Gross ultrastructural changes and necrotic fiber segments in elbow flexor muscles after maximal voluntary eccentric action in humans

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Lauritzen F, Paulsen G, Raastad T, Bergersen LH, Owe SG. Gross ultrastructural changes and necrotic fiber segments in elbow flexor muscles after maximal voluntary eccentric action in humans. J Appl Physiol 107: 1923–1934, 2009. First published October 1, 2009; doi:10.1152/japplphysiol.00148.2009.—Eccentric muscle actions are associated with ultrastructural changes. The severity and types of change depend on the nature of the stimulation protocol, and on the method for assessing such changes, and can be regarded as a continuum from mild changes to pathological-like changes. Most studies describing more severe changes have been performed on animals and only a few in humans, some using electrical stimuli. Hence, a debate has emerged on whether voluntary actions are associated with the pathological-like end of the continuum. The aim of this study was to determine whether severe muscle damage, i.e., extensive ultrastructural changes, is confined to animal studies and studies on humans using electrical stimuli. Second, because there is no generally approved method to quantify the degree of muscle damage, we compared two published methods, analyzing the Z disks or sarcomeres, as well as novel analyses of pathological-like changes. A group of untrained subjects performed 70 voluntary maximal eccentric muscle actions using the elbow flexors. On the basis of large reductions in maximal force-generating capacity (on average, −62 ± 3% immediately after exercise, and −35 ± 6% 9 days later), five subjects were selected for further analysis. Biopsies were taken from m. biceps brachii in both the exercised and nonexercised arm. In exercised muscle, more disrupted (13 ± 4 vs. 3 ± 3%) and destroyed (15 ± 6 vs. 0%) Z disks were found compared with nonexercised muscle. A significant proportion of exercised myofibers had focal (85 ± 5 vs. 11 ± 7%) moderate (65 ± 7 vs. 11 ± 6%), and extreme (38 ± 9 vs. 0%) myofibrillar disruptions. Hypercontracted myofibers, autophagic vacuoles, granular areas, central nuclei, and necrotic fiber segments were found to various degrees. The present study demonstrates that the more severe end of the continuum of ultrastructural changes occurs in humans after voluntary exercise when maximal eccentric muscle actions are involved.

Accordingly, muscle damage; streaming; eccentric exercise; myofibrillar disruptions; electron microscopy

THE OCCURRENCE OF DAMAGE IN skeletal muscle activated and stretched beyond the optimal length for the generation of force was originally proposed by Sir Bernard Katz in 1939. Later, in the 1960s and 1970s, distinct ultrastructural changes in the myofibrillar structure were observed in patients with various skeletal muscle dystrophies (19, 20, 70). Finally, similar ultrastructural changes were also observed after in vivo human experiments using eccentric muscle actions (29, 31, 55). During the last 25 years, factors related to skeletal muscle damage after physical exercise have been investigated in a number of studies. Particularly, eccentric muscle actions (i.e., lengthening contractions) have proven to cause more damage than isometric or concentric action (3, 49, 55). Damage has been measured directly using histological methods, and indirectly by relying on changes in skeletal muscle force, plasma creatine kinase (CK) levels, and self-reported skeletal muscle soreness as damage markers. Among these markers, change in force-generating capacity is considered to be the most reliable (74), although indirect methods can never be used as indisputable evidence (27). Consequently, indirect markers of muscle damage should ideally always be supported by direct measures (22). Nevertheless, in human studies, only minuscule parts of the experimental muscle can be studied by obtaining biopsies (~100 mg); hence markers that reflect overall muscle damage can yield valuable information. Among histological methods, electron microscopic analyses are preferable, since damage can be distinguished from other structural changes known to occur, i.e., separate myofibrillar damage from misalignment. Furthermore, structural features can be directly visualized by electron microscopy, independent of the use of markers.

As a locus for eccentric muscle action-induced damage, the majority of morphological studies conclude that the Z disk is the most vulnerable structure (e.g., Ref. 26). Alteration of the Z disk includes streaming and disintegration, first described as a central core disease by Engel et al. (20) and Gonatas et al. (32) as irregular Z disks with a widened and zigzagged appearance. More severe lesions are associated with patches of dense material located in the midst of sarcomeres. Alterations in Z-disk morphology have been reported after both electrically activated eccentric muscle actions in animals (43) and humans (17), and after voluntary eccentric muscle actions in humans (23, 31, 33). Other ultrastructural myofibrillar alterations reported after eccentric muscle actions include disorganized myofilaments, hypercontracted and/or overstretched sarcomeres, and myofibrils out of register (29).

A problem when comparing studies is the lack of a common method to quantify muscle damage. Hence, based on the histological data presented, comparing the outcome of different damage protocols has been difficult. For instance, in studies where muscle damage has been quantified as myofibrillar disruptions, more extensive damage is reported than in studies where only random Z-disk disruptions are quantified (17, 31, 33). However, because different exercise protocols are used in these studies, deciding whether the discrepancy is a result of differences in the analytical approach or a result of different exercise protocols is not feasible. Whether the ultrastructural changes demonstrated after in vivo human experiments apply...
ing voluntary muscle actions actually are not signs of damage, but rather remodeling of the myofibrillar structure (i.e., adaptation, Refs. 47, 77), has also been discussed. The lack of damage in some studies could be due to the use of only mild stimuli. In this study, the aim was to thoroughly investigate muscle ultrastructure after extreme voluntary stimuli, causing very large reductions in maximum force. Hence, we address whether muscle damage rather than remodeling occurs with exercise. Two previously published methods for quantitative assessment of damages at the ultrastructural level were compared. We also qualitatively and quantitatively report novel observations of a number of pathological-like changes possibly associated with maximal voluntary exercise.

**Materials and Methods**

Subjects. Five healthy students (3 women and 2 men, 25 ± 2 yr, 169 ± 9 cm, 66 ± 4 kg; means ± SD) voluntarily participated in the study. All subjects signed a consent document after being informed about the possible risks of conducting unaccustomed, maximal eccentric muscle actions. The subjects were physically active, but none was engaged in regular heavy strength training of the elbow flexors 6 mo before the study. Subjects were instructed not to take any form of medications or supplements. The study complied with the standards set by the Declaration of Helsinki and was approved by the Regional Ethics Committee of Southern Norway. The 5 subjects were chosen from a total of 33 subjects who performed voluntary eccentric muscle actions based on a >50% reduction in force-generating capacity. In a study where data from all 33 subjects were published (59), the 5 subjects included here are referred to as high responders.

**Experimental design.** Changes in force-generating capacity were monitored 9 days after two identical bouts of unilateral, voluntary maximal eccentric muscle actions. Test of muscle function was followed by blood sampling. The dominant or nondominant arm was randomly chosen to perform the exercise protocol. The same arms were exercised at both occasions, and the nonexercised arm functioned as control for all tests and measurements. The subjects were familiarized with the strength tests before day 1 of the experiment. Baseline values were obtained immediately before exercise. Biopsies were taken at a total of six different time points: at 1 h, 48 h, 4 days, 7 days, 21 days, and 23 days after exercise, from both control and exercised skeletal muscle. However, biopsies were only taken at three time points from each subject. Since our study presents 5 high responders selected from a total of 33 subjects, the number on each biopsy time point was low. Consequently, biopsies were pooled into three groups for further statistical analysis: early (1 + 48 h), middle (4 + 7 days) and late (21 + 23 days). The three biopsy time points of each subject were matched with these groups, so that all five subjects had one biopsy time point in each temporal group.

**Eccentric muscle actions.** The subjects performed 70 unilateral, maximal isokinetic eccentric muscle actions (30°/s) with the elbow flexors applying a REV 9000 dynamometer (Technogym, Gambettola, Italy). The subjects were positioned in the chair and fastened with belts over the hip, chest, and shoulder, and the upper arm was stabilized by a cushion. Thus the shoulder joint was kept in a slightly flexed position (30–35° from the vertical axis) and prevented from moving during the elbow exercise. The work consisted of 14 sets of 5 repetitions with 30-s rest in between sets. The range of motion in the elbow joint was 40–175° (180° equals full extension of the elbow joint), and the forearm (elbow) was kept in a supinated position during the extension. At the completion of each eccentric muscle action, the arm was immediately returned passively to the starting position. The exercise bout lasted for ~15 min.

**Skeletal muscle function.** The force-generating capacity was measured as voluntary maximal isometric torque at 90° in the elbow joint. Preceding the test, subjects warmed up on an arm ergometer (Loda, Groningen, The Netherlands) for 3 min at 30–50 W, followed by four submaximal repetitions of concentric contractions in the dynamometer. Thereafter, subjects performed two maximal isometric repetitions, lasting 5 s, separated by 30-s rest. Peak isometric torque was registered and used for further analyses.

**Blood sampling and CK activity.** Blood was drawn from an antecubital vein into a 10-mL serum vacutainer tube. After coagulating for 30–45 min at room temperature (~20°C), the blood was centrifuged at 2,700 g for 10 min at 4°C. Serum was immediately pipetted into Eppendorf tubes and stored at ~80°C until analysis. CK was analyzed with an automated chemistry analyzer (Modular P, Hitachi High-Technologies, Tokyo, Japan), with analytic coefficient of variation being <5%. Values are given as units of enzyme activity per liter of serum (U/L).

**Muscle soreness.** Muscle soreness [delayed onset muscle soreness (DOMS)] was rated on a visual analog scale, where 0 represented “not sore at all” and 100 mm “extremely sore”. DOMS was assessed in m. biceps brachii and m. brachialis after the subjects had stretched, contracted, and palpated the elbow flexors.

**Skeletal muscle biopsy procedure.** A 6-mm Pelomi needle (Albertsson, Sweden, Denmark) with manual suction was used to obtain tissue samples (100–150 mg) from the middle section (central) of m. biceps brachii. Subjects lay in supine position, while the procedure was done under local anesthesia (Xylocain adrenaline, 10 mg/ml + 5 μg/ml; AstraZeneca). For the three biopsies, each needle insertion was placed 1–2 cm lateral or medial to the previous insertion to avoid affected tissue from earlier biopsies. Visible blood contamination and adipose and connective tissue were carefully removed in saline. A smaller tissue section with intact unidirectional fibers (~10 mg) was dissected, dried on filter paper, and immersed in fixative (4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4) and stored at +4°C until analysis.

**Tissue embedding.** Fixed samples were embedded with the use of freeze substitution, as described by Bergersen et al. (5). Briefly, the specimens were cryoprotected by immersion in increasing concentrations of glycerol (10, 20, and 30%) in phosphate buffer for 30 min at each step, and overnight at 4°C in the 30% solution. Then samples were plunged into liquid propane cooled to ~170°C by liquid nitrogen in a Universal Cryofixation System KP80 (Reichert-Jung, Vienna, Austria). The samples were then immersed in a solution of 0.5% uranylacetate in anhydrous methanol overnight at ~90°C in a cryo-substitution unit (AFS, Reichert). The following day, temperature was raised stepwise in 4°C increments per hour from ~90 to ~45°C. Samples were washed multiple times with anhydrous methanol to remove residual water and uranylacetate and infiltrated with Lowicryl HM20 resin at ~45°C, with a stepwise increase in the ratio of resin to methanol (1:2, 1:1, and 2:1 to pure Lowicryl). Resin polymerization was catalyzed by ultraviolet light at a wavelength of 360 nm for 2 days at ~45°C, followed by 1 day at room temperature (~20°C). The skeletal muscle tissue samples were longitudinally oriented before ultrathin sections (80 nm) were cut with a diamond knife (ultra 45°, Diatome) on a Reichert-Jung ultramicrotome (Reichert ultracut S, Vienna, Austria) and mounted on nickel grids (200 mesh TB, Electron Microscopic Sciences) using an adhesive pen (Electron Microscopy Sciences). Ultrathin sections were contrasted in uranyl acetate (5% in 40% ethanol for 90 s) and lead citrate (0.3% for 90 s) before they were analyzed in a Tecnai 12 FEI transmission electron microscope.

**Electron microscopic analysis.** Changes in myofibrillar ultrastructure were analyzed by two different methods: 1) method modified from Gibala et al. (31); and 2) from Cramer et al. (17).

In brief, using the method from the Gibala study, ~10 fibers with a length of more than 3 grid holes (365 μm) from each sample were scanned for sarcomeres with nonlinear Z lines and disorganized myofilaments, using ×1,700 to ×2,550 original magnification. A fiber was considered to be disrupted if it contained any apparent disturbances in the normal myofibrillar banding pattern (see Figs. 2, A and B). Fibers were classified as having focal damages when ~2 sarco-
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meres in parallel and/or in series were disrupted, moderate when 3–9 sarcomeres in parallel and/or in series were disrupted, and extreme if ≥10 sarcomeres in parallel and/or in series had changes in morphology. Fibers with five or more centrally placed skeletal muscle fiber nuclei and extensive structural changes were termed degenerative/regenerative. In addition, fibers with >10 focal disruptions were termed moderate, and fibers with >10 moderate disruptions were termed extreme.

With the method adapted from Crameri et al. (17), 10 random pictures were taken in the electron microscope with a ×2,550 original magnification from each biopsy (the "search" function in the electron microscope was used to randomly choose 10 different locations within each sample). The numbers of total Z disks in each picture were estimated by multiplying the number of Z disks along the x- and y-axis, and then the Z disks were classified as intact (normal straight Z disk), disrupted (Z disks still present but showing morphological changes from the intact Z disks), or destroyed (Z disks no longer recognizable as an intact structure) (see Fig. 2, C–E). In addition to these criteria from Crameri et al. (17), an overlay with a central 100-nm-wide zone and two lateral zones of 100-nm width was constructed above each picture to more precisely evaluate the straightness of each Z disk and reduce observer bias (Z-disk lateral deviations from the central zone on one or both sides = disrupted; lateral deviations of >100 nm from the central zone or no clear lateral boundary of the Z disk = destroyed). Additionally, using the same pictures, mitochondrial area, the area occupied by mitochondria in each electron micrograph, was measured and averaged for control and exercised muscle. During all analyses, the sample identity was concealed from the investigator until all quantifications were completed.

Identification of other intracellular alterations. The following structural alterations were identified according to the literature: hypercontraction and contraction clumps (19, 58), granular sarcoplasm and leukocytes (19), autophagic vacuoles (21), central nuclei (38), and traction and contraction clumps (19, 58), granular sarcoplasm and leukocytes (19), autophagic vacuoles (21), central nuclei (38), and rounded nuclei as a sign of abnormal myonuclear shape.

The following criteria were used for quantification. Hypercontraction was recognized when the parallel I-bands of the Z disks were no longer discernable and subdivided into two categories, covering areas of more than two sarcomeres, but <100 μm², and covering areas of >100 μm². Granules were recognized as light stained, small circular objects, and granular areas were quantified when having a width of >5 μm to separate granular areas from more common intermyofilbrilar granules. Autophagic vacuoles were defined as light stained, round objects with membrane-like interior structures, and only those with a diameter of >0.5 μm were considered. To separate these findings from more commonly appearing vacuoles, a cutoff of >10 vacuoles per 100 μm² were chosen. Myonuclei interior to at least one myofibril from the cell membrane were quantified as internal. Nuclei with a length-to-width ratio of <1.5 were quantified as rounded. When foreign cells were present inside of the muscle cell membrane, the fiber was termed as having one or more necrotic segments. When all specimens had been analyzed, the number of subjects with each of these findings was summarized, as well as the percentage of fibers that had the given finding among the affected specimens.

Statistics. Paired Student’s t-tests were used on data, where values from all time points were compared (see Fig. 3), and results are presented as means ± SE. Bivariate relationships were examined with the Pearson’s product-moment correlation coefficient test. When comparing values that had been subdivided into time points, nonparametric tests were used because of the low number of subjects. To compare the percentages of affected fibers in specimens between exercise and control, Mann-Whitney U-tests were used. For comparison of repeated measurements, Friedman tests with Dunn posttests were used. Results from data treated as nonparametric are presented as median ± minimum/maximum. Statistical significance was set at P ≤ 0.05.

RESULTS

Skeletal muscle function. Force-generating capacity during maximal isometric elbow actions was reduced by 62 ± 3% immediately after exercise (P < 0.01; Fig. 1). The recovery was slow, and force-generating capacity was still reduced by 35 ± 6% 9 days later (P < 0.01), and by 24 ± 6% (P < 0.01) 21 days after exercise. In control skeletal muscles, peak isometric torque was reduced 2–7% after exercise, and it was significantly different from preexercise values 1 and 4 days after exercise. Compared with the exercised arm, the reductions in force-generating capacity in the control arm were minor at all time points (P < 0.01).

Muscle soreness and serum CK activity. Muscle soreness peaked 2 and 3 days after exercise, with values of 56 ± 12 and 55 ± 11, respectively (P < 0.01). Serum CK activity increased exponentially from 129 ± 23 U/l preexercise to a peak of 9,698 ± 4,270 U/l 4 days after exercise (P < 0.05) before it declined to 3,653 ± 1,582 U/l 1 wk after exercise.

Muscle damage. Muscle damage, as described in Gibala et al. (31) (Fig. 2, A and B, as myofibrillar disruptions) and in Crameri et al. (17) (Fig. 2, C–E, as Z-disk disruptions), was identified in the biopsies taken after the exercise protocol. When quantified, the proportion of disruptions (Fig. 3, A and B) was higher in the exercised muscle compared with the control muscle, independent of quantification method used (P < 0.01 in both analyses). However, large differences in amount and severity of disruptions were registered between the two methods (Fig. 3C). For further analysis, the quantitative ultrastructural data from all time points after exercise were pooled into either exercised or unexercised control skeletal muscle (Fig. 3).
investigated from each subject. A mean of 3,446 et al. (17). Ten randomly taken electron micrographs were compared with 96 3,881/H11006/H11021 disruptions (P moderate (r exercise (pooled) correlated with the percentage of fibers with reduction in force-generating capacity at 1 and 48 h after exercised skeletal muscle, 72 3/H11006/H11021). More Z disks categorized as disrupted (13 B disruptions (Fig. 3 11/H11006/H11002). In contrast, in exercised muscle, 85 6% of all of the fibers contained focal or moderate disruptions (Fig. 3A). In contrast, in exercised muscle, extreme disruptions (38 9%, P 0.01) were found. The reduction in force-generating capacity at 1 and 48 h after exercise correlated with the percentage of fibers with moderate (r = −0.93) and extreme (r = −0.94) myofibrillar disruptions (P 0.05).

Z-disk disruptions. This method was adapted from Crameri et al. (17). Ten randomly taken electron micrographs were investigated from each subject. A mean of 3,446 ± 264 and 3,881 ± 226 Z disks from each subject were analyzed in exercised and control skeletal muscle, respectively. In exercised skeletal muscle, 72 ± 10% of the Z disks were intact, compared with 96 ± 3% in control skeletal muscle (P < 0.01) (Fig. 3B). More Z disks categorized as disrupted (13 ± 4 vs. 3 ± 3%) and destroyed (15 ± 6 vs. 0.4 ± 0.2%) were found in exercised skeletal muscle compared with control skeletal muscle (P < 0.01). The reductions of isometric torque 1 and 48 h after exercise correlated with the level of disrupted (r = −0.90) and destroyed (r = −0.92) Z disks (P < 0.05). The two different approaches to quantify muscle damage were correlated (Fig. 3C) when Z disks categorized as disrupted or destroyed were compared with the level of fibers with extreme myofibrillar disruption (r = 0.82, P < 0.01 and 0.7, P < 0.05, respectively).

Mitochondrial density. A 32.5% reduction in average mitochondrial area was observed in the electron micrographs used for Z-disk disruption analysis when exercised muscle from the early biopsy time point (1 ± 48 h after exercise) was compared with control skeletal muscle (1.33 and 1.97 μm² respectively, P < 0.05).

Hypercontractions. At all time points and in both exercised and control muscles, hypercontractions were observed (Fig. 4). In control specimens, two subjects had small hypercontractions of <100 μm² at early, 9% (6–33%) of the fibers, and middle, 13% (8–18%) of the fibers, time points. In one of these subjects, 10% of the fibers also had contractions at the late time point. In exercised muscles, three subjects had small hypercontractions at the early time point, 11% (6–36%) of the fibers, and two subjects at the middle, 17% (9–25%) of fibers, and late, 33% (8–58%) of the fibers. In only one subject were small hypercontractions found in all exercised muscle specimens. Large hypercontractions of >100 μm² were found in three controls, 18% (6–33%) of the fibers, and in all five exercised

Fig. 2. Sample electron micrographs of exercised skeletal muscle illustrating six categories of muscular disruption. A: first, based on the ultrastructural skeletal muscle damage quantification criteria adopted from Gibala et al. (31), focal (*) and moderate (**) disruption were identified and are shown [X = 6,000, scale bar (sb) = 2 μm]. B: an extreme disruption is shown (X = 4,200, sb = 5 μm). C–E: second, electron micrographs of exercised skeletal muscle illustrating three categories of Z-disk morphology, according to ultrastructural skeletal muscle-damage quantification criteria adopted from Crameri et al. (17), are shown. C: example shown of a Z disk (Z), A-band (a), I-band (I), M-line (M), and a mitochondria (m) have been labeled in a sarcomere with intact Z-disk. Also shown categories of Z-disk morphology, according to ultrastructural skeletal muscle-damage quantification criteria adopted from Crameri et al. (17), are shown. C: example shown of a Z disk (Z), A-band (a), I-band (I), M-line (M), and a mitochondria (m) have been labeled in a sarcomere with intact Z-disk. Also shown are disrupted (D) and destroyed (E) Z disks. In addition to the original criteria in Crameri et al., objective values for the width of Z-disks were chosen to reduce observer bias (lateral deviations of >25 nm from a 100-nm-wide zone constructed above the Z disk = disrupted; lateral deviations of >100 nm from Z-disk zone, or no clear lateral boundary of the Z disk = destroyed). X = 26,500, sb = 0.5 μm.

Myofibrillar disruptions. This method was adapted from Gibala et al. (31). An average of 9.8 ± 0.2 fibers were investigated from each biopsy, yielding a total of 148 fibers in both groups, and a mean fiber length of 525 ± 61 and 647 ± 37 μm from exercised and control skeletal muscle, respectively. Control muscles were primarily intact, but 11 ± 7 and 11 ± 6% of all of the fibers contained focal or moderate disruptions (Fig. 3A). In contrast, in exercised muscle, 85 ± 5% of all fibers contained focal disruptions, and 65 ± 7% moderate disruptions (P < 0.01). In the exercised muscle only, extreme disruptions (38 ± 9%, P < 0.01) were found. The two different approaches to quantify muscle damage were correlated (Fig. 3C) when Z disks categorized as disrupted or destroyed were compared with the level of fibers with extreme myofibrillar disruption (r = 0.82, P < 0.01 and 0.7, P < 0.05, respectively).
muscles, 18% (10–56%) of the fibers at the early time point, whereas, at the middle time point, four control specimens, 18% (8–50%) of the fibers, and three exercised specimens, 58% (11–100%) of the fibers, were affected. At the late time point, large hypercontractions were found in one control (10% of the fibers) and four exercised muscles [30% (6–100%) of the fibers]. Notably, all maximum values for exercised muscles were higher than corresponding controls, although the median did not differ significantly. The number of subjects that had small or large hypercontractions did not differ significantly between groups.

Granular areas. At the early time point, granular areas were found in all five control subjects, 13% (7–18%) of the fibers, and in two exercised muscles, 8% (6–10%) of the fibers (Fig. 5). At the middle time point, only two subjects had granular areas affecting 33% (11–55%) and 23% (8–39%) in control and exercised muscle, respectively. At the late time point, granular areas were found in one control (11% of the fibers) and in one exercised muscle (13% of the fibers).

Autophagic vacuoles. Early, autophagic vacuoles were found in exercised muscle from all five subjects and in four subjects at the middle and late time point, affecting 22% (9–63%), 40% (9–85%), and 17% (7–18%) of the fibers at the early, middle, and late time points, respectively (Fig. 6). By comparison, one control muscle with 13% of the fibers affected was found at the early time point, and three subjects, 18% (8–22%) of the fibers affected, and one subject with 10% of the fibers affected were found at the middle, and late time points, respectively. Significantly more exercised than control muscle specimens were affected at the early time point.

Internal myonuclei. Three, two, and none of the control specimens had internal nuclei in 18% (10–20%), 13% (13–14%), and none of the fibers at the three time points, respectively (Fig. 7, A–C). Observations in exercised muscles differed in that two, four, and four subjects had 10%, 24% (11–36%), and 25% (0–60%) of the fibers at early, middle, and late time points, respectively. There are significantly more specimens with internal nuclei among the exercised than the controls at the late time point.

Rounded myonuclei. In only one control muscle, at the early time point, were rounded nuclei found in 19% of the fibers. Similar findings appeared to be more common in exercised muscles and were found in three, two, and three subjects at the early, middle, and late time points, and in 10% (9–13%), 16% (8–23%), and 12% (8–27%) of the fibers, respectively (Fig. 7, D–F).

Necrotic segments. Three exercised muscle specimens were confirmed to have necrotic segments, one at the middle and two at the late time point, with the two most severely affected specimens being from the same subject. The percentage of affected fibers was 33 and 34% (12–56%) (Fig. 8).

DISCUSSION

The aim of this study was to challenge the view that severe muscle damage, i.e., extensive ultrastructural changes seen together with an inflammatory reaction and segmental necrosis, is confined to animal studies or studies using electrical stimulation. Exercised skeletal muscles were examined, in regard to both the magnitude and the types of damage, after 70 voluntary maximal eccentric muscle actions, using the elbow flexors and a large range of motion. Claims that only minor muscle damage or no damage at all after maximal voluntary eccentric muscle actions occurs in lower limbs in humans (17) are not supported by the present findings of ultrastructural disruptions and necrotic segments of muscle in the fibers from the upper body when using high responders. Neither are claims that ultrastructural changes are rather signs of remodeling and adaptations (77). Rather, in the most extreme cases, the findings were similar to those reported in patients with skeletal
muscle dystrophies (19, 20) and to studies using animal models and electrical stimulation to initiate eccentric muscle actions (43).

Muscle damage and changes in force-generating capacity. One could claim that the first prerequisite to discuss whether muscle damage occurs in humans after voluntary eccentric muscle actions is a thorough evaluation of the exercise protocol and of the exercise-induced changes in force-generating capacity in investigations of muscle damage. Generally, we argue that the authors who have spoken against the existence of muscle damage in humans have used exercise protocols that have to be considered as “mild”. In fact, several of the studies report reductions in force-generating capacity that were either small/moderate (<30%; Refs. 17, 48) and/or the protocol did not include maximal/near maximal eccentric muscle actions over a large range of motion; e.g., Yu et al. (76, 77) and Malm et al. (48). Moreover, the use of DOMS to indicate muscle damage (e.g., Refs. 76, 77) has no support in the literature (56, 73). We found only moderate muscle soreness associated with a high degree of muscle damage. In studies in which muscle damage has been claimed, the changes in force-generating capacity have generally been large (11, 34; T. Raastad, unpublished observations). In line with these studies, we found a 62% reduction in force-generating capacity, which was even larger than reported in the classical studies using similar methods (16, 35, 53). Most likely, this was due to the selection of five subjects from a larger group based on their reduction in force-generating capacity. Actually, our subjects responded similar to so-called “high-responder subjects” reported on in recently published studies (10, 60, 65). Hence, long-lasting reductions in force-generating capacity is well established as a marker for muscle damage, and the assumption that changes in force-generating capacity reflect the degree of muscle damage evaluated at the ultrastructural level (22, 31; T. Raastad, S. G. Owe, G. Paulsen, D. Enns, K. Overgaard, R. M. Crameri, S. Kiil, A. N. Belcastro, L. H. Bergersen, J. Hallen, unpublished observations) is supported by the present study. Care was taken to fulfill known criteria to induce muscle damage: 1) a large range of motion (13, 22, 40, 54, 68); and 2) high force in each muscle action (6, 12, 46, 71). In support of criteria 1, eccentric muscle actions with a large range of motion by direct comparison yields a more pronounced reduction in peak isometric torque than using a small range of motion in human subjects (57). Second, Black et al. (6) have recently verified that force

Fig. 4. Two degrees of hypercontraction in exercised skeletal muscle fibers. A: small hypercontraction: a number of myofibrils show hypercontracted sarcomeres (h) underneath the plasma membrane (p), with swollen Z disks and invisible I-bands, covering an area of <100 μm² (time t = 48 h after exercise, X = 4,200, sb = 5 μm). B: histogram of the number of subjects in whom small hypercontractions were found at the three time points. C: box plot showing median, maximum, and minimum percentages of fibers in which small hypercontractions are found. D: large hypercontraction. Noticeably, there is also clumping of myofibrils (contraction clumps). Z disks are not aligned or perpendicular to the plasma membrane. Overstretched areas are present between contraction clumps (t = 7 days after exercise, X = 1,700, sb = 10 μm). E: histogram of the number of subjects in whom large hypercontractions were found at the three time points. F: box plot showing median, maximum, and minimum percentages of fibers in which large hypercontractions are found.
generation per se dictates the muscle damage outcome in humans. The biopsy sampling procedure or central fatigue seems to have little effect on the force-generating capacity, as evidenced by the small reduction seen in control skeletal muscles.

Furthermore, because of the small changes seen in the control muscle when quantifying muscle damage, changes in ultrastructure could not be caused only by the needle biopsy procedure, as has previously been proposed (63). Possible negative effects of the anesthetics were omitted by the used Xylocain with adrenaline injected in the skeletal muscle fascia and by taking biopsies 2–3 cm from the area of injection. Successive biopsies were taken 1–2 cm laterally or medially from the initial sample in both control and exercised arms. In other human studies, successive biopsies from the same skeletal muscles are often taken days and weeks after the initial exercise protocol, and mechanical force is exerted on the muscle, potentially inflicting morphological damage. However, in a study by Staron et al. (66), two biopsies were taken every week, in 8 wk. Still, degenerative changes were not found in the control arm before the 7th and 9th wk (1.7 and 4.9%, respectively). At this time point, >10 biopsies had been taken from the same skeletal muscle.

**Direct comparison of two quantitative methods.** A considerable amount of publications have addressed the issue of ultrastructural changes after exercise (23, 24, 28–30; e.g., Ref. 33), but comparisons of morphological outcome after different damage protocols are difficult, because there is no unison method on how to quantitatively assess the degree of muscle damage. We have compared two approaches (17, 31) on the same samples. Although a correlation was found between methods, the method quantifying myofibrillar disruptions (31) yielded much higher values than the Z-disk disruption method (17). A reason for concern about using the Z-disk disruption method is that areas with disruption are normally intermingled with intact areas. Hence, a random sampling as in the Z-disk disruption method can be expected to overlook some of the damaged areas by chance. Looking into how big an area is being analyzed, 10 pictures, each ~30 × 40 μm in size, are taken randomly, representing a total of ~12,000 μm² of total surface area analyzed in each specimen. The whole surface area of the same specimen is ~1–2,000,000 μm²; thus the
randomly chosen areas constitute only 0.6–1.2% of the specimen. The specimen itself already represents a limited selection: 10 fibers of a total of 250,000 fibers in the biceps brachii muscle (36) or 500,000 fibers in the vastus lateralis muscle (39). Hence, delimitating the analyzed area by a factor of 100 could be detrimental, taking the heterogeneity of muscle fibers in consideration. When compared, myofibrillar disruptions were always associated with Z-disk disruptions, but Z-disk disruptions could be seen without myofibrillar disruptions. Hence, Z-disk disruption could represent a milder ultrastructural change than myofibrillar focal disruptions, whereas destroyed Z disks are observed in areas together with myofibrillar disruptions. Z-disk streaming is known to be a common structural alteration in healthy, young adults (51).

Severity of muscle damage can be compared between studies using the same methods of quantification. In the study by Gibala et al. (31), disruption in the biceps brachii muscle was evident in 3% of the baseline fibers, 33% of the fibers subjected to concentric muscle actions, and 82% of the fibers subjected to eccentric muscle actions. In the present study, 11 and 85% of the fibers were classified with disruptions in the control and exercised arm, respectively. Of all fibers analyzed, extreme disruptions were evident in 41% immediately after and 50% 48 h after eccentric muscle actions in the Gibala study, compared with 38% at all time points combined in the present study. How submaximal eccentric exercise performed in the Gibala et al. (31) study can induce a similar amount of extreme disruptions as a maximal eccentric exercise protocol is unclear, because the force developed during each action seems to be critical for the resulting muscle damage (71). Fibers with disruption after the concentric work were relatively numerous, considering the small changes in force-generating capacity found and compared with other studies (18, 55).

Crameri et al. (17) found that 30% of all Z disks analyzed could be classified as disrupted 5 h after voluntary exercise with eccentric muscle actions, and the number of disrupted Z disks decreased rapidly, to 15% 24 h after exercise. Interestingly, the findings in the electrically stimulated leg were opposite as the number of disrupted Z disks slowly increased to an amount of 30% 4 days after the exercise. When looking at the more severe alterations, destroyed Z disks, none was found in the leg performing voluntary exercise, whereas, in the electrically stimulated leg, an increase peaking at 22% of all Z disks was found 1 day after exercise. In comparison, we found an average of 13% of disrupted Z disks (all time points), not very different from what Crameri et al. (17) found after their voluntary exercise protocol. This similarity was unexpected, given that Crameri et al. registered 16 and 24% reduction of force-generating capacity immediately and 24 h after voluntary exercise, respectively, and that full recovery

Fig. 7. Exercised skeletal muscles with extensive damage and internally placed or rounded myonuclei. A: two central myonuclei in a muscle fiber with several moderate disruptions as classified in Fig. 2. B: histogram of the number of subjects in whom internal myonuclei were found at the three time points (*P < 0.05). C: box plot showing median, maximum, and minimum percentages of fibers in which internal myonuclei are found. D: two rounded myonuclei (n) in a muscle fiber where striation is nearly abolished. E: histogram of the number of subjects in whom rounded myonuclei were found at the three time points. F: box plot showing median, maximum, and minimum percentages of fibers in which rounded myonuclei are found. A and B: t = 7 days after exercise, X = 1,700, sb = 10 μm.
was reported 48 h after exercise, whereas, 3 wk after exercise, our high-responder subjects still showed impairment in force-generation capacity of \( \sim 24\% \). However, in our studies, 15% of the Z disks in the exercised arm were classified as destroyed compared with none in the voluntary exercise and 22% in the electrical stimulation exercise performed by Crameri et al. (18). Hence, using the same methods for analysis but a more severe voluntary exercise protocol, the results obtained from the present study are most similar to the electrically stimulated fibers reported in the former study. Caution should be made when comparing due to our selection of subjects and due to the very limited sample area that is analyzed using this method.

Pathological-like changes. In addition to Z-disk streaming and integration, first described in central core disease, and myofibrillar disruptions, early linked to eccentric muscle actions, several other features previously linked to disease and animal models were observed. First, hypercontraction, as described by Lotz and Engel (44), to various degrees, is shown in Fig. 4. These analyses are prone to artifacts caused by the biopsy procedures (9), as evidenced in the frequent findings in control specimens. Albeit not statistically significant, large hypercontractions appeared to progress over time in the exercised muscle and to decrease in the controls. Exercised muscles systematically showed the most severe cases at all time points.

The appearance of autophagic vacuoles has been reported after strenuous, long-lasting running in mice (64) and is described in the inflammatory muscle disease, inclusion body myositis (37). Central nuclei are not normally present in healthy, young subjects (52). Significantly more subjects were found to have densities of vacuoles above cutoff at the early time point in the exercised muscle (Fig. 6), and, although not statistically significant, the upper values recorded in exercised muscle were up to threefold higher than in the controls. This is in line with animal studies (2) and similar to findings from the mutant mdx mouse (69). The percentage of exercised muscle fibers that displayed internal nuclei appeared to increase at the middle and late time points, whereas the percentage of affected fibers was low in all control samples.

When analyzing for rounded myonuclei, some nuclei are expected to be rendered round by chance as an effect of the sectioning technique. However, rounded nuclei were infrequently encountered and only seen in one control sample. Possibly the cutoff at a ratio of 1.5 could have contributed to keep the numbers low. Rounded nuclei were seen in approximately one-half of the exercised muscle samples in relatively few fibers. Necrotic segments were also encountered and were exclusively found in exercised muscle. Of the two subjects affected, one had necrotic segments in around one-half of the fibers analyzed. From light and electron microscopic studies (25), necrotic segments can be seen repeatedly over the length of a fiber; hence there is a risk of sampling areas that are either

Fig. 8. Immune cell infiltration in exercised skeletal muscle. A: extravascular erythrocytes (arrows) and immune cells in the intercellular space between two myofibers 7 days after exercise. B: immune cells resembling macrophages (one circled in red) underneath the basal lamina 23 days after exercise. These and other cells were numerous in the necrotic segments identified (A and B: X = 1,700, sb = 10 \( \mu \)m). C: higher magnification of the border between an immune cell and intact sarcomeres (t = 7 days after exercise, X = 4,200, sb = 5 \( \mu \)m). D: histogram of the number of subjects in whom necrotic segments were found at the three time points. E: box plot showing median, maximum, and minimum percentages of fibers in which necrotic segments are found.
completely well or completely necrotic when dissecting the small tissue blocks for electron microscopy.

Contrary to the above findings, granular areas were found in all control specimens at the early time point, but not in all of the exercised muscle specimens. Findings in the control specimens could either be an artifact from the biopsy sampling technique, but could also be due to systemic effects of the exercise. Little is known about such effects. The percentage of affected fibers appeared to be highest at the middle time point (Fig. 5). In summary, most findings, except for necrotic segments, were done in both exercise and control muscles, but appeared to be more severe in the exercised muscles, as indicated by higher maximum values. Also, at the late time point, few findings were done in the control specimens (only hypercontractions), whereas all categories, except from granular areas, were found in the exercise specimens. In conclusion, data are scarce due to the low number of subjects and findings and should be further investigated, but indicate that several pathological hallmarks are also involved in eccentric muscle action and recovery.

Support for similarities between animal and human studies have previously been found using the indirect marker CK in blood, since damage to the myofibrils and to the plasma membrane is thought to induce an outward flux of muscle proteins (1, 27). CK, to some extent, reflects the amount of myofibrillar damage, especially when the damage becomes severe (T. Raastad, S. G. Owe, G. Paulsen, D. Enns, K. Overgaard, R. M. Cramer, S. Kiil, A. N. Belcastro, L. H. Bergeners, J. Hallen, unpublished observations). In Clarkson et al. (14), muscle damage is discussed as a possible reason for rhabdomyolysis and possibly renal failure, which is hard to explain unless voluntary contractions could lead to substantial muscle damage. The observed biphasic increase in blood CK activity may reflect 1) an initial increase in sarcolemmal permeability caused by mechanical strain during exercise; and 2) a following peak in blood CK activity observed 4–5 days after exercise, possibly due to segmental myofiber necrosis (as observed morphologically 5 and 7 days after exercise). Inflammatory events like the presence of macrophages (Fig. 7) in necrotic segments of muscle fibers are commonly reported in animal studies (50) and muscle dystrophy (69).

Muscle damage as a cause of peripheral fatigue. If we assume that central fatigue (reduced ability to activate the exercised muscles) is of relatively minor importance for the observed changes in muscle function (8, 31, 53), the loss of force-generating capacity observed was comparable to some animal models (4, 45, 75). However, in other animal models, the degree of damage (e.g., Ref. 49) seems to be ethically out of reach for human studies. Nevertheless, the degree of muscle damage after high-force exercise must be viewed as a continuum from no damage to devastating damage, and, to compare studies, we ought to know where we are at this continuum. Correspondingly, muscle damage could play an incremental role in peripheral fatigue, as the loss of force-generating capacity increase.

Damage to a few, individual sarcomeres will probably not result in significant impairments of skeletal muscle function, since force can be transmitted laterally through intermediate filaments to the sarcolemma and extracellular matrix (7). Hence, nonfunctional, damaged sarcomeres can be bypassed. Multiple small focal disruptions were found in exercised skeletal muscle fibers. While such small lesions do not necessarily affect the skeletal muscle’s ability to generate and transmit force, larger lesions likely will. In the present study, we often observed fibers with extreme damage; in fact, 65 and 55% of the exercised skeletal muscle fibers contained this degree of disruption 0–2 and 5–7 days after exercise, respectively. Often the lesions covered the whole fiber diameter and extended several micrometers longitudinally. In addition, eccentric muscle actions may result in loss of intermediate filaments (4, 25, 42), and loss of intermediate filaments have been observed to correlate with reduction in force-generating capacity (41). Consequently, if we assume that the same myofibers have both myofibrillar disruptions and disruptions of intermediate filaments affecting lateral force transmission, the resulting reduction in force-generating capacity should reflect the number of affected fibers.

Ultrastructural findings after eccentric muscle actions have been suggested to be primarily remodeling and not damage. However, based on the intrinsic differences in exercise protocols, this does not exclude the possibility that what we and others observe is preferentially damage, and not remodeling (e.g., Ref. 31). Importantly, the initial component of recovery in force-generating capacity is faster than can be explained by repair of sarcomeres; hence damage is not the single cause of reduced force-generating capacity. Rather, disturbances seen in the myofibrillar ultrastructure are one manifestation of damage to force-generating and force-transmitting structures, which also include other components, such as extracellular structures (67). Alternatively, damage to the excitation-contraction coupling has been regarded as the primary cause of the reduced force-generating capacity after eccentric muscle actions (72), while others have proposed that excitation-contraction coupling dysfunction is secondary after disruption of sarcomeres (61). Indicative of such dysfunction, we observed a significant low-frequency fatigue (20:100 Hz ratio) (data not shown), repeatedly reported in previous studies (15).

Conclusions. The present study shows that maximal voluntary eccentric muscle actions can result in considerable myofibrillar and Z-disk damage, as well as pathological-like findings, including necrotic fiber segments. The severity of damage apparently depends on individual differences, on the exercise protocol in use (exercise protocols that include high-force eccentric muscle actions), and on the choice of methods to quantify damage. Importantly, in view of the findings presented, the substantial amount of data previously reported using animal models should still be taken into account in the understanding of human response to unaccustomed eccentric muscle actions.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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