Raastad, T., Owe, S. G., Paulsen, G., Enns, D., Overgaard, K., Crameri, R., Kiil, S., Belcastro, A., Bergersen, L. H., Hallén, J. Changes in calpain activity, muscle structure, and function after eccentric exercise. *Medicine & Science in Sports & Exercise,42*, 86-95.

Title:

Changes in calpain activity, muscle structure and function after eccentric exercise

Authors: Truls Raastad[1], Simen Gylterud Owe[1, 6], Gøran Paulsen[1], Deborah Enns[2], Kristian Overgaard[3], Regina Crameri[4], Steinar Kiil[5], Angelo Belcastro[2], Linda Bergersen[6] and Jostein Hallén[1]

[1]Norwegian School of Sport Sciences, Oslo, Norway

[2]School of Kinesiology, Faculty of Health Sciences, University of Western Ontario, London, Canada

[3]Department of Sport Science, University of Aarhus, Denmark

[4]Institute of Sports Medicine, Copenhagen Bispebjerg Hospital, Copenhagen, Denmark

[5]Department of Vascular Surgery, Aker University Hospital, Oslo, Norway

[6]Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Norway

Running head:

Calpain activity and myofibrillar disruptions

No external funding was received for this work

Correspondence:

Truls Raastad

Norwegian School of Sport Sciences

P.O. Box 4014 U.S.

N- 0806 Oslo, Norway

Fax: +47 22 23 42 20 E-mail: truls.raastad@nih.no

Keywords: Proteolytic activity; muscle damage; fatigue; force-generating capacity; extracellular matrix; Tenascin-C

Abstract

PURPOSE: The aim of this study was to investigate mechanisms behind muscle damage and the relationship between changes in muscle structure and function after high-force eccentric exercise. METHODS: Eleven healthy males performed 300 maximal voluntary eccentric actions with knee extensors in one leg. Maximal force-generating capacity was measured before exercise and regularly during the next seven days. Biopsies from m. vastus lateralis were taken in both control and exercised legs 0.5, 4, 8, 24, 96, and 168 hours after exercise for evaluation of myofibrillar structure, extracellular matrix proteins, and calpain activity. RESULTS: In the exercised leg, peak torque was reduced by 47±5% during exercise and was still 13±4% lower than baseline one week after the exercise. Calpain activity was three times higher in the exercised leg compared to the control leg 30 min after exercise and was still significantly higher 96 hours after the exercise. Myofibrillar disruptions were observed in 36±6% of all fibers in exercised muscle and in 2±1% of fibers in control muscle. The individual reductions in peak torque correlated with the proportion of fibers with myofibrillar disruptions (r=0.89). The increase in calpain activity was not correlated to the proportion of fibers with myofibrillar disruptions. Nevertheless, the characteristics of the myofibrillar disruptions mimicked calpain-mediated degradation of myofibrils. Tenascin-C and PIIINP showed increased staining intensity on cross sections 4-7 days after the exercise. CONCLUSION: Myofibrillar disruptions appear to be a main cause for the long-lasting reduction in force-generating capacity after high-force eccentric exercise. The increase in calpain activity, but the lack of a relationship between calpain activity and the amount of muscle damage, suggests multiple roles of calpain in the damage and repair process.

Introduction

Paragraph 1 Although exercise-induced muscle damage has been studied extensively over the last 25 years, the etiology is still to a large extent unknown (29). However, a mechanical tearing of force-bearing structures is thought to occur during eccentric exercise (25) and this initial damage may activate protein degradation within the muscle cell. Activation of the Ca^{2+}-dependent protease, calpain, is suspected because Ca^{2+} homeostasis is disturbed following eccentric exercise and several proteins connected to Z-disks are known calpain substrates (6). In addition, other proteolytic systems, such as the ubiquitin-proteosome system, are likely involved (34). The suggested sequence of events in the degradation process is that calpains degrade and release proteins from myofibrillar structures, and degradation is thereafter completed by the ubiquitin-proteosome system (37).

Paragraph 2 Myofibrillar disruptions following eccentric exercise have been well documented (e.g. (15)). However, a clear relationship between the magnitude of myofibrillar disruptions and changes in muscle function has not always been observed (36). Normally, only a few sarcomeres are disrupted during eccentric exercise (14;15), and since force not only is transmitted longitudinally, but also laterally towards the sarcolemma and extracellular matrix, it is possible to transmit force around a few disrupted sarcomeres (8). If the structural elements involved in lateral force transmission remain intact, only minor changes in muscle function are expected. However, if there is a concomitant destruction of the lateral force transmitters between myofibrils (e.g. desmin (21)) or muscle fibers (e.g. extracellular matrix (33)), a close relationship between the magnitude of myofibrillar disruptions and reductions in force-generating capacity is expected. The extracellular matrix protein, tenascin-C, is rapidly upregulated in the endomysium in both animal and human models after increased loading on skeletal muscles, and has been suggested as an indicator of disruptions in the

extracellular matrix (11;13). The N-terminal propeptide of procollagen type III (PIIINP) is also upregulated after a single bout of high-force eccentric exercise in humans (11). Tenascin-C and PIIINP may therefore be used as markers for remodeling of the extracellular matrix after exercise.

Paragraph 3 In addition to the observed myofibrillar disruptions, overstretching of sarcomeres and damage to the components involved in excitation-contraction (E-C) are also potential mechanisms underlying the reductions in force-generating capacity (for reviews, see (31;35). Overstretched sarcomeres may be indicated by a shift in the optimal muscle length for peak tension in the direction of longer muscle lengths (31), while changes in E-C coupling may be indicated by greater reductions in force with low frequency stimulations compared to high frequency stimulations (e.g. 20 vs. 50 Hz stimulation) (35). Interestingly, Gregory et al. (19) observed that increased series compliance, induced by eccentric contractions (overstretched sarcomeres) or by inserting a compliant spring, also affects twitch:tetanus force ratio. Therefore, the different contractile measurements indicating muscle damage may interact with another and thereby complicate the interpretation from one single measurement after eccentric exercise.

Paragraph 4 Since a tight coupling exists between myofibrils, the cytoskeleton and membranes, damage to the sarcolemma and other membrane structures is expected to accompany sarcomere disruptions. A possible consequence of ruptures in both sarcolemmal and sarcoplasmic reticulum membranes is an increase in the levels of intracellular Ca^{2+}, as observed in mice muscles after downhill running (22). In addition, activation of stretch sensitive Ca^{2+} -channels might also contribute to increased cytosolic Ca^{2+} levels after eccentric exercise (1). Increased levels of Ca^{2+} activate calcium-dependent proteases such as

calpains (6). Calpains are suspected to play an important role in exercise-induced muscle damage because these proteases are largely localized to the I- band and Z-disk regions of myofibrils and many important myofibrillar structural proteins such as desmin are known calpain substrates (6). Calpains have been shown to disrupt the integrity of Z-disk structures and release myofibrillar proteins during a number of physiological conditions (9;18), but to date calpain activity has not been measured after eccentric exercise in humans.

Paragraph 5 The mechanisms suggested to play a role in exercise-induced muscle damage have been studied separately in different studies with rather large variations in exercise protocols and study designs. Therefore, the aim of this study was to investigate the mechanisms behind muscle damage through studies of muscle structure and function in the same subjects after high-force eccentric exercise. Care was taken to control for possible artifacts of repeated biopsies by taking biopsies both from the exercised muscle and from the contra lateral non-exercised control muscle. The main hypothesis was that the eccentric exercise would result in myofibrillar and membrane disruptions with a concomitant calcium overload and increased calpain activity. Furthermore we expected to find close relation between muscles with substantial signs of myofibrillar disruptions and reductions in force-generating capacity.

Materials and methods

Paragraph 6 ***Subjects.*** Eleven healthy male students (28±4 years, 180±8 cm, 83±6 kg; means±SD) gave written, informed consent to participate in the study. The subjects' level of fitness varied due to a variable level of daily physical activity; three subjects were sedentary, whereas the other eight were physically active to very active (exercising 4-7 days a week), but none of the subjects were engaged in heavy strength training. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway.

Paragraph 7 ***Experimental design.*** Muscle structure and function were monitored for seven days after a bout of maximal eccentric exercise with the m. quadriceps (Figure 1). Biopsies were collected 30 minutes, 4, 8, 24, 96, and 168 hours after exercise (each subject was biopsied at four time points). Recovery of muscle function was assessed with repeated tests of maximal isokinetic knee extensions. Baseline values were obtained before the eccentric exercise, the second test started approximately 3 min after exercise, and tests were thereafter repeated 6, 23, 28, and 47, 71, 95, and 167 hours after exercise. The frequent biopsy sampling during the first day was included to get a detailed time line for the initial events including myofibrillar disruptions and a concomitant activation of calpains. The later biopsies and the frequent testing of muscle function were chosen in order to relate the changes in force-generating capacity to changes in muscle structure.

(Fig. 1, near here)

Paragraph 8 ***One-legged eccentric exercise.*** The subjects performed 300 unilateral, voluntary maximal isokinetic eccentric actions ($30^{\circ}\cdot s^{-1}$) with the m. quadriceps using a Cybex^{6000}

(Lumex, Ronkonkoma, NY, USA). The workout lasted 40 minutes and consisted of 30 sets of 10 repetitions (30 sec rest between each set). The range of motion was from 35-105˚ (0˚ equals full extension of the knee joint).

Paragraph 9 ***Muscle function.*** Maximal force-generating capacity was measured as maximal isokinetic concentric knee extension peak torque at $60^{\circ}\cdot s^{-1}$. In addition, the knee angle of peak torque was registered. All subjects participated in at least four familiarization tests on separate days before entering the study. The coefficient of variation (CV) for repeated measures was <5%.

Paragraph 10 After the isokinetic knee extensions, the subjects remained seated in the Cybex^{6000} and were prepared for electrical stimulation (S11 Stimulator, Grass Instruments, MA, USA). The leg was fixed at a knee angle of 90˚ and electrodes (5 x 10 cm, Polartrode™, Medi-Stim, Oslo, Norway) were placed longitudinally on the m. vastus medialis. Electrode position during each trial was standardized by marking the skin with indelible ink. The stimulation protocol consisted of two trains of 200 ms at 20 Hz and two trains of 200 ms at 50 Hz; each square-wave pulse lasted 0.5 ms and the voltage was fixed at 150 V. Force was measured by a load cell (HBM, U2A, Darmstadt, Germany) connected to the Cybex^{6000} level arm and sampling frequency was 1 kHz. The 20/50 Hz force ratio (CV<5%) was used in the data analysis. Stimulation with 50 Hz produced a force that was equivalent to 5-10% of the subjects' maximal voluntary isometric knee extension.

Paragraph 11 The resting tension in the knee extensors was measured as the force against the load cell when the subjects were relaxed before the electrical stimulation. The mean force during the last 20 ms before the first pulse was used in the data analysis.

Paragraph 12 *Muscle biopsies.* A 5 mm Pelomi needle (Albertslund, Denmark) with manual suction was used to obtain tissue samples (3 x 30-100 mg) from the mid-section of the m. vastus lateralis. Subjects were in the supine position and the procedure was performed under local anaesthesia (Xylocain® adrenaline, 10 $mg \cdot ml^{-1}$ + 5 $\mu g \cdot ml^{-1}$, AstraZeneca). For each subject, four biopsies were collected from both legs at different time points. With 11 subjects and six time points a total of seven biopsies from each leg was analyze at each time point. Each needle insertion was placed 3 cm proximal to the last insertion to avoid affected tissue from earlier biopsies. Muscle samples were rinsed in saline, fat and connective tissue were removed and the best bundles were selected for immunohistochemistry and electron microscopy. In addition, 50 mg of tissue was selected for calpain analysis, 20-30 mg was used for calcium analysis and the rest was used for tissue homogenate. Bundles selected for electron microscopy were placed in a fixing buffer while all other samples were frozen in isopentane on dry ice and stored at -70°C until analysis.

Paragraph 13 *Immunohistochemistry.* Transverse serial sections of the muscle biopsy samples were cut at 5 μm thickness using a cryostat microtome (Microm, Germany) at -22°C and mounted. Serial sections were immunohistochemically stained for PIIINP (Chemicon AB764P, USA) and tenascin C (DAKO M 0636, Denmark). A negative and positive control was included in each staining batch for all antibodies assessed. A routine hematoxylin and eosin stain was also performed to assess for morphological changes. The percentage of the staining area was calculated for both PIIINP and tenascin C as follows. Muscle sections were visualized using a light microscope, and four random images were saved as tagged-image format (TIFF) files without compression. Each field covered an area of 0.64 x 0.48 mm of section. The percent area of red staining within each section was calculated by applying an

intensity threshold in Image J image analysis software (National Institutes of Health, USA). The number of highlighted pixels was then expressed relative to the total number of pixels being evaluated.

Paragraph 14 ***Electron microscopy.*** Biopsy tissue sections with intact, unidirectional fibers were perfused in a fixative prepared from glutaraldehyde (0.1 %) and paraformaldehyde (4%) in a 0.1 M sodium phosphate buffer, pH 7.4 (NaPi), for at least 3 days. Samples were then embedded by freeze substitution as described by Bergersen *et al.* (7). Briefly, rectangular specimens (approx. 0.5 mm x 0.5 mm x 1 mm) were dissected for longitudinal analysis and cryoprotected by immersion in graded concentrations of glycerol (10%, 20% and 30%) in NaPi for 30 min at each step and then overnight at 4^{o}C in the last bath. Samples were then plunged into liquid propane cooled to -190^{o}C by liquid nitrogen in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). Tissue samples were then immersed in a solution of 0.5% uranyl acetate in anhydrous methanol overnight at -90^{o}C. The following day, temperature was raised stepwise in 4^{o}C increments per hour from -90 to -45^{o}C, where it was kept for the subsequent steps. Tissue samples were washed several times with anhydrous methanol to remove residual water and uranyl acetate. Lowicryl HM20 resin infiltration went stepwise from Lowicryl/methanol 1:2, 1:1 and 2:1 (1 hr each) to pure Lowicryl (overnight). Resin polymerization was catalyzed by ultraviolet light at a wavelength of 360 nm for two days at -45°C followed by one day at room temperature (~21º C).

Paragraph 15

Ultrathin sections (80 nm) were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on nickel grids (200 mesh TB, Electron Microscopic Sciences, Fort Washington, USA) using an adhesive pen (David Sangyo). Sections were further contrasted

in uranyl acetate (20 min) and lead citrate (90 sec) before they were observed in a Philips CM100 electron microscope.

Paragraph 16 Methods for analysis were modified from Gibala *et al.* (16). All fibers with a length of more than 3 gridholes (365µm) were scanned for sarcomeres with nonlinear Z-lines and corresponding filaments that were not perpendicular. Areas with disrupted sarcomeres were further investigated at a primary magnification of 3400x. Disruptions were classified as focal when less than 3 sarcomeres in both directions were affected, moderate when 3 to 9 sarcomeres were affected, and extreme if more than 10 sarcomeres in both directions had changes in morphology.

Paragraph 17 *Calpain activities.* Calpain activities were determined using a colourimetric assay as described previously (32) with minor modifications. Briefly, tissue samples were homogenized for 3 x 20 s at two-thirds maximal power in a buffer containing 80 mM KCl, 20 mM Tris (pH 7.5), 5 mM EGTA and 20 mM DTT. The mixture was centrifuged (4°C) at 20 000 *g* for 15 min and the supernatant (soluble fraction) was removed. The pelleted material was resuspended in the same buffer (with 0.33% Triton X-100 added) and recentrifuged as before. The supernatant from the second centrifugation step (particulate fraction) was removed and stored on ice.

Paragraph 18 The Ca^{2+}-dependent proteolytic activities were assessed using a microplate assay. Soluble or particulate fractions were added to reaction mixtures containing 1 mg/ml casein, 50 mM Tris (pH 7.5) and 20 mM DTT (in duplicate). Following a 5-min preincubation at 30°C, 5 mM $CaCl_2$ was added to one duplicate, while 5 mM EGTA was added to the other. After 30 min at 30°C, an aliquot of each sample was assayed for

proteolysis using diluted Bradford protein dye reagent concentrate (Bio-Rad Laboratories) and an incubation time of 10 minutes. One unit of calpain activity represented a change in A_{595} of 0.1 absorbance units.

Paragraph 19 *Total calcium content*. Biopsy samples weighing 20-30 mg (wet weight) were dried to constant weight at 60ºC and subsequently soaked overnight in 2.5 ml of 0.3 M trichloroacetic acid. Ca^{2+} content was determined by atomic absorption spectrometry as described elsewhere (17).

Paragraph 20 *Statistics.* A one-way repeated measures ANOVA with Dunnett's post-hoc test was performed to identify significant changes. We assessed the differences from baseline for both exercised and control legs, as well as differences between the legs. Since different subjects were biopsied at each time point, Student's paired t-test or Wilcoxon signed rank test was used to analyze differences between exercised and control muscle for all variables analyzed on muscle tissue. Selected bivariate relationships were examined with the Pearson product-moment correlation coefficient test. A p-value of ≤ 0.05 was used for establishing statistical significance. Data are presented as means±SEM.

Results

Paragraph 21 *Exercise load.* During the 300 maximal eccentric actions with the knee extensors in one leg, subjects performed a total negative work of 50±4 kJ. Total negative work in each set of 10 maximal eccentric repetitions was reduced from 2.2±0.1 kJ in the 2nd set to 1.3±0.1 kJ in the 30th set (42±5% reduction, $p<0.01$; n=11). The reduction was linear during the first 20 sets, but seemed to plateau during the last ten sets.

Paragraph 22 *Fatigue and recovery of force-generating capacity.* Peak torque and total work in maximal concentric knee extensions was 6±2% higher in exercised than in control leg at the baseline test ($p<0.05$, table 1). Peak torque was reduced by 47±5% in the exercised leg immediately after exercise (figure 2 A). During the next six hours a rapid phase of recovery was observed, but thereafter recovery was halted and a secondary drop in force-generating capacity was observed the day after exercise. From 48 hours after exercise peak torque recovered slowly, and seven days after exercise peak torque was still 13±4% lower than baseline values ($p=0.01$). The individual acute reductions in peak torque varied from 19 to 73%.

(Table 1 and figure 2 near here)

Paragraph 23 The 20/50 Hz force ratio was reduced by 48±4% immediately after exercise ($p<0.01$) and recovered gradually thereafter. After three days the 20/50 Hz force ratio was no longer significantly different from baseline (figure 2 B). A significant correlation between the individual reductions in voluntary peak torque and 50 Hz stimulated force was observed immediately after exercise ($r=0.88$, $p<0.01$).

Paragraph 24 The knee angle of peak torque during the maximal concentric knee extensions increased by 9±2° after exercise and this increase persisted for the following three days ($p<0.01$, figure 2 C). Thereafter, the knee angle of peak torque recovered towards baseline values and was normalized seven days after exercise.

Paragraph 25 Resting tension in the quadriceps, measured as the force against the load cell before electrical stimulation, increased from 5.2±0.5 N before exercise to 9.3±0.9 N immediately after exercise ($p<0.01$, figure 2 D). Thereafter, resting tension gradually recovered and baseline values were reached seven days after exercise.

Paragraph 26 *Calpain activity.* The total calpain activity in the exercised muscle was 326±71% of the activity in the control muscle 30 min after exercise ($p<0.05$; n=5). Thereafter, calpain activity in the exercised leg seemed to decrease gradually, but the activity was still significantly higher in the exercised leg compared to the control leg 96 hours after exercise (209±37% of control, $p<0.05$, figure 3). The largest increase in calpain activity was observed in the particulate fraction of the muscle homogenate where myofibrillar structures are present. Thirty min after exercise 60% of the total calpain activity was registered in the particulate fraction of the exercised muscle while only 20% of the total activity was found in the particulate fraction in the control muscle.

Paragraph 27 *Ca^{2+} content.* There were no significant changes in Ca^{2+} content in the exercised leg compared to the non-exercised leg at any of the time points (figure 3).

(Figure 3 near here)

Paragraph 28 *Myofibrillar disruptions.* The proportion of fibers with moderate to extreme myofibrillar disruptions was higher in the exercised muscle compared with the control muscle at all time points after exercise ($p<0.05$, figure 4, lower panel). Moderate disruptions were evident throughout the length of the fibers examined, and disruptions were repeated at several locations within a positive fibre. Sarcomere disruptions were characterized by myofilament disorganization and loss of Z-disk integrity (figure 4, upper panel). The variation with the biopsy technique was indicated by the finding of 50, 0 and 80% of fibers with disruptions at 30 min, 24 and 96 hours, respectively, in one subject, and by a high coefficient of variation for the repeated biopsies (CV=51%).

Paragraph 29 *Extracellular matrix.* The area positive for tenacin C and PIIINP immunoreactivity on cross sections increased in the exercised muscle the days after exercise (figure 5). The tenacin C positive area was increased in exercised muscle as soon as eight hours after exercise and was significantly higher than in the control until 96 hours after exercise ($p<0.05$; n=6-7). The PIIINP positive area in exercised muscle was significantly different from control muscle 96 hours after exercise ($p<0.05$; n=6).

(figure 5 near here)

Paragraph 30 *Correlations.* No significant correlations between calpain activity and myofibrillar disruptions or reductions in force-generating capacity were observed (figure 6), but the correlations were heavily influenced by the result from one subject showing a much higher calpain activity than the other subjects. When this subject was excluded, the correlations between calpain activity and myofibrillar disruption and between calpain activity and force deficit, were significant ($r=0.69$ and $r=0.68$, respectively, $p<0.05$). The mean

proportion of fibers with myofibrillar disruptions throughout all time points was correlated with the reduction in force-generating capacity ($r=0.89$, $p<0.01$, figure 7A) and with the change in angle of peak torque ($r=0.72$, $p<0.05$, figure 7C). A significant correlation was also observed between the change in knee angle of peak torque and the acute change in peak torque after exercise was observed ($r=0.84$, $p<0.01$, figure 7B).

(Fig 6 and 7, near here)

Discussion

Paragraph 31 For the first time we report increased calpain activity in human muscle after eccentric exercise, and the observed disruptions in the myofibrillar structure mimicked changes observed after increased calpain activity in experimental muscles. As expected, the high-force eccentric exercise resulted in a large and long lasting reduction in force-generating capacity, and the individual reductions in force-generating capacity were closely related to the proportion of fibers with myofibrillar disruptions. The observed increase in total calpain activity strengthens the hypothesis that disturbances in intracellular Ca^{2+} homeostasis with concomitant activation of calcium-dependent proteolytic pathways play a role in exercise-induced muscle damage and recovery (28).

Paragraph 32 The acute reduction in force-generating capacity of 47±5% is in line with earlier observations using the same exercise protocol (23). However, in our subjects a rather large variation in force deficit was observed, ranging from 19 to 73%. The variation seemed to be related to the subject's training status, since the three most inactive subjects (evaluated with a physical activity diary) experienced the largest force deficits (63-73%). These three subjects also had the most extreme results in all variables related to muscle damage.

Paragraph 33 The individual reductions in force-generating capacity immediately after exercise was highly correlated to the proportion of fibers with myofibrillar disruptions ($r=0.89$, $p<0.01$). This indicates that force deficit after this type of eccentric exercise is directly related to myofibrillar disruptions and is in line with observations on stretched single fibers showing a similar reduction in force-generating capacity (3). Other mechanisms, including metabolic disturbances and changes in E-C coupling, may also have contributed to the force loss after exercise, as indicated by the rapid recovery phase in the first hours after

exercise and the decrease in the 20/50 Hz force-ratio. The contribution from other causes of muscle weakness immediately after exercise, is also indicated by the fact that the proportion of fibers with disruption correlated even better with the force-deficit measured six hrs after exercise (r=0.92). In contrast to our observations, some studies have found no relationship between the amount of fibre disruptions and force deficit (35;36). However, the large variation in the proportion of fibers with disruptions between biopsies from the same subject observed by us (CV=51%) and others (4), indicates that the relationship between myofibrillar disruptions and changes in muscle function may be difficult to detect with single biopsies. In our material, a gradual better correlation between the proportion of fibers with disruptions and the reduction in force-generating capacity was observed when going from including one single biopsy (r=-0.47) to two (r=-0.80) or three (r=-0.89) biopsies from each subject in the equation. Therefore, in order to get a representative number of fibers with disruption with this EM analysis technique, it seems like at least two biopsies taken from different parts of the muscle should be included. It should also be noted that the extent of fibre disruptions was correlated with the change in knee angle of peak torque ($r=0.84$, $p<0.01$), but not with the reduction in the 20/50 Hz force ratio or the rise in passive tension after the eccentric exercise protocol.

Paragraph 34 The observed increase in knee angle of peak torque of 9° indicates an increase in the optimum length of force production, as reported in quadriceps and elbow flexors after eccentric exercise (10;30). A rightward shift in the length-tension curve after eccentric exercise has been explained by acutely overstretched sarcomeres (popping sarcomeres) or by the addition of sarcomeres in series (25). Yu et al. (39) suggested an increased number of sarcomeres in series as part of the remodeling process after a single bout of eccentric exercise in humans (indicated by histochemical findings on longitudinal sections). However, the

observation that the knee angle of peak torque returned to baseline levels after one week in our subjects does not support incorporation of new sarcomeres as the most likely explanation for the transient increase in knee angle of peak torque. Therefore, in line with observations on stretched fibre bundles we believe that "popping sarcomeres" is the main reason for the observed shift in angle of peak torque (27).

Paragraph 35 Warren et al. (35) have suggested that changes in E-C coupling explain most of the reductions in force-generating capacity immediately following eccentric exercise. We observed a 48±4% reduction in the 20/50 Hz force ratio, indicating that reduced E-C function contributed to the reduction in force-generating capacity. However, the observation of a gradual recovery of the 20/50 Hz force-ratio during the first four days, with little or no recovery of voluntary peak torque, indicates that E-C coupling dysfunction was not the cause of the long lasting reductions in force-generating capacity. This is supported by the observation that voluntary peak torque was still reduced by 31±7% three days after exercise, at the time when the 20/50 Hz ratio was recovered. In addition, there was no significant correlation between the individual reductions in peak torque and the 20/50 Hz force ratio after exercise. In line with our findings, Martin et al. (24) found that the 20/80 Hz force ratio was depressed after eccentric exercise of the knee extensors, but recovered within 48 hours, whereas MVC force remained depressed at this point. The late force depression in that study was suggested to be caused by structural damage of the involved muscles. It should also be mentioned that the reduction in the 20/50 Hz force ratio might be a result of increased series compliance due to overstretched sarcomeres and not only to changes in E-C coupling (19). Consequently, the possible interaction between the different contractile measurements makes it difficult to draw definitive cause-effect conclusions based on changes in one of the indicators of muscle damage.

Paragraph 36 The relationship between local myofibrillar disruptions and changes in force-generating capacity was likely dependent on concomitant disruptions of lateral force-transmitting structures both between myofibrils and between muscle fibers. In animal models, eccentric exercise have been shown to destroy the regular pattern of desmin in muscle fibers (21). Although we have no direct evidence of disruptions in desmin or in extracellular structures, the increase in tenascin-C and PIIINP staining in the endomysium after exercise indicates that the extracellular matrix was affected. Similar changes in tenascin-C and PIINP staining intensity have been observed 2-8 days after a comparable bout of eccentric exercise (11). In addition, we observed an increase in the size of the intercellular space and the presence of many round fibers on cross sections from biopsies taken 8-24 hours after exercise (~15% of all fibers). This is in line with the findings of Stauber *et al.* (33) and Koh & Escobedo (20), who suggested that the extracellular matrix was "pulled away" from the fibers after eccentric exercise.

Paragraph 37 The three-fold increase in total calpain activity 30 min after exercise supports the hypothesis that disturbances in calcium homeostasis and concomitant calpain activation are present in exercise-induced muscle damage. Although we did not observe significant increases in total calcium content, this does not exclude changes in cytosolic calcium levels. The increase in resting tension in the exercised muscle may indicate increased levels of calcium in parts of the exercised muscle (31). Although the cause of the increase in resting tension may be multi-factorial, local contractures in fibre segments following a rise in myoplasmic Ca^{2+} caused by membrane damage is one likely explanation (22;31). Furthermore, an increase in total muscle calcium content has been observed both *in vitro* (17) and *in vivo* in studies using electrical stimulation in rat muscles (12).

Paragraph 38 Similar to Arthur *et al.* (2), who measured calpain activity after running exercise in rats, we observed the largest increases in calpain activity in the particulate fraction of the muscle homogenate from the biopsies taken 30 min after exercise (data not shown). This finding suggests an increase in calpain-mediated proteolysis of myofibrillar structures, which is not surprising since myofibrillar disruptions were visible and a large proportion of calpains in striated muscles are located near Z-disks (18). Interestingly, it has been estimated that all Z-disks in a muscle fibre would be destroyed in less than 5 minutes if all calpains in the Z-disks were active (18). This fits with the observed loss of Z-disk integrity in the disrupted sarcomeres from all time points in our electron micrographs. In addition, calpain-mediated cleavage of titin and nebulin near the sites of the Z-disks together with cleavage of desmin have been shown to release the Z-disks and leave a space in the myofibril similar to that observed in our electron micrographs (18). Following calpain-mediated release of myofilament proteins, the degradation is thereafter probably completed by the ubiquitin-proteosome system (37).

Paragraph 39 Calpain activation in areas with myofibrillar disruptions is probably necessary for the release of disrupted filaments so that complete degradation in the ubiquitin-proteosome system can occur. It is however unclear how well-regulated the calpain activation is. Does the calpains only release filaments from already mechanically disrupted sarcomeres, or are additional intact sarcomeres affected? We did not observe a significant correlation between individual rises in calpain activity and the amount of myofibrillar disruptions. This could be due to methodological limitations, or the fact that calpains exert other important effects in muscles related to processes involved in recovery from muscle damage (6). One interesting observation was that the largest increases in calpain activity were found in the

subject who recovered fastest from the exercise. It could be speculated that in this subject, who also was the best trained subject, calpain activity was regulated to the areas of mechanical disruptions and consequently cleaned the area for the coming remodeling process. It is also worth noticing that when we removed this subject from the correlation analysis a significant correlation between calpain activity and the amount of fibers with myofibrillar disruptions was evident. The documentation for the "disturbance in Ca^{2+} homeostasis – calpain hypothesis" in muscle damage is still not overwhelming in humans. However, administration of a calcium channel blocker prior to eccentric contractions reduced desmin disruptions and Z-band streaming in on human study (5), and the amount of autolyzed (activated) calpain-3 was observed to increase 24 hrs after eccentric exercise in another human study (26). On the other hand, the calcium blocker had no effect on other aspects of muscle damage. In animal models administration of both calcium chelators, calcium channel blockers and calpain inhibitors has been shown to reduce intracellular calcium concentrations and attenuate muscle injury from lengthening contractions (38;40). A possible role of calpains in the recovery process might be an "uncoupling" of disrupted myofibrils, which may facilitate the rebuilding of sarcomeres while the whole muscle maintains its normal function.

Paragraph 40 In conclusion, the significant and long-lasting increases in total calpain activity in the exercised muscle, supports a role for this protease in exercise-induced muscle damage and recovery. However, the lack of a significant correlative relationship between individual levels of calpain activity and the extent of fibre disruptions makes it difficult to draw definitive conclusions on the importance of this protease for the observed myofibrillar disruptions. The strong relationship between the proportion of fibers with myofibrillar disruptions and changes in force-generating capacity in the vastus lateralis indicates the

myofibrillar disruptions concomitant with changes in the extracellular matrix is the main cause for the long lasting changes in force-generating capacity after high-force eccentric exercise.

Reference List

1. Allen DG, Whitehead NP, Yeung EW. Mechanisms of stretch-induced muscle damage in normal and dystrophic muscle: role of ionic changes. *J Physiol* 2005 Sep 15;567(Pt 3):723-35.

2. Arthur GD, Booker TS, Belcastro AN. Exercise promotes a subcellular redistribution of calcium-stimulated protease activity in striated muscle. *Can.J Physiol Pharmacol.* 1999 Jan;77(1):42-7.

3. Balnave CD, Davey DF, Allen DG. Distribution of sarcomere length and intracellular calcium in mouse skeletal muscle following stretch-induced injury. *The Journal of Physiology Online* 1997 Aug 1;502(3):649-59.

4. Beaton LJ, Tarnopolsky MA, Phillips SM. Variabilty in estimating eccentric contraction-induced muscle damage and inflammation in humans. *Can.J Appl.Physiol* 2002;27(5):516-26.

5. Beaton LJ, Tarnopolsky MA, Phillips SM. Contraction-induced muscle damage in humans following calcium channel blocker administration. *J Physiol* 2002 Nov 1;544(Pt 3):849-59.

6. Belcastro AN, Shewchuk LD, Raj DA. Exercise-induced muscle injury: a calpain hypothesis. *Mol.Cell Biochem* 1998 Feb;179(1-2):135-45.

7. Bergersen LH, Storm-Mathisen J, Gundersen V. Immunogold quantification of amino acids and proteins in complex subcellular compartments. *Nat.Protoc.* 2008;3(1):144-52.

8. Bloch RJ, Gonzalez-Serratos H. Lateral force transmission across costameres in skeletal muscle. *Exerc.Sport Sci Rev.* 2003 Apr;31(2):73-8.

9. Blunt BC, Creek AT, Henderson DC, Hofmann PA. H2O2 activation of HSP25/27 protects desmin from calpain proteolysis in rat ventricular myocytes. *Am.J Physiol Heart Circ.Physiol* 2007 May 18.

10. Bowers EJ, Morgan DL, Proske U. Damage to the human quadriceps muscle from eccentric exercise and the training effect. *J Sports Sci* 2004 Nov;22(11-12):1005-14.

11. Crameri RM, Langberg H, Teisner B, Magnusson P, Schroder HD, Olesen JL, Jensen CH, Koskinen S, Suetta C, Kjaer M. Enhanced procollagen processing in skeletal muscle after a single bout of eccentric loading in humans. *Matrix Biol.* 2004 Jul;23(4):259-64.

12. Everts ME, Lomo T, Clausen T. Changes in K+, Na+ and calcium contents during in vivo stimulation of rat skeletal muscle. *Acta Physiol Scand* 1993 Apr;147(4):357-68.

13. Fluck M, Chiquet M, Schmutz S, Mayet-Sornay MH, Desplanches D. Reloading of atrophied rat soleus muscle induces tenascin-C expression around damaged muscle fibers. *Am.J Physiol Regul.Integr.Comp Physiol* 2003 Mar;284(3):R792-R801.

14. Friden J, Sjostrom M, Ekblom B. A morphological study of delayed muscle soreness. *Experientia* 1981 May 15;37(5):506-7.

15. Friden J, Sjostrom M, Ekblom B. Myofibrillar damage following intense eccentric exercise in man. *Int.J Sports Med.* 1983 Aug;4(3):170-6.

16. Gibala MJ, MacDougall JD, Tarnopolsky MA, Stauber WT, Elorriaga A. Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *J Appl.Physiol* 1995 Feb;78(2):702-8.

17. Gissel H, Clausen T. Excitation-induced Ca2+ uptake in rat skeletal muscle. *Am.J Physiol* 1999 Feb;276(2 Pt 2):R331-R339.

18. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev.* 2003 Jul;83(3):731-801.

19. Gregory JE, Morgan DL, Allen TJ, Proske U. The shift in muscle's length-tension relation after exercise attributed to increased series compliance. *Eur.J Appl Physiol* 2007 Mar;99(4):431-41.

20. Koh TJ, Escobedo J. Cytoskeletal disruption and small heat shock protein translocation immediately after lengthening contractions. *Am J Physiol Cell Physiol* 2004 Mar;286(3):C713-C722.

21. Lieber RL, Schmitz MC, Mishra DK, Friden J. Contractile and cellular remodeling in rabbit skeletal muscle after cyclic eccentric contractions. *J Appl.Physiol* 1994 Oct;77(4):1926-34.

22. Lynch GS, Fary CJ, Williams DA. Quantitative measurement of resting skeletal muscle [Ca2+]i following acute and long-term downhill running exercise in mice. *Cell Calcium* 1997 Nov;22(5):373-83.

23. MacIntyre DL, Reid WD, Lyster DM, Szasz IJ, McKenzie DC. Presence of WBC, decreased strength, and delayed soreness in muscle after eccentric exercise. *J Appl.Physiol* 1996 Mar;80(3):1006-13.

24. Martin V, Millet GY, Lattier G, Perrod L. Effects of recovery modes after knee extensor muscles eccentric contractions. *Med Sci Sports Exerc* 2004 Nov;36(11):1907-15.

25. Morgan DL, Allen DG. Early events in stretch-induced muscle damage. *J Appl.Physiol* 1999 Dec;87(6):2007-15.

26. 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* 2007 Sep;103(3):926-31.

27. Patel TJ, Das R, Friden J, Lutz GJ, Lieber RL. Sarcomere strain and heterogeneity correlate with injury to frog skeletal muscle fiber bundles. *J Appl Physiol* 2004 Nov;97(5):1803-13.

28. Paulsen G, Crameri RM, Benestad HB, Fjeld JG, Mørkrid L, Hallen J, Raastad T. Time course of leukocyte accumulation in human muscle after eccentric exercise. *Med Sci Sports Exerc* 2009;Submitted.

29. Peake J, Nosaka K, Suzuki K. Characterization of inflammatory responses to eccentric exercise in humans. *Exerc Immunol.Rev.* 2005;11:64-85.

30. Philippou A, Bogdanis GC, Nevill AM, Maridaki M. Changes in the angle-force curve of human elbow flexors following eccentric and isometric exercise. *Eur.J Appl Physiol* 2004 Oct;93(1-2):237-44.

31. Proske U, Allen TJ. Damage to skeletal muscle from eccentric exercise. *Exerc Sport Sci Rev.* 2005 Apr;33(2):98-104.

32. Raj DA, Booker TS, Belcastro AN. Striated muscle calcium-stimulated cysteine protease (calpain-like) activity promotes myeloperoxidase activity with exercise. *Pflugers Arch.* 1998 May;435(6):804-9.

33. Stauber WT, Clarkson PM, Fritz VK. Extracellular matrix disruption and pain after eccentric muscle action. *J.Appl.Physiol* 1990;69:868-74.

34. Thompson HS, Scordilis SP. Ubiquitin changes in human biceps muscle following exercise-induced damage. *Biochem Biophys.Res.Commun.* 1994 Nov 15;204(3):1193-8.

35. Warren GL, Ingalls CP, Lowe DA, Armstrong RB. Excitation-contraction uncoupling: major role in contraction-induced muscle injury. *Exerc.Sport Sci.Rev.* 2001 Apr;29(2):82-7.

36. Warren GL, Lowe DA, Armstrong RB. Measurement tools used in the study of eccentric contraction-induced injury. *Sports Med* 1999 Jan;27(1):43-59.

37. Wei W, Fareed MU, Evenson A, Menconi MJ, Yang H, Petkova V, Hasselgren PO. Sepsis stimulates calpain activity in skeletal muscle by decreasing calpastatin activity but does not activate caspase-3. *Am.J Physiol Regul.Integr.Comp Physiol* 2005 Mar;288(3):R580-R590.

38. Willems ME, Stauber WT. Streptomycin and EDTA decrease the number of desmin-negative fibers following stretch injury. *Muscle Nerve* 2005 Sep;32(3):310-5.

39. Yu JG, Furst DO, Thornell LE. The mode of myofibril remodelling in human skeletal muscle affected by DOMS induced by eccentric contractions. *Histochem.Cell Biol.* 2003 May;119(5):383-93.

40. Zhang BT, Yeung SS, Allen DG, Qin L, Yeung EW. The role of the calcium-calpain pathway in cytoskeletal damage after eccentric contractions. *J Appl Physiol* 2008 May 22.

Acknowledgments: The authors would like to acknowledge Professor Torben Clausen and Tove L Andersen for providing facilities for and technical assistance with the Ca^{2+} measurements. The results of the present study do not constitute endorsement by ACSM

Table 1: Morning (baseline) values obtained in the isokinetic knee-extension strength test before the eccentric exercise protocol.

Test:		**Exercise leg**	**Control leg**
Peak torque $60°\cdot s^{-1}$	(Nm)	267 ± 9	256 ± 11*
Total work $60°\cdot s^{-1}$	(J)	229 ± 7	218 ± 9*

* = Significant difference between exercise and control leg ($p < 0.05$)

Figure legends:

Figure 1. The experimental design. Knee extension strength was measured by voluntary isokinetic knee extensions.

Figure 2. Changes in peak torque during maximal voluntary isokinetic knee extensions (A), changes in the 20/50 Hz force ratio during electrically evoked isometric contractions (B), changes in the knee angle of peak torque (C), and the pressure against the force transducer when the leg was relaxed before electrical stimulation (at a knee angle of 90°) (D). Values are means ± SEM, n = 11. # = significantly different from control leg, $p<0.05$.

Figure 3. Total calpain activity (upper panel) and total calcium content (lower panel) in exercised muscle given as the percentage of the levels in the control muscle. # = significantly different from control muscle, $p<0.05$. N = 6-7 at each time point.

Figue 4. Upper panels: Electron micrograph of longitudinally oriented skeletal muscle fibers exhibiting a focal myofibrillar disruption occupying a single sarcomere and associated Z-disks (left) and a moderate myofibrillar disruption occupying several adjacent sarcomeres and myofibrils (right) (magnification x 16,000). Note the lack of Z-disks in the disrupted sarcomeres. Lower panel: The proportion of fibers showing moderate to extreme myofibrillar disruptions in the exercised and control muscles. At least 10 fibers were investigated in each subject. # = significantly different from control muscle, $p<0.05$. N = 6-7 subjects at each time point and the total number of fibers examined was 210 in the exercised muscle and 200 in the control muscle.

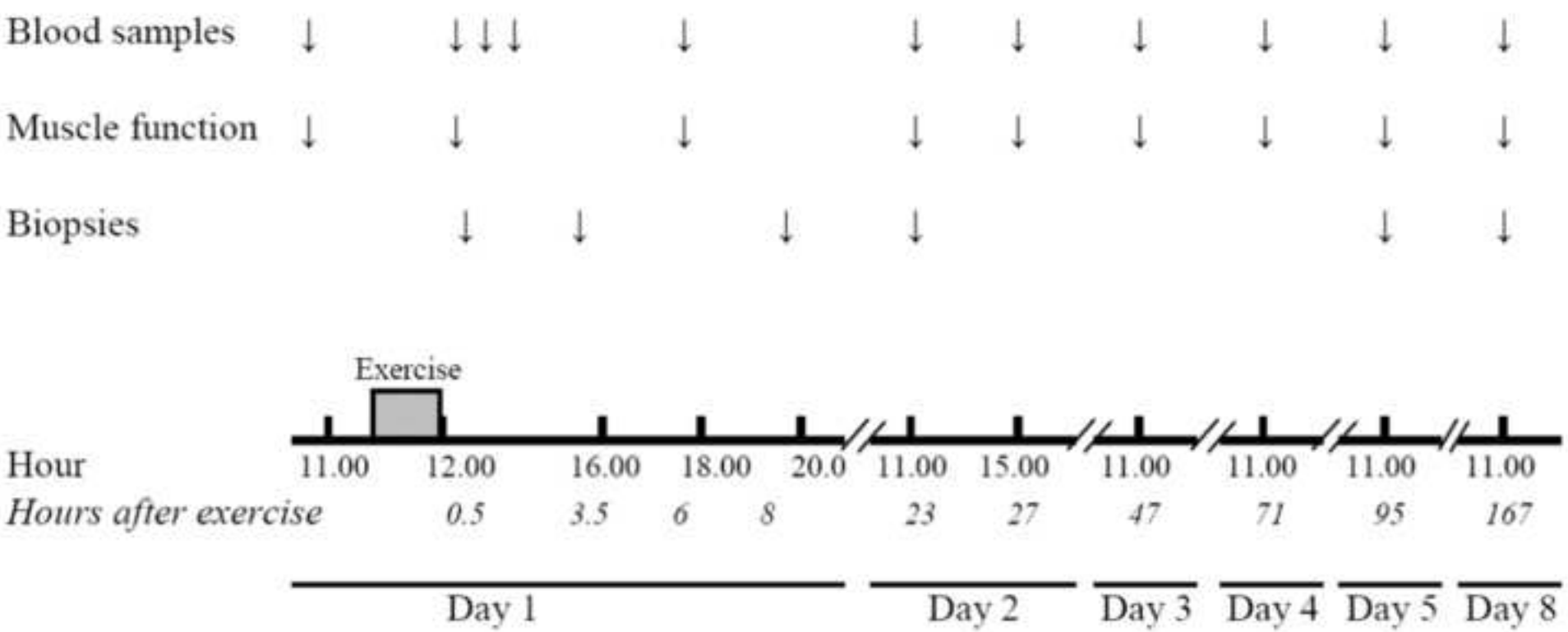
Figure 5. Left panels: Tenascin C (upper panel) and PIIINP (lower panel) area of positive immunoreactivity on cross sections given as the percentage of the total photomicrograph area. Right panels: Cross sections stained for Tenacin C (upper panel) and PIIINP (lower panel) from biopsies taken 96 hours after exercise # = significantly different from control muscle, $p<0.05$. N = 6-7 at each time point.

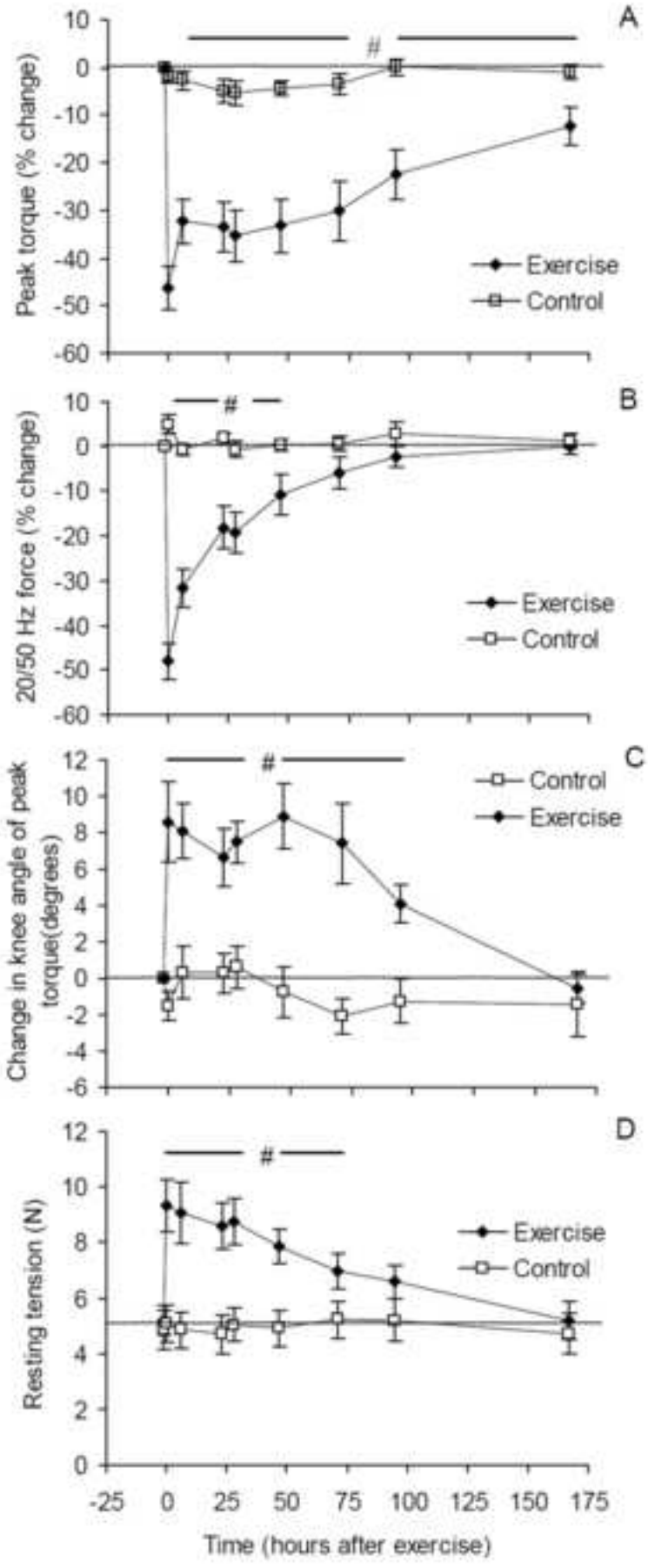
Figure 6. X-Y plots showing the relationship between calpain activity and mean number of fibers with myofibrillar disruptions (lower panel) and between calpain activity and the force

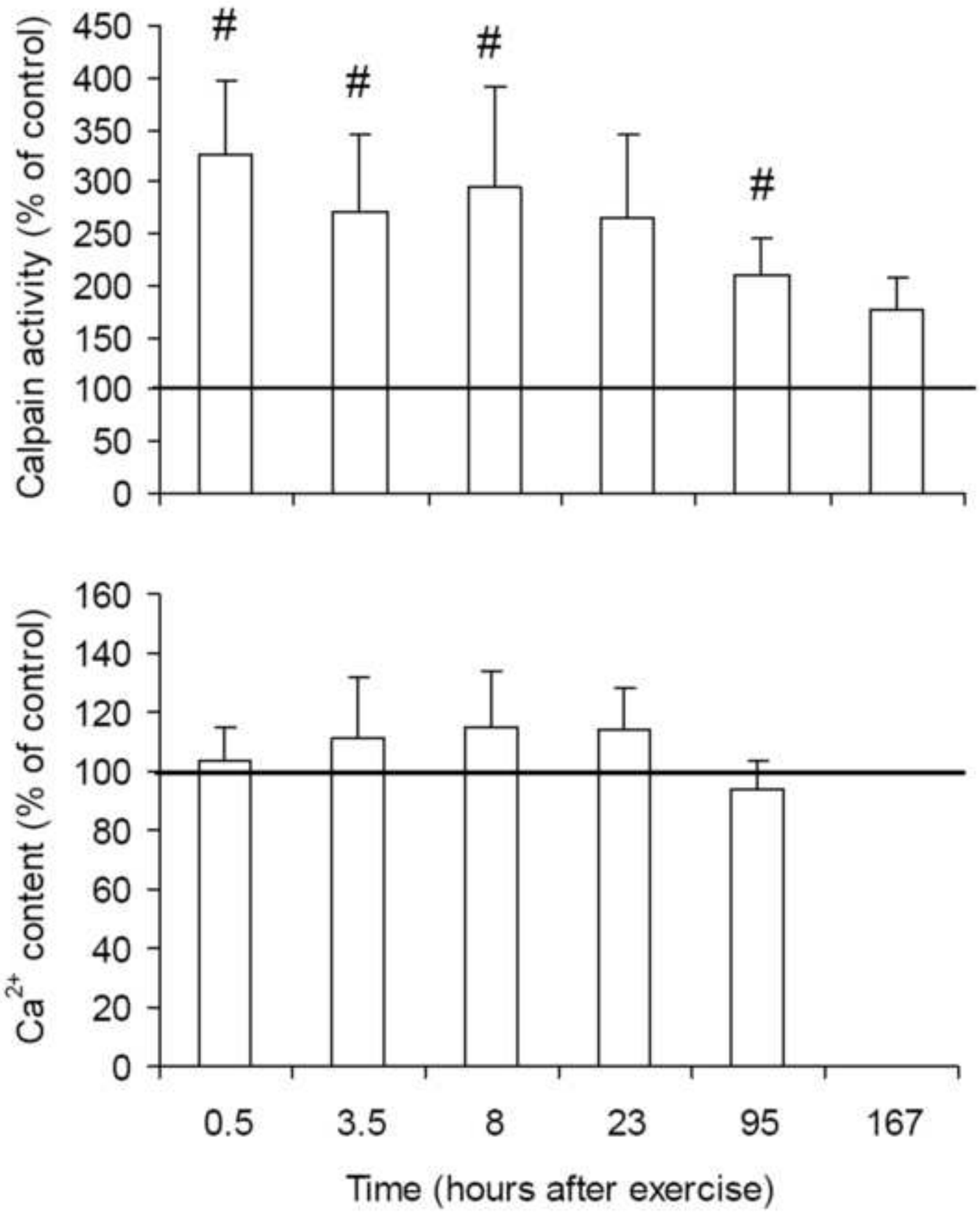
deficit measured six hours after exercise.

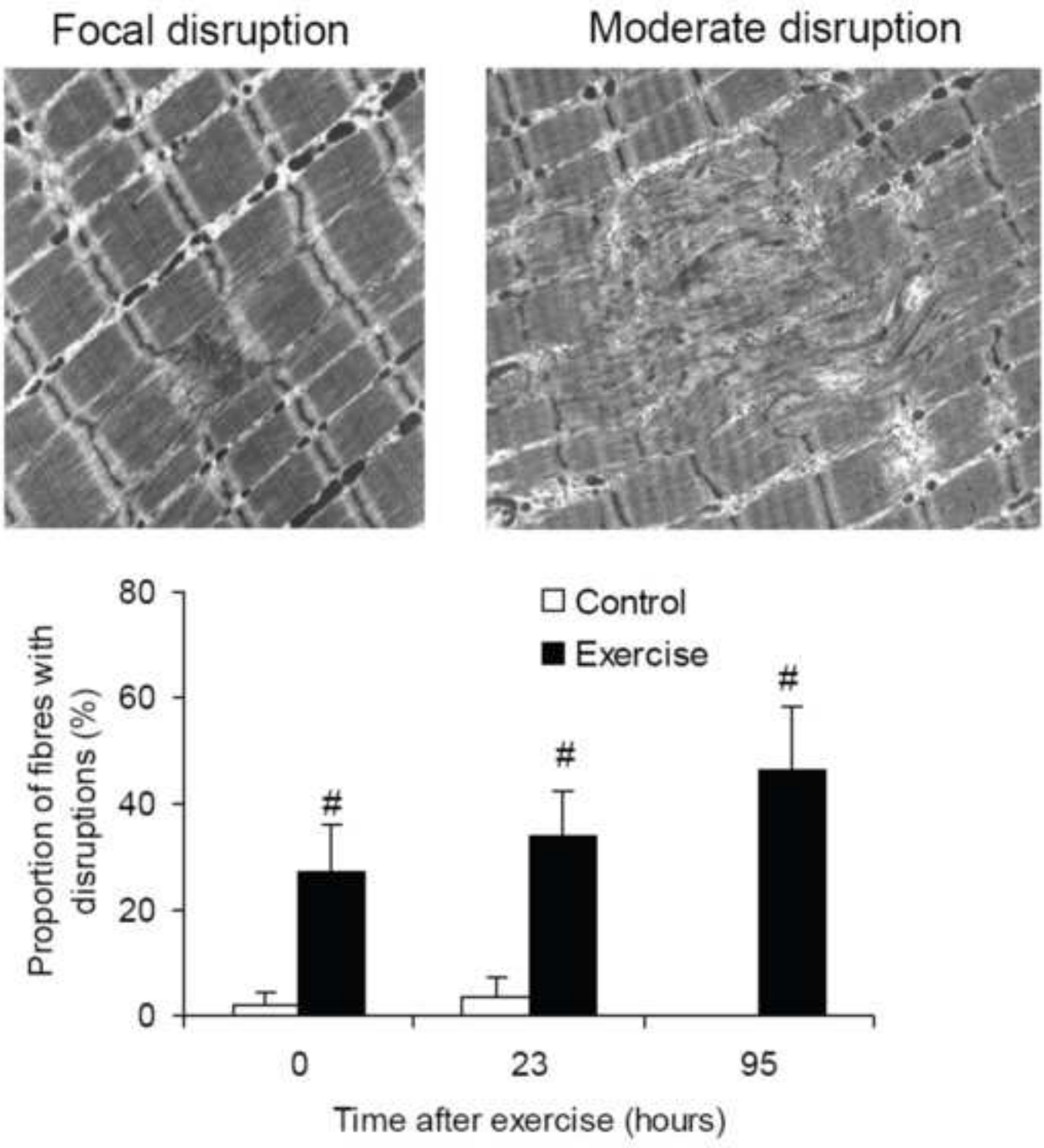
Figure 7. X-Y plots showing the relationship between the mean proportion of fibers with myofibrillar disruptions in biopsies taken 0-95 h after exercise vs. the acute change in force-generating capacity (0 h) (A), the peak change in the knee angle of peak torque (0-48 h after exercise) vs. the acute change in force-generating capacity (B), and the mean proportion of fibers with myofibrillar disruptions vs. the peak change in knee angle of peak torque (C).

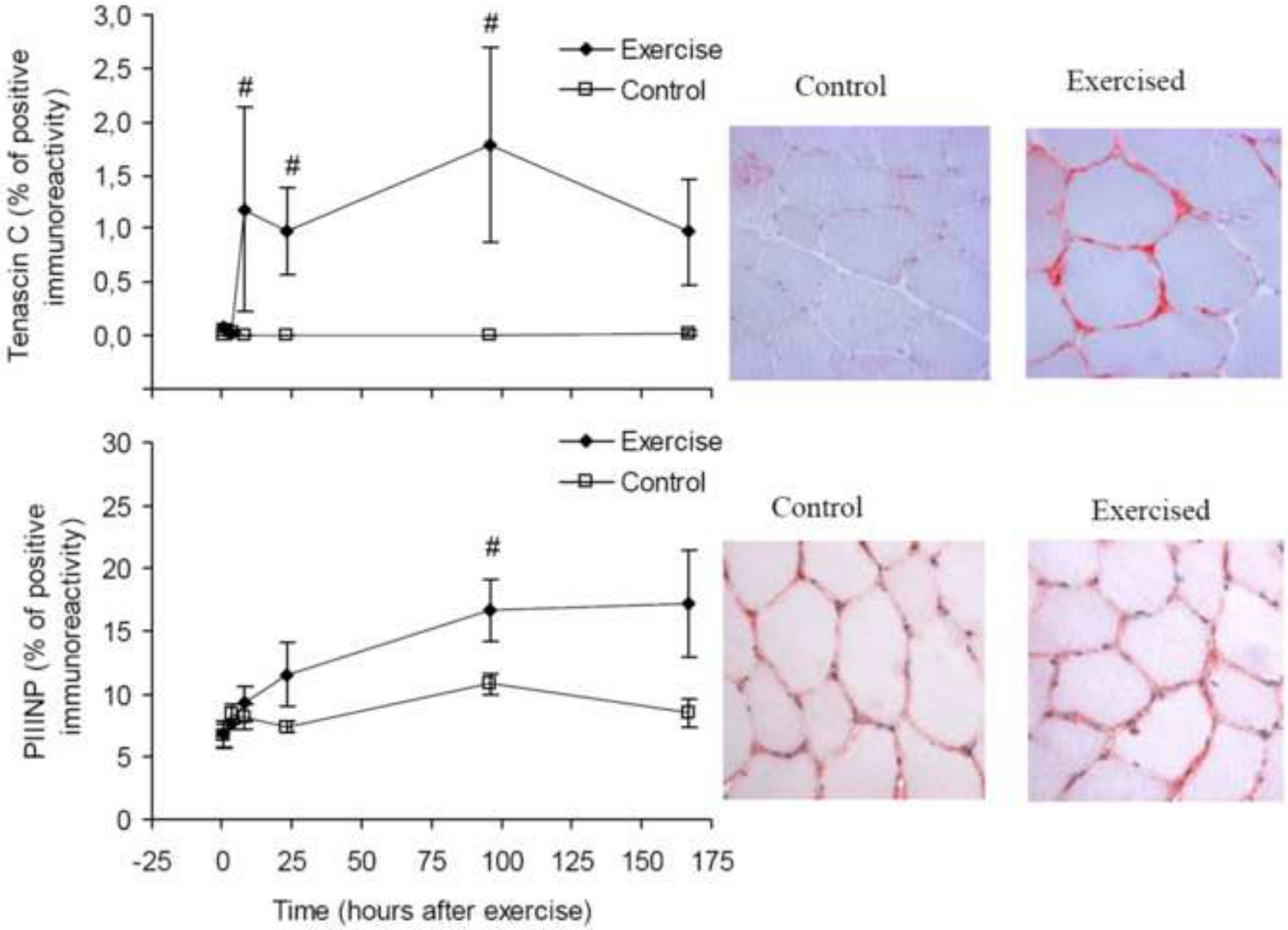
C

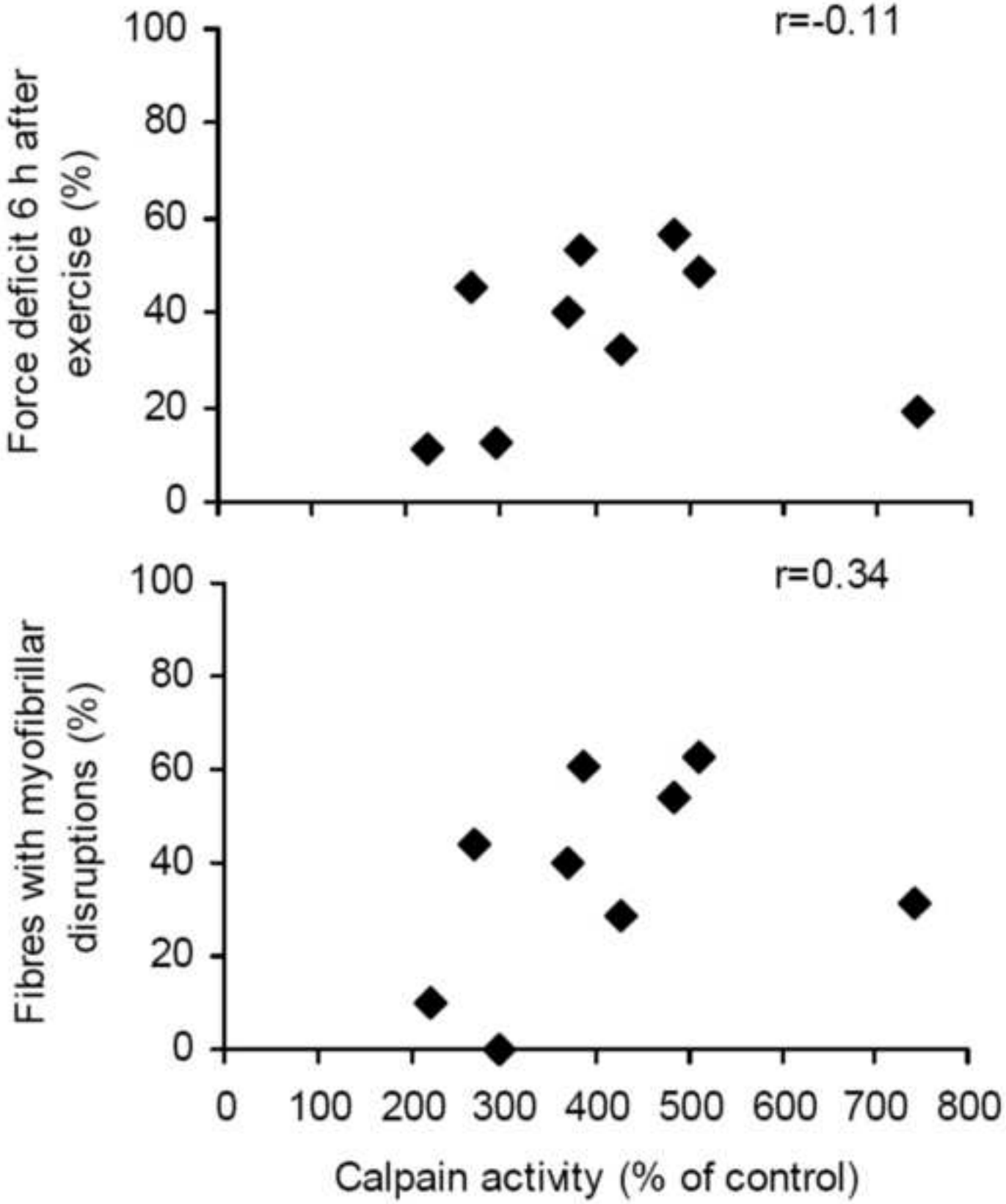












Click here to download high resolution image