Calpain-3 is autolyzed and hence activated in human skeletal muscle 24 h following a single bout of eccentric exercise

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Murphy RM, Goodman CA, McKenna MJ, Bennie J, Leikis M, Lamb GD. Calpain-3 is autolyzed and hence activated in human skeletal muscle 24 h following a single bout of eccentric exercise. J Appl Physiol 103: 926–931, 2007. First published June 21, 2007; doi:10.1152/japplphysiol.01422.2006.—The function and normal regulation of calpain-3, a muscle-specific Ca2+-dependent protease, is uncertain, although its absence leads to limb-girdle muscular dystrophy type 2A. This study examined the effect of eccentric exercise on calpain-3 autolytic activation, because such exercise is known to damage sarcomeric structures and to trigger adaptive changes that help prevent such damage on subsequent exercise. Six healthy human subjects performed a 30-min bout of one-legged, eccentric, knee extensor exercise. Torque measurements, vastus lateralis muscle biopsies, and venous blood samples were taken before and up to 7 days following the exercise. Peak isometric muscle torque was depressed immediately and at 3 h postexercise and recovered by 24 h, and serum creatine kinase concentration peaked at 24 h postexercise. The amount of autolyzed calpain-3 was unchanged immediately and 3 h after exercise, and returned to preexercise levels within 7 days. In contrast, the eccentric exercise produced little autolytic activation of the ubiquitous Ca2+-activated protease, µ-calpain. Eccentric exercise is the first physiological circumstance shown to result in calpain-3 activation in vivo.

µ-calpain; proteolysis; autolysis; muscle damage

CALPAINS ARE NONLYSOSOMAL, Ca2+-activated cysteine proteases. Skeletal muscle fibres contain not only the ubiquitous calpains, µ-calpain and m-calpain (also referred to as calpain-1 and calpain-2), but also a muscle-specific calpain known as calpain-3 or p94 (32), for which the mRNA expression is 10-fold higher than for the other calpains in muscle (34). There is some understanding of the physiological functions of the ubiquitous calpains, which among other things appear to be involved in initial proteolytic dismantling of the sarcomere that occurs before the major degradation of proteins via ubiquitin-proteasomal pathways (6, 15, 16). In contrast, the role of calpain-3 remains an enigma. Complete absence of calpain-3 from skeletal muscle and/or the inability of calpain-3 to undergo autolysis (from its full-length 94-kDa form to 60-, 58-, and 56/55-kDa forms) results in limb-girdle muscular dystrophy type 2A in humans (10, 31, 33). Also, calpain-3 needs to be bound to the large elastic sarcomeric protein, titin, to maintain its integrity. Mutations in the titin gene adjacent to the calpain-3 binding site, which inhibit calpain-3 binding there, result in tibial muscular dystrophy (17). Somewhat surprisingly, overexpression of this protease has no noticeable effect on muscle function (35). Furthermore, there was contention for many years about whether or not the activation of calpain-3 in muscle is Ca2+-dependent, because it appeared to autolyze even in the absence of Ca2+. It is now clear, however, that activation of calpain-3 is indeed Ca2+-dependent and that the earlier findings were due to the particularly high sensitivity of the process to very low levels of Ca2+ (8, 9, 13, 26, 28). One recent suggestion is that calpain-3 normally plays an important role in sarcomere maintenance in mature muscle and that its absence or loss of proteolytic activity results in degeneration and death of muscle cells (9, 21). However, there is no evidence to date as to precisely when or why calpain-3 is normally activated. Given that autolysis of calpain-3 is necessary for its normal function as a protease (30), it is important to know what conditions result in its autolysis. Studies to date have found that calpain-3 remains in its full-length form and thus inactive in healthy human muscle (8, 26) at rest as well as after typical concentric exercise (26), and no studies have reported a physiological intervention or circumstances that results in calpain-3 autolysis (9).

Our laboratory has found recently that neither calpain-3 nor µ-calpain were autolyzed in human skeletal muscle following an “all-out” sprint exercise bout or endurance cycling in trained subjects (26). Unless it is particularly excessive, such exercise is not normally deleterious to the muscles, and muscle performance typically recovers within an hour. In contrast, eccentric exercise, where muscles are stretched while contracting, such as occurs in downhill walking, can result in muscle weakness lasting 24 h or more (14, 18, 36). Following such exercise there is often overt damage of the sarcomeric structure, typically involving Z-disk streaming and marked widening of the I band (2, 29). Although many fibers may show some damage (2, 14), usually only a comparatively small percentage of the total fiber volume is affected (2, 18), and the muscle weakness is due in large part to a reduction in excitation-induced Ca2+ release from the sarcoplasmic reticulum (5, 18). Significantly, it has been observed in mouse fibers that the resting intracellular Ca2+ concentration ([Ca2+]i) remains elevated for hours (5, 18) and even days (22) following eccentric contraction. A common marker of muscle damage is serum creatine kinase (CK), which leaks from muscles if the surface membrane is damaged, something that might occur during the exercise itself or sub-

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Because sex does not affect either the relative changes in muscle force of all test procedures and associated risks before giving written training or had recent trauma to the knee joint. Subjects were informed active, and none had in the previous 6 mo participated in resistance strapped to the dynamometer chair across the hips and chest to restrict ately before use, and all data were corrected for gravity. Subjects were dynamometer was calibrated for angle, torque and velocity immedi-
etric dynamometer (Cybex Norm 770, Henley Health Care). The isometric torque of the knee extensors were performed on an isoki-
both the eccentric exercise bout and measurements of maximal
exercise. Venous blood samples were taken to determine increases in
subsequent measurements occurred at 24 h and 7 days after eccentric
exercise. Following injection of a local anesthetic (1% Xylocaine) into the skin and subcutaneous tissue, a small incision was made and biopsies were taken from the midportion of the vastus lateralis muscle of the right leg. Subjects were supine for the pre and 3 h, 24 h, and 7 days after biopsies, and they were seated for the immediately after eccentric exercise biopsy. Each biopsy was taken by the same experienced medical practitioner from separate incisions, at a constant depth. Samples were immediately blotted on filter paper and then frozen in liquid N2 and stored at −80°C until analyzed for calpains.

The biopsy immediately preceded the torque measurements.

Muscle biopsies. A muscle biopsy was taken at pre, post, and at 3 h, 24 h, and 7 days after eccentric exercise. Following injection of a local anesthetic (1% Xylocaine) into the skin and subcutaneous tissue, a small incision was made and biopsies were taken from the midportion of the vastus lateralis muscle of the right leg. Subjects were supine for the pre and 3 h, 24 h, and 7 days after biopsies, and they were seated for the immediately after eccentric exercise biopsy. Each biopsy was taken by the same experienced medical practitioner from separate incisions, at a constant depth. Samples were immediately blotted on filter paper and then frozen in liquid N2 and stored at −80°C until analyzed for calpains.

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Overview of testing. Subjects underwent an eccentric exercise bout to induce damage of the knee extensor muscles. A vastus lateralis muscle biopsy was taken for measurement of calpain autolysis at five time points: preexercise (pre), immediately postexercise (post), and at 3 h, 24 h, and 7 days postexercise. Peak isometric torque of the knee extensors was measured on 6 different days to initially determine variability and then deterioration in muscle function following eccentric exercise. Peak torque was measured on 3 separate days preceding the eccentric exercise bout, which comprised an initial familiarization test and repeat tests on the next two visits to determine variability, enabling interpretation of the subsequent postexercise and recovery data. On the day of eccentric exercise, peak isometric torque was measured pre, immediately post, and at 3 h after eccentric exercise; subsequent measurements occurred at 24 h and 7 days after eccentric exercise. Venous blood samples were taken to determine increases in serum CK as a marker of muscle damage, at the times corresponding to biopsy sampling.

Maximal eccentric knee extension and isometric muscle strength test. Both the eccentric exercise bout and measurements of maximal isometric torque of the knee extensors were performed on an isoki-
netic dynamometer (Cybex Norm 770, Henley Health Care). The dynamometer was calibrated for angle, torque and velocity immedi-
ately before use, and all data were corrected for gravity. Subjects were strapped to the dynamometer chair across the hips and chest to restrict upper body movement, and they were strapped across the thigh to stabilize the active leg. During the initial test, each subject’s positions were recorded and were replicated during each subsequent isokinet test session. The dynamometer’s axis of rotation was aligned with the anatomic axis of rotation of the knee, while the lever arm was extended with the pad covering the distal shin. All measurements were conducted on the right leg, as all subjects were right-hand dominant. A real-time visual display of torque and strong verbal support were provided to encourage subjects to exert maximal force during each contraction.

Each trial of maximal isometric knee extensor torque consisted of a standard warm-up (except the immediately postexercise measure) and three maximal 5-s contractions, at a knee joint angle of 45°, with a 30-s recovery between contractions.

The eccentric exercise test was based on previous studies that induced muscle damage of the knee extensors (3, 7, 24, 25). Briefly, subjects performed 300 repetitions of maximal eccentric knee extension, consisting of 10 sets of 30 repetitions at 30°/s. Each set was separated by a 1-min rest period. The subject’s leg was initially fully extended. At commencement of the eccentric bout, the subject was instructed to maximally resist the downward movement of the lever arm, through the full range of motion. When the leg was fully flexed, the subject was then instructed to relax the leg, while the investigator returned the lever arm (leg) to a fully extended position. This procedure ensured that the mode of muscle contraction was purely eccentric. All torque data are expressed relative to the preexercise value for each individual.

Muscle biopsies. A muscle biopsy was taken at pre, post, and at 3 h, 24 h, and 7 days after eccentric exercise. Following injection of a local anesthetic (1% Xylocaine) into the skin and subcutaneous tissue, a small incision was made and biopsies were taken from the midportion of the vastus lateralis muscle of the right leg. Subjects were supine for the pre and 3 h, 24 h, and 7 days after biopsies, and they were seated for the immediately after eccentric exercise biopsy. Each biopsy was taken by the same experienced medical practitioner from separate incisions, at a constant depth. Samples were immediately blotted on filter paper and then frozen in liquid N2 and stored at −80°C until analyzed for calpains.

The biopsy immediately preceded the torque measurements.

Muscle homogenate preparation. Skeletal muscle samples [9 ± 5 (SD) mg] were homogenized (10:1 wt/vol) in 0.4 M Tris–Cl, pH 6.8, and 25 mM EGTA ([Ca2+] < 10 nM), following which SDS was added to a final concentration of 4% and homogenates were incubated at 4°C for 20–40 min. Samples were spun (3,000 g, 5 min), and the supernatant added (2:1 vol/vol) to SDS loading buffer (0.125 M Tris–HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8). Samples were heated to 95°C for 4 min and stored at −20°C until analyzed by Western blotting.

Western blotting of μ-calpain and calpain-3 in muscle homoge-

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expressing the density of the bands of the autolyzed products relative to the total density of all the bands for that calpain (i.e., autolyzed and unautolyzed) in the sample. This indicated what proportion of the given calpain was autolyzed in that particular sample, irrespective of any minor differences in protein loading.

Biopsy calpain was not available for one subject at both the pre and immediately postexercise time points, and so no protein data are presented for this subject. For another subject, there was no sample immediately postexercise, so data presented are for \( n = 5 \) for pre, 3-h, 24-h and 7-day samples and \( n = 4 \) for immediately postexercise samples.

\( \text{CK} \). Serum CK concentration ([CK]) was determined pre, post, and 3 h, 24 h, and 7 days after eccentric exercise. Arterialized blood was sampled from a dorsal hand vein for the pre and post samples, with all subsequent samples obtained from an antecubital vein. Each sample was placed into a plain evacuated test tube, and blood allowed to coagulate for 30 min at room temperature and then centrifuged at 1,500 g for 10 min. The serum layer was removed and frozen at \(-20^\circ\text{C}\) until analyzed in duplicate for [CK] using an Olympus GmbH AU1000 analyzer (Olympus Diagnostics, Clare). The normal reference range of [CK] using this method is 45–130 U/l.

Statistics. Data are expressed as means \( \pm \) SE, unless otherwise indicated. Data were analyzed using a one-way ANOVA with repeated measures. When the ANOVA revealed a significant effect, Newman-Keuls post hoc analyses were performed. Pre and post samples were compared using a Student’s \( t \)-test (paired, 2-tailed). All analyses were performed using Prism V 4.01. A probability value of \( P < 0.05 \) was deemed to indicate significant difference.

RESULTS

Torque and [CK] after the eccentric exercise. As expected, peak isometric torque was decreased following the eccentric exercise (Fig. 1). Immediately after exercise, relative torque was 76 \( \pm \) 8% \( (P < 0.05) \) of the preexercise levels and was 84 \( \pm \) 7% \( (P < 0.05) \) at 3 h after exercise. At 24 h and 7 days after eccentric exercise, peak isometric torque did not differ from preexercise levels. Before exercise, [CK] was 133 \( \pm \) 37 U/l, and the value at 3 h postexercise \((235 \pm 25 \text{ U/l}; P < 0.05)\) was significantly greater than before exercise, immediately postexercise, and 7 days postexercise (Fig. 2). Serum [CK] peaked at 24 h after exercise \((339 \pm 49 \text{ U/l}; P < 0.05\) greater than all other time points). By 7 days after the exercise bout, serum [CK] had returned to the “pre” exercise levels.

Amount of calpain-3 and its autolysis. Figure 3A, A and B, shows representative Western blots for calpain-3 for two subjects. Before the exercise (pre), calpain-3 existed in the muscle of the subjects predominantly as a 94-kDa protein (top band), which requires autolysis (to the 58- and 56-kDa forms) to be proteolytically active (13, 30). Figure 3C shows the proportion of calpain-3 that was autolyzed (i.e., amount in 58- and 56-kDa forms compared with sum of all calpain-3 bands) at each time after exercise expressed relative to that present before exercise in the same subject. On average, the proportion of calpain-3 that was autolyzed was increased more than threefold at 24 h after the eccentric exercise bout compared with that present either before exercise (pre) or 3 h and 7 days after exercise (Fig. 3C). To indicate how much of the total muscle calpain-3 was autolyzed on average, the percentage of calpain-3 in autolyzed forms was expressed as a percentage of the total calpain-3 present in the same muscle sample, without normalizing to the preexercise levels in the subject. Preexercise 16 \( \pm \) 2% of the calpain-3 was autolyzed, and this amount increased substantially 24 h after exercise to 35 \( \pm \) 12%.

Amount of \( \mu \)-calpain and its autolysis. The effects of the eccentric exercise on \( \mu \)-calpain were also investigated. Figure 4A and B, shows representative Western blots for \( \mu \)-calpain for the same two subjects as shown for calpain-3 in Fig. 3. A and B, \( \mu \)-calpain exists as an 80-kDa protein (top band) and once activated autolyzes to 78- and 76-kDa bands. Analysis of the pooled data (Fig. 4C) showed no significant difference in the amount of autolysis of \( \mu \)-calpain across the trial, irrespective of whether or not the values were normalized to the levels present in each subject before exercise. However, there did appear to be noticeable autolysis in some individual cases, such as in the immediate postexercise sample shown in Fig. 4B. There was no correlation between the percentage of autolyzed \( \mu \)-calpain and the percentage of autolyzed calpain-3 across the different subjects and time points (linear regression analysis, \( r^2 = 0.114, P = 0.16 \)).

Extent of autolysis of calpain-3 and \( \mu \)-calpain in preexercise samples. Finally, the small level of \( \mu \)-calpain autolysis observed in the subjects here in preexercise state (pre samples, 9 \( \pm \) 3% of the total \( \mu \)-calpain) was not significantly different from that found previously (26) in a group of healthy, active men before exercise \((10 \pm 2\%, n = 11; P > 0.05, 2\text{-tailed, unpaired } t\text{-test})\). The amount of autolyzed calpain-3 in the preexercise samples in the present study \((16 \pm 2\%)\) was slightly greater than in that previous study \((9 \pm 1\%, n = 11; P > 0.05, 2\text{-tailed, unpaired } t\text{-test})\). It should be noted that this slightly higher baseline level of calpain-3 autolysis would, if
anything, be expected to have detracted from, rather than exaggerated, the relative changes in calpain-3 autolysis observed here after exercise (Fig. 3C).

**DISCUSSION**

In this study, we report the first example of physiological circumstances that result in calpain-3 autolysis. We have shown that a single bout of eccentric exercise resulted in calpain-3 autolysis and hence in its activation. At 24 h postexercise ~35% of the total calpain-3 present in the muscle was in the autolyzed state. Given that 1) the total proportion of sarcomeres showing noticeable disruption after eccentric exercise is relatively small (<10%) (2, 18) (which is also likely the case here because subjects were able to produce near-maximal

![Fig. 3. Effect of eccentric exercise on the extent of calpain-3 autolysis. A and B: Western blots of calpain-3 for 2 subjects. For each Western blot, the Coomassie blue stain of the SDS-PAGE gel showing myosin heavy chain (MHC) following transfer indicates the relative loading of the samples. The 94-kDa calpain-3 (top band) can be seen autolyzed to 58- and 56-kDa bands at various time points. C: amount of 58- and 56-kDa calpain-3 (i.e., in autolyzed forms) expressed as a proportion of the total calpain-3 present at each time, normalized to the proportion of autolyzed calpain-3 present before exercise in the same subject (*pre* time sample) (see METHODS) (n = 5 subjects except n = 4 at post). Values are means ± SE. 7d, 7 days. *P < 0.05 different from all other time points (1-way ANOVA with repeated-measures, Newman-Keuls post hoc analyses).](#)

![Fig. 4. Effect of eccentric exercise on the extent of μ-calpain autolysis. A and B: Western blots of μ-calpain for same 2 subjects as in Fig. 3. For each Western blot, the Coomassie blue stain of the SDS-PAGE gel showing MHC following transfer indicates the relative loading of the samples. μ-calpain exists predominantly in its unautolyzed 80-kDa form (top band) at most time points, with only a little autolysis to 78- and 76-kDa isoforms at any time point. C: percentage of total μ-calpain in autolyzed forms (n = 5 subjects, except n = 4 for “post” as indicated). Values are means ± SE.](#)
torque within 24 h after the exercise), and 2) all of the calpain-3 within a muscle fiber is normally tightly bound (27) [most likely to titin (19, 33)]; the high proportion of calpain-3 autolysis suggests that the autolysis was a widespread process within the muscle fibers and that a substantial amount of autolysis must have taken place in many and perhaps in all of the fibers in the exercised muscles and was not limited solely to the localized regions of overt damage. We note that there are both type I and type II muscle fiber types in human vastus lateralis samples, and the findings here do not reveal whether calpain-3 activation occurred in a fiber type-dependent manner.

Of note, increased calpain-3 autolysis was apparent 24 h after the eccentric exercise protocol, but it was not detectable either immediately after or 3 h after the exercise. This makes it unlikely that the peak in calpain-3 autolysis was caused directly by the exercise itself or by the acute effects associated with it, such as the repeated rises in cytoplasmic [Ca^{2+}] eliciting the muscle contractions. Nevertheless, as explained below, it is quite likely that this autolysis of calpain-3 is caused by raised cytoplasmic [Ca^{2+}] but that it primarily occurs in response to a very prolonged rise in resting [Ca^{2+}] rather than to the acute rises during muscular activity.

Overall, μ-calpain showed a different response to the exercise protocol compared with calpain-3, there being no significant change in the proportion of autolyzed μ-calpain at any time postexercise. Interestingly though, in two subjects there did appear to be an increased amount of autolyzed μ-calpain immediately postexercise (e.g., Fig. 4B), possibly indicating that the eccentric exercise caused some μ-calpain activation in those particular subjects. It could be that those two subjects were slightly different in their training status or lifestyle compared with the rest of the group or that their muscles were more sensitive to the nature of the eccentric exercise regime.

There was no significant correlation between the level of calpain-3 autolysis and that of μ-calpain across the subjects and time course of the study. This indicates that the factor(s) leading to the increased level of calpain-3 autolysis at 24 h were relatively specific for calpain-3 compared with μ-calpain. One plausible scenario is that the autolysis of calpain-3 is due to a prolonged rise in the resting [Ca^{2+}] in the muscle fibers following the eccentric contractions. Such a rise has been observed in mouse skeletal muscle after eccentric contractions, with the resting [Ca^{2+}] being increased from its normal level of ~100 nM to ~250 nM for more than 24 h (22). At submicromolar levels of Ca^{2+} there is virtually no autolysis or activation of μ-calpain (15, 26, 27), but calpain-3 does show autolysis and activation (13, 26), although the process proceeds extremely slowly at such low Ca^{2+} levels and can take up to 24 h for much of the calpain-3 to become autolyzed (13). This slowness in the Ca^{2+}-dependent autolysis of calpain-3 is also apparent at higher [Ca^{2+}], because it was found that exposure to 2.5 μM Ca^{2+} caused a small amount of autolysis of μ-calpain within 1 min but there was no detectable autolysis of calpain-3 unless the period of exposure was increased (26). This latter finding also can account for the fact that the eccentric exercise regime here appeared to cause proportionately more autolysis of μ-calpain than of calpain-3 during the actual course of the exercise period. However, in view of the variability in the measurements and the relatively small number of subjects examined, it is possible that there was some level of calpain-3 autolysis occurring during the exercise, but this was not enough to reach significance levels; in any case it was far smaller than the amount of autolysis present 24 h after the exercise. Serum [CK] increased significantly in the 3 h following the exercise and was maximal at the 24 h time point (Fig. 2), which could be due to both acute damage to the muscle and subsequent membrane damage caused by raised cytoplasmic [Ca^{2+}] (1). Thus the prolonged rise in resting intracellular [Ca^{2+}] following eccentric exercise may be responsible for both calpain-3 autolysis and CK loss.

We did not examine whether the other ubiquitous calpain, m-calpain, was autolyzed during or following the exercise, although given the potential role of m-calpain in muscle regeneration (15), a future study should examine its autolysis and activity following eccentric exercise, particularly given that a twofold increase in the mRNA levels for m-calpain has been observed a day after eccentric exercise (11).

Finally, on a speculative note, we suggest that the rise in calpain-3 autolysis seen 24 h after the eccentric exercise is reflecting an important role of calpain-3 in sarcomeric repair and remodeling following the eccentric exercise. This would be consistent with previous suggestions, based on studies with calpain-3 knockout mice, that calpain-3 plays a vital role in regulating and remodeling sarcomeric structure in mature muscle (9, 21). Furthermore, one of the unique features of eccentric exercise experienced by individuals unaccustomed to the exercise protocol is that the muscle undergoes changes that help prevent damage on a subsequent similar exercise bout. Thus it is possible that calpain-3 may be involved not only in the repair and reassembly of the original sarcomeric structure following damage by eccentric contractions, but also in any subsequent adaptive changes, such as adding extra sarcomeres to lengthen the myofibrils (23, 29). Given that calpain-3 contains a putative nuclear translocation domain (32) and it has been found localized to the nucleus (34), it may have a role not only as a sarcomeric protease but also as a signaling molecule (31).

The present study showed increased calpain-3 autolysis in a cohort of healthy individuals, who likely possessed the normal adaptive mechanisms to eccentric exercise. Although not examined in the present study, we expect that if limb-girdle muscular dystrophy type 2A patients had performed the same eccentric exercise they would have experienced a similar level of acute damage to their muscles as normal individuals [as is the case with calpain-3 knockout and normal mice; (12)], but their muscle fibers may not be able to subsequently undergo the normal repair mechanisms because of the absence of functional calpain-3 (21). Consequently, we speculate that the progressive dystrophic changes that occur in limb-girdle muscular dystrophy type 2A patients over their life (and also in calpain-3 knockout mice) may be at least in part due to inadequate repair of the cumulative minor damage that likely arises from the mild eccentric muscle contractions that are a normal component of daily activity, particularly because this effect would be compounded if the muscles also fail to show the normal adaptation to eccentric exercise. This could be explored in future experiments examining the long-term effects and adjustments that occur in muscles of normal and calpain-3 knockout mice following eccentric exercise.

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