in these analyses, effectively all the constituents in the fractions were examined, allowing direct comparison of the amount of calpain-3 in a given fraction with the amount in whole muscle (Fig. 4A). Rat tissue was also included in this analysis to allow comparison with results from our previous study of calpain-3 localization (16).

Characterization of the crude fractions is presented in Fig. 5. The presence of the protein lamin A/C in the nuclear fraction and the absence of the myofibrillar protein tropomyosin, as well as the cytosolic proteins GAPDH and creatine kinase, is shown. Lamin A/C has been reported to exist as oligomers and is seen here in the nuclear fraction at ~200 kDa (25). As expected, tropomyosin is shown in the myofibrillar fraction, while GAPDH and creatine kinase are shown in the cytosolic crude fraction (Fig. 5). The crude nuclear fraction contained some of the fast-twitch SERCA isoform (SERCA1) (Fig. 4B, lane 1). This was likely due to sarcoplasmic reticulum (SR) contamination of the crude nuclear fraction; however, it also raised the following question: Was the calpain-3 in the crude nuclear fraction due to calpain-3 localized to the SR? It has been suggested by others that some calpain-3 is associated with the SR (11). To ascertain such an association, we obtained highly purified SR vesicles (kindly provided by Prof. Angela Dulhunty, Australian National University) and analyzed them for the presence/absence of calpain-3. The purity of the vesicles was established by the presence of SERCA1 and also the absence of GAPDH and tropomyosin (Fig. 4B, lanes 4 –7). No calpain-3 was detectable in the purified SR vesicles (Fig. 4B, lanes 6 and 7). Also, in the extremely overloaded lanes containing enriched SR vesicles (Fig. 4B, lanes 4 and 5), no 94– or 55-kDa calpain-3 bands (i.e., those bands identified in the nuclear fractions, lane 1) were detected, even when the membrane was overexposed (not shown).

The great majority of the calpain-3 was present in the enriched myofibrillar fractions (Fig. 4A, lanes 6, 12, and 13), and, of this, 4% (rat preparations) and ~34% (human preparations) was in an autolyzed form. Typically, there is some degree of autolysis in tissue frozen prior to extraction that is not seen in freshly prepared tissue (Fig. 3C). This basal level of calpain-3 autolysis in frozen samples did not interfere with the present findings, as the amount of calpain-3 autolysis was always compared between biopsies obtained from the same individuals and, importantly, treated in the same way after sampling. In contrast, in the enriched nuclear fractions, the majority of the calpain-3 was present almost entirely in its 55-kDa autolysed form (Fig. 4A, lanes 7–10); across a number of samples, an average of ~80% was in the autolyzed form in nuclei from rat and human samples (Fig. 4C). In rat skeletal muscle, however, the basal level of calpain-3 autolysis was always ~5% (Fig. 3C).
muscle, the 12A2 antibody also detects m-calpain at ~82 kDa, which was enriched in the cytosolic (lane 5) and nuclear (lanes 7 and 8) fractions, but very little was present in the enriched myofibrillar fraction (Fig. 4A, lane 6). About 5% (rat EDL) and ~8% (human) of calpain-3 in the total homogenates was present in the nuclear fraction (Fig. 4D).

Cytosolic calpain-3 following eccentric exercise. To establish whether calpain-3 translocates to the cytosol following exercise-induced activation, it was necessary to obtain enriched cytosolic fractions from the frozen tissue samples. The diffusibility experiments on freshly obtained tissue described above (Fig. 3) established that only a small proportion of calpain-3 is localized to the cytosolic compartment of human skeletal muscle, and this was also evident using the alternate technique of enriched cytosolic preparations (Fig. 6B, compare lanes 1–3 with lanes 4 and 5). While the enriched cytosolic preparations contained a high proportion of the predominantly cytosolic μ-calpain (20) (Fig. 6C), they contained considerable actin (Fig. 6D) and a small amount of the major myofibrillar protein MHC (Fig. 6A). These myofibrillar proteins would not normally be expected in a truly pure cytosolic fraction and likely represent myofibrillar contamination of this enriched fraction due to sample preparation or damage during the biopsy procedure (Fig. 3F). Despite this amount of contamination, the amount of calpain-3 in the cytosolic fraction (full-length and autolyzed calpain-3) was not detectably altered as a consequence of the eccentric exercise intervention (Fig. 6E). The amount of calpain-3 autolysis in the cytosolic fraction was not different before or after exercise (n = 7 subjects, P = 0.44, by 1-way ANOVA). For visualization of the cytosolic calpain-3 in the context of the total cellular calpain-3 pool, the data in Fig. 6E are shown as the proportion of the total calpain-3 pool represented by this cytosolic fraction (i.e., 5%), which is illustrated in Fig. 3E.

Nuclear calpain-3 following eccentric exercise. Calpain-3 in enriched nuclear fractions was examined before and after eccentric exercise. Similar to the crude nuclear fractions (Fig. 4, A and C), calpain-3 was almost entirely in its 55-kDa autolyzed form, with some of the 58-kDa isoform also present before exercise and 3 and 24 h after exercise (>95% autolyzed; Fig. 7, A and B). When the density of total calpain-3 was calculated for all isoforms in the enriched nuclear fractions (94, 60, 58, and 55 kDa), the amount of calpain-3 at 3 and 24 h postexercise was not different from that before exercise (Fig. 7C). Similar to the representation of cytosolic calpain-3, for visualization of the nuclear calpain-3 in the context of the total cellular calpain-3 pool, the data in Fig. 6C are shown as the proportion of the total calpain-3 pool represented by this nuclear fraction in humans (i.e., 8%), which is shown in Fig. 4D.

DISCUSSION

The main novel findings of the present study are as follows: 1) of the total calpain-3 pool in nonexercised human skeletal muscle, only a small proportion is localized to the nuclear (~8%) and cytosolic (~5%) compartments of muscle fibers; 2) although eccentric exercise resulted in increased calpain-3 activation at 3 and 24 h following cessation of exercise, there was no detectable change in the amount of full-length or autolyzed calpain-3 in enriched nuclear or cytosolic preparations; and 3) >80% of the nuclear calpain-3 was in its autolyzed form at all times. Thus, as we hypothesized, although calpain-3 is activated by eccentric exercise, it does not translocate appreciably to the cytosol or the nucleus to exert its regulatory functions.

Calpain-3 Is Activated Following Eccentric Exercise

In order for calpain-3 to be active, it must first proteolyze (autolyze) itself through an intramolecular event (7, 29). This results in cleavage of its full-length 94-kDa isoform to the 60-kDa isoform. Two further steps result in the appearance of 58- and 55-kDa isoforms. Autolysis of calpain-3 was detected 3 and 24 h postexercise (Fig. 2). In our previous study, significant calpain-3 autolysis was observed 24 h after eccentric exercise (15). We have also demonstrated in vitro that
A Small Proportion of Calpain-3 Is Localized to the Nuclei of Quiescent Skeletal Muscle Fibers; However, It Does Not Translocate Following Activation

Calpain-3 was detected in highly enriched nuclear fractions from skeletal muscle samples. With use of the two different types of nuclear fractions (i.e., enriched and crude preparations), it was possible to address two key issues. 1) Does calpain-3 translocate to the nucleus following activation? 2) How much of the total pool is present in the nuclei? When the total amount of calpain-3, i.e., the densities of all isoforms detected, was examined, there was no difference between the amount of calpain-3 in enriched nuclear fractions before and after exercise (Fig. 7, A and C). Crude nuclear fractions were prepared to determine the amount of the total calpain-3 pool present in nuclei as a proportion of that present in whole muscle samples. With this approach, it was consistently seen that 1) at least some calpain-3 was always present in these nuclear fractions and 2) of the calpain-3 present, ≥80% of the pool was in its autolyzed form (predominantly as the 55-kDa isoform; Fig. 4C). This was the case for nuclei prepared from human and rat skeletal muscle (Fig. 4). Previously, with use of immunohistochemical analyses, it was reported that 45% of the nuclei detected in cross sections of healthy individuals contained calpain-3 (2). The findings presented here suggest that the amount of calpain-3 in the nuclei actually constitutes a very small proportion of the total calpain-3 in skeletal muscle and that most of the calpain-3 is autolyzed (Fig. 7).

As mentioned above, calpain-3 must be autolyzed to be proteolytically active (7, 29). There are no reports detailing the regulation of calpain-3 inactivation or, indeed, the functional relevance of further calpain-3 breakdown. Baghdiguian et al. (2) reported that the NF-κB pathway is perturbed in limb-girdle muscular dystrophy type 2A. Specifically, an accumulation of IκBα in cross sections of skeletal muscle of limb-girdle muscular dystrophy type 2A patients compared with barely detectable levels in healthy individuals led Baghdiguian et al. to speculate that calpain-3 might play a role in the mediation of apoptosis through regulation of the level of the apoptotic factor NF-κB in the nucleus. Since Baghdiguian et al. (2, 3) proposed a putative role for calpain-3 in apoptosis via the NF-κB pathway (2, 3), the findings presented here suggest that this involvement would be via the calpain-3 intrinsically present in the nuclei. While the findings suggest that a role for calpain-3 in signaling for muscle remodeling and repair via direct activation on nuclear signaling events is unlikely, they cannot rule out an important role for the autolyzed calpain-3 localized in the nuclei and, perhaps, the translocation of substrates for proteolytic cleavage. Additionally, very small changes, which might be physiologically relevant, could be beyond the detection limitations of the techniques used here, and, thus, we cannot rule out that they occurred, nor can we rule out their functional importance.

while stretching a muscle fiber does not result in calpain-3 activation, a small, yet sustained, increase in [Ca\textsuperscript{2+}] does result in activation of calpain-3 (16). Together, these data suggest that the expected small, yet sustained, increase in cytosolic [Ca\textsuperscript{2+}] following eccentric exercise in murine animals (12) also occurs in humans. While no cytosolic [Ca\textsuperscript{2+}] data are available for human muscle after eccentric exercise, the likelihood that cytosolic [Ca\textsuperscript{2+}] is increased following certain types of exercise is supported by the observation that total muscle [Ca\textsuperscript{2+}] was increased in humans immediately and up to 48 h following prolonged long-distance running in healthy individuals (22, 23). The activation of calpain-3 seen here, as well as previously (15), supports the suggested role for calpain-3 in sarcomeric repair and remodeling. It is quite well documented that eccentric exercise results in some muscle damage, one indication of which is a decrease in the maximal voluntary contraction (MVC) of the muscles that have been lengthened during contraction. The individuals examined here displayed this typical decline in MVC 3 and 24 h postexercise (31). Consistent with previous investigations, this decline in MVC occurred prior to any elevation in the serum markers creatine kinase and myoglobin, which occurred 3–4 days following the exercise intervention, when MVC had returned to near-preexercise levels (31).
Myofibrillar Calpain-3 Remains Bound to the Myofibrillar Compartment Following Activation and Likely Plays an Important Role in Muscle Repair/Remodeling Following Eccentric Exercise

Given the apparent lack of calpain-3 translocation, the N2A line region of titin must be further considered. With calpain-3 bound tightly in this region, it is highly likely that substrates are already localized in the vicinity of calpain-3 of the N2A line or nearby at the Z-disk region. Alternatively, but perhaps less likely, substrates could translocate to the N2A line for cleavage by calpain-3. In either situation, a possible role for calpain-3 could be exertion of an initial proteolytic function on myofibrillar substrates (e.g., damaged myofilaments) prior to further degradation, such as through the ubiquitin-proteasome system. A recent study has detailed a cleavage motif recognized by calpain-3, and of the many potential substrates of calpain-3, a common thread among a large number of the candidates was a functional role within the cytoskeletal domain of skeletal muscle (5). Among the plausible calpain-3 substrates within this area are muscle ankyrin repeat protein (MARP) and/or myopalladin, which, along with calpain-3, have been localized to the N2A line of titin in muscle and are likely involved in muscle stress response pathways (13). Interestingly, in response to stretch in fetal cardiomyocytes, MARPs have been shown to translocate to the nucleus (13). Further investigation of a possible in vivo calpain-3-mediated cleavage of proteins localized at or near the N2A line of titin, such as MARP and myopalladin, is warranted.

Very Little Calpain-3 Is Found in the Cytosol of Quiescent Skeletal Muscle

We have shown that, in 30 min, only a small proportion of calpain-3 (5% of the total pool) is able to wash out of quiescent muscle fibers from fresh human skeletal muscle biopsies. In contrast to this finding and as expected, >90% of the cytoplasmic \( \mu \)-calpain was found in the same wash solutions (Fig. 3), thus validating that our measures are representative of a true cytosolic pool. Importantly, with use of this method of mechanical skinning of single fibers, only cytoplasmic proteins diffuse out of the fiber (16, 18, 20), and there is typically no contamination of other organelles or the abundant myofibrillar proteins actin and myosin in the diffusible fractions (Fig. 3F). These findings are similar to those we previously reported following examination of the free diffusion of calpain-3 using mechanically skinned single fibers from rat muscle, where calpain-3 was barely detectable in the diffusible fraction following a 10-min wash step (16). In the same study, we also showed that if Triton X-100 was present in the wash solution, >80% of membrane-associated proteins (e.g., ryanodine receptor and SERCA1) appeared in the wash after 10 min, whereas only ~10% of the calpain-3 appeared in the wash from the same fiber. This indicated that very little calpain-3 was associated with membranes but, rather, likely associated with the myofibrillar network (16). In further support of those findings, we demonstrate here that no calpain-3 is detectable in enriched or purified SR preparations (Fig. 4B). A further finding of the present study was that the small amount of calpain-3 that was diffusible in skeletal muscle remained predominantly in its full length (i.e., was not autolyzed). We cannot rule out that an amount of autolyzed calpain-3 was also diffusible but not detectable because of the threshold limits of the detection system, although this could only account for a very small proportion of the total calpain-3 pool, since there was no apparent loss of calpain-3 signal (i.e., measured density) when the calpain-3 density in Diff + F-sk was compared with F-in from the same fiber (Fig. 3).

Calpain-3 Does Not Translocate to the Cytosol Following Activation in Response to Eccentric Muscle-Damaging Exercise

Finally, we have shown that, in human skeletal muscle, activation of calpain-3 does not result in any detectable change in the very small amount of calpain-3 in the cytosol. As shown in Fig. 3, myofibrillar contamination is not seen when truly diffusible, hence cytoplasmic, proteins are identified using mechanically skinned fibers. While there was some myofibrillar contamination in the enriched cytosolic preparations (Fig.
4), it would seem unlikely that this contamination was due to
the small amount of exercise-induced muscle damage that
likely occurred in each muscle fiber as a proportion of its fiber
volume (8). In any case, considering that all preparations were
made identically, we can conclude that there was no obvious
translocation of calpain-3 to the cytosol following activation in
muscle.

Model Depicting Calpain-3 Localization in Healthy Human Skeletal Muscle

We have provided a comprehensive analysis of the localizations of calpain-3 in human skeletal muscle at rest and following activation. The findings are incorporated into the proposed model (Fig. 8), which shows the large majority of calpain-3 in the myofibrillar compartment, with ~5% and 8% of the total pool in the cytosol and nuclei, respectively. After exercise-induced ~1.7-fold activation of calpain-3 in healthy individuals, the amount of calpain-3 in the cytosol and nuclei is not changed. Of the total calpain-3 pool, typically, a small amount of autolyzed calpain-3 is always present, and we hypothesize that this proportion is, in part, attributable to the almost totally autolyzed nuclear pool of calpain-3.

Conclusions

In conclusion, the present study has furthered our understanding of the functional role for calpain-3 in skeletal muscle. It seems plausible that, at least under nonpathological muscle-damaging conditions, such as eccentric exercise, calpain-3 exerts its primary role on cytoskeletal substrates. This is consistent with the skeletal muscle repair and remodeling that is known to occur as a consequence of eccentric exercise and likely partly dependent on the activation of calpain-3. As such, this exercise intervention, which remains the only physiological intervention that results in the activation of calpain-3 in vivo, should be further utilized in healthy individuals to understand the functional role of calpain-3 in skeletal muscle. Finally, to make further advances in the field of calpain-3 biology, it will be crucial to enhance our understanding of not only the activation of calpain-3 but also, importantly, its inactivation and target substrates.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


