The effects of eccentric contraction on myofibrillar proteins in rat skeletal muscle

Running head: ECC-induced reductions in myofibrillar ATPase

Keita KANZAKI[1], Mai KURATANI[1], Takaaki MISHIMA[2], Satoshi MATSUNAGA[3], Noriyuki YANAKA[4], Sachio USUI[1] and Masanobu WADA[1]

[1] Graduate School of Integrated Arts and Sciences, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima-shi, Hiroshima, Japan, 739-8521

[2] Faculty of Human Health Science, Hachinohe University, 13-98 Mihono, Hachinohe-shi, Aomori, Japan, 031-8588

[3] Faculty of Education and Culture, Miyazaki University, 1-1 Gakuenkihanadainishi, Miyazaki-shi, Miyazaki, Japan, 558-8585

[4] Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima-shi, Hiroshima, Japan, 739-8528

Correspondence to: Masanobu Wada
Graduate School of Integrated Arts and Sciences, Hiroshima University
1-7-1 Kagamiyama, Higasihiroshima-shi, Hiroshima, Japan 739-8521
TEL: +81 82 424 6584
FAX: +81 82 424 0759
E-mail: wada@hiroshima-u.ac.jp

ABSTRACT

The present study investigated the effects of eccentric contractions (ECC) on the content of myofibrillar proteins (my-proteins) and the catalytic activity of myofibrillar ATPase (my-ATPase) in skeletal muscles. Rat extensor digitorum longus and tibialis anterior muscles were exposed to 200-repeated ECC or isometric contractions (ISC) and used for measures of force output and for biochemical analyses, respectively. Whereas in ISC-treated muscles, full restoration of tetanic force was attained after 2 days of recovery, force developed by ECC-treated muscles remained depressed ($P < 0.05$) after 6 days. The total my-protein content and the relative content of myosin heavy chain (MHC) in total my-proteins were unaltered during 4 days of recovery after ECC, but fell ($P < 0.05$) to 55.9 and 63.4% after 6 days of recovery, respectively. my-ATPase activity expressed on a my-protein weight basis was unaltered immediately after ECC. However, it decreased ($P < 0.05$) to 75.3, 45.3, and 49.3% after 2, 4 and 6 days of recovery, respectively. Total maximal calpain activity measured at 5 mM Ca^{2+} was significantly augmented ($P < 0.05$) after 2 days of recovery, reaching a level of 3-fold higher after 6 days. These alterations were specific for ECC and not observed for ISC. These results suggest that depressions in my-ATPase activity contribute to ECC-induced decreases in force and power which can take a number of days to recover.

Key Words: myosin heavy chain, muscle damage, stretched contraction, proteolysis, myofibrillar ATPase

INTRODUCTION

Muscles exposed to repeated isometric or concentric contractions show a progressive decline of performance, which normally recovers within several hours (Mishima et al. 2009). This reversible phenomenon is denoted muscle fatigue. In contrast, eccentric muscle contraction (ECC), in which muscles are stretched while contracting, can result in muscle weakness lasting 24 h or more (Allen 2001). Following ECC, there is often muscle damage that is characterized by ultrastructural disruption (Gibala et al. 1995), degradation of cytoskeletal proteins (Zhang et al. 2008), inflammation (Fielding et al. 1993) and increased serum creatine kinase activity (Clarkson et al. 1992). It has widely been accepted that long-lasting muscle weakness observed after ECC reflects the time necessary for de novo synthesis of disrupted and/or degraded proteins and for insertion of newly synthesized proteins into the appropriate region (Ingalls et al. 1998a and 1998b; Corona et al. 2010).

Myosin, the most abundant protein in myofibril, is a hexameric protein consisting of two heavy chains (MHC) and four light chains. Dimerization of MHC forms an asymmetrical molecule with distinct “head” and “tail” domains. According to present models of cross-bridge force production, the myosin head and actin are initially in weak-bound low-force state. With the release of inorganic phosphate from the nucleotide-binding pocket of the myosin head, the cross-bridge transforms into strongly bound high-force state (Fitts 2008). The determinants of force output of the myofibril are the number of strongly bound cross-bridges and the force per strongly bound cross-bridge. The myofibrillar ATPase (my-ATPase) which resides in the head

region of MHC plays a central role in the cross-bridge cycle (Fitts 2008).

It is conceivable that depressions in my-ATPase activity would occur *in vivo* in muscles fatigued by intense exercise since my-ATPase activity is deleteriously affected by metabolites, such as ADP, inorganic phosphate and H^+, which accumulate during anaerobic muscle contraction (Fitts 2008). In contrast, there are no published reports showing that acute exercise is capable of changing my-ATPase activity as measured *in vitro*. Given that ECC causes increased protein degradation and disruption in sarcomeric architecture (Talbot and Morgan 1996; Zhang et al. 2008), it is envisaged that myofibrillar proteins (my-proteins) could be disturbed with ECC and that disturbances in myofibrillar function could be responsible, at least in part, for prolonged muscle weakness. In the only study on this point, Ingalls et al. (1998a) found an approximately 20% decline in the my-protein content 5 days after ECC. Although their finding implies that an appreciable decay of my-ATPase activity expressed on a muscle wet weight basis would occur as a consequence of quantitative changes in my-proteins, the enzyme activity was not measured in that study. Furthermore, they addressed alterations only in muscles undergoing ECC and did not compare ECC with isometric or concentric contractions (Ingalls et al. 1998a). It is accordingly equivocal as to whether the alterations observed in my-proteins are specific only for ECC.

These findings prompted us to investigate time-dependent changes in the catalytic activity of my-ATPase in muscles exposed to ECC. In this study, rat extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were exposed to 200-repeated ECC or isometric contractions

(ISC) and compared with the contralateral, untreated muscles. Given the observations from previous studies on the effects of calpain and protein oxidation on muscle damage (Matsunaga et al. 2001; Murphy et al. 2007; Zhang et al. 2008), measurements of calpain activity and carbonyl groups were included in the present study. The present experiments conducted with *in situ* muscle contraction confirm previous findings of Ingalls et al. (1998a) and reveal that depressions in my-ATPase activity occur in conjunction with elevated activity of calpain a few days after ECC.

MATERIALS AND METHODS

Animal care and experimental protocol

The experiments were performed on adult male Wistar rats (14-17 weeks old) weighing 366 ± 14.2 g (mean ± SD). The rats were given water and laboratory chow ad libitum and housed in a thermally controlled room maintained at 20-24°C with a 12-h light/dark cycle. All procedures were approved by the Animal Care Committee of Hiroshima University. The rats were randomly divided into ISC and ECC groups (n = 24 animals per each group). Under anaesthesia using an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body weight), the animal was placed in a supine position and the left foot was secured in a home-made foot holder attached to the rim of servomotor. The knee was secured using a strap, so that the foot was perpendicular to the lower leg. Sterilized needle electrodes were inserted through aseptically prepared skin for stimulation of the peroneal nerve. Muscle contractions of the anterior crural muscles (EDL and TA muscles) were elicited by stimulating the peroneal nerve using a 1-s train of 1-ms pulse at 50 Hz and supramaximal voltage. For ECC protocol, the experimental foot was forcibly extended by servomotor at an angular velocity of 150º s^{-1} from an ankle flexed 30º to a fully extended 180º position during stimulation for 1 s. For ISC protocol, the ankle joint was fixed at 90º. ECC and ISC were repeated every 4 s for 200 cycles. Immediately, 2, 4 and 6 days (n=6 animals per each time point; ECC0, ECC2, ECC4, ECC6, ISC0, ISC2, ISC4 and ISC6 groups) after contraction protocols, experimental (left leg) and the contralateral control (right leg) EDL and TA muscles were excised under anesthesia using pentobarbital sodium (6 mg/100

g body weight) and were used for measures of force output and for biochemical analyses, respectively. At the end of the experiment, the rats were killed by an overdose of diethyl ether, followed by cervical dislocation.

Measurement of isometric force output

Isolated EDL muscles were mounted vertically in a chamber (30°C) between two platinum plate stimulation electrodes and connected to an isometric force transducer. The chamber contained 200 ml of Krebs–Ringer solution consisting of (in mM) 115 NaCl, 5 $KHCO_3$, 1 $MgCl_2$, 20 $NaHCO_3$, 2 $CaCl_2$, 5 *N*,*N*-bis(2-hydroxyethl)-2-aminoethanesulfonic acid, 11 glucose, 0.3 glutamic acid and 0.38 glutamine. The solution was continuously bubbled with 95% O_2-5% CO_2, which gives a bath pH of 7.4. The muscles were allowed to equilibrate for 10 min, during which time optimal length was determined. Tetanic forces were elicited by direct stimulation at 100 Hz using supramaximal voltage, 1-ms pulses, and trains of 1.5 s. Forces were recorded on a personal computer, analyzed using dedicated software and normalized to cross-sectional area, where cross-sectional area was computed as muscle wet weight divided by the product of muscle length and density (1.07 g/ml).

Myofibril extraction

my-proteins were prepared by the methods of Tsika et al. (1987). Approximately 250 mg of tissue were diluted in 10 volumes (mass vol^{-1}) of ice-cold homogenizing buffer composed of

250 mM sucrose, 100 mM KCl, 20 mM imidazole and 5 mM EDTA (pH 6.8) and homogenized on ice for 3×30 s at 5,000 rpm using a hand-held glass homogenizer. After centrifugation at 1,000 *g* for 10 min at 4°C, the supernatant was discarded. The resulting pellet was rehomogenized in 10 volumes (mass vol^{-1}) of 175 mM KCl containing 0.5% (vol vol^{-1}) Triton X-100 (pH 6.8). This homogenizing-centrifugation cycle was repeated total three times. These procedures were designed to remove membrane bound ATPase. The homogenizing-centrifugation cycle was then repeated two more times using a solution (solution 1) of 150 mM KCl and 20 mM imidazole (pH 7.0). The resulting pellet was suspended in solution 1 and protein concentrations were determined by the Bradford assay (Bradford 1976) using bovine serum albumin as a standard. The protein content contained in the suspension was calculated as milligrams per mL of suspension. The absolute values measured for the my-protein content were lower in this study than in other studies (Tsika et al. 1987; see RESULTS), suggesting some loss of proteins during the extraction step. For the my-protein content, there were no significant differences between the eight groups (ISC0-6 and ECC0-6) in the control muscles and between the experimental and the contralateral control muscles immediately after ISC or ECC (see RESULTS), suggesting that the contraction protocol per se or the time of muscle collection may not affect yield of my-protein. All samples were treated in the same way during the extraction step, and therefore were comparable.

my-ATPase activity

my-ATPase activity was spectrophotometrically determined in myofibril extracts at 37°C

using a modification of the assay of Tsika et al. (1987). The reaction mixture consisted of 30 mM KCl, 30 mM Tris, 2 mM sodium azide, 1 mM $MgSO_4$,, 1 mM EGTA, 1.1 mM $CaCl_2$, 0.4 mM NADH, 10 mM phosphoenolpyruvate, 18 U ml^{-1} pyruvate kinase and 18 U ml^{-1} lactate dehydrogenase. The reaction was started by adding ATP to give a final concentration of 1 mM. The oxidation of NADH was monitored in a spectrophotometer for 3 min (340 nm). my-ATPase activity was calculated as micromoles per minute per mg my-protein.

Contents of MHC and actin in total myo-proteins

We found significant decreases in my-ATPase activity only in ECC-treated muscle (see RESULTS). In order to examine the possibility that the decreased activity could be attributed to decreases in a specific protein contained in my-proteins, the contents of MHC and actin, abundant proteins in my-proteins, were estimated in ECC-treated muscle. To separate my-proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10-20% (mass vol^{-1}) gradient separating gel, as previously described (Yamada et al. 2006). Aliquots of myofibril extracts containing 5 μg of protein were subjected to electrophoresis at 22°C for 5 h, applying a current of 20 mA. The gels were stained with Coomassie blue R. The bands of MHC and actin were identified according to our previous study in which the same method to separate my-proteins was employed (Yamada et al. 2006). The relative contents occupied by MHC and actin in total my-proteins were densitometrically

estimated using NIH Image software. The sum of the individual values for all my-proteins was set to 100%, and the MHC and actin content was expressed as percentages.

Total calpain activity

Muscle pieces of approximately 100 mg were diluted in 9 volumes (mass vol^{-1}) of ice-cold homogenizing buffer composed of 5 mM EDTA, 5 mM EGTA, 20 mM Tris, 10 μg ml^{-1} pepstatin A, 10 μg ml^{-1} 4-(2-aminoethyl)-benzenesulfonyfluoride (AEBF), 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (pH 7.4) and homogenized on ice for 3×30 s at 5,000 rpm using a hand-held glass homogenizer. After centrifugation at 2,000 *g* for 10 min at 4°C, the resultant supernatant was collected. Total calpain activity was measured using the assay of Levine et al. (1990) and Sutan et al. (2000). N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) served as a substrate for calpain. Muscle extract was incubated for 10 min at 37°C in a buffer solution (pH 7.4) containing 20 mM Tris, 1 mM DTT, 10 μg ml^{-1}AEBF, 0,014 mM pepstatin A, and 5 mM $CaCl_2$. The reaction was started by adding SLY-AMC to give a final concentration of 125 μM. Fluorescence of the liberated AMC was monitored in a fluorometer for 15 min (excitation 380 nm, emission 460 nm). Control assay was performed without $CaCl_2$ in the presence of 10 mM EDTA and 10 mM EGTA.

Casein zymography

Casein is a good substrate for calpain. In a casein zymographic assay, calpain digests

casein comprised in nondenaturing polyacrylamide gels. Therefore, calpain activity can be estimated by measuring the intensity of the caseinolytic bands on the gels (Raser et al. 1995; Sultan et al. 2000). The values obtained from this assay represent the maximal activities, but not *in vivo* activities as the enzyme is incubated at high Ca^{2+} concentration ($[Ca^{2+}]$). Casein zymography was performed using procedures described by Raser et al. (1995) and Sultan et al. (2000). Casein (0.2%, mass vol^{-1}) was copolymerized in a 10% (mass vol^{-1}) polyacrylamide gel (pH 8.6) in the absence of SDS. The casein gel was subjected to 15 min of preelectrophoresis (100 V) with a buffer containing 25 mM Tris, 192 mM glycine, 1 mM EGTA and 1 mM DTT in a cold room. An amount of 40 μg protein in the supernatant used for measures of total calpain activity was loaded on the gel and electrophoresis lasted 4 h at constant voltage (170 V). The gel was washed twice for a buffer consisting of 20 mM Tris-HCl (pH 7.4) and then incubated at room temperature overnight in activation buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM DTT and 4 mM $CaCl_2$. Then, the casein gel was stained with Coomassie blue R and the intensity of the caseinolytic bands was densitometrically evaluated using NIH Image software.

Skeletal muscles contain both the ubiquitous calpains, μ- and m-calpains, as well as a muscle-specific calpain, calpain-3 (Dargelos et al. 2008). Calpain-3 binds tightly to titin at the N2A line, whereas the ubiquitous calpains are able to translocate to several intracellular domain such as endoplasmic reticulum, golgi apparatus and plasma membranes (Keira et al. 2003; Dargelos et al. 2008). A proportion associating with the plasma membranes increases with a rise in the cytoplasmic $[Ca^{2+}]$ (Dargelos et al. 2008). Furthermore, it has been reported that much of

diffusible pool of μ-calpain binds to the site within the fiber when [Ca^{2+}] is raised (Murphy et al. 2006). It is reasonable to assume that a higher proportion of calpain exists in a bound form in muscles undergoing ECC than in quiescent muscles since ECC leads to a long-term rise in the resting cytoplasmic [Ca^{2+}] (Lynch et al. 1997). In this study, debris contained in homogenate samples was sedimented by centrifugation. The possibility cannot be excluded, therefore, that the certain amounts of bound forms of calpains were discarded in the centrifugation step, and that as a result, the values measured for total calpain activity and casein zymography were affected by a different portion of calpain being present in the supernatant and were underestimated, in particular for ECC-treated muscles.

Carbonyl content

Because enhanced protein oxidation has been shown to play a role in the degradative process of proteins (Matsunaga et al. 2001), 2,4-dinitrophenylhydrazine (DNPH) -reactive carbonyl contents, an indicator of protein oxidation, were assessed in this study. Approximately 150 mg of tissue was diluted in 9 volumes (mass vol^{-1}) of ice-cold homogenizing buffer containing 50 mM K-phosphate buffer and 1 mM EDTA (pH 7.4) and homogenized on ice for 3×30 s at 5,000 rpm using a hand-held glass homogenizer. After centrifugation at 16,000 *g* for 10 min at 4°C, the supernatant was collected. The carbonyl content in the supernatant was determined by spectrophotometry methods, as described by Levine et al. (1990). Briefly, homogenates containing 200 μg protein were incubated for 30 min at room temperature in 10

mM DNPH in 2 N HCl. Derivatization was stopped by the addition of 20% (mass vol^{-1}) trichloroacetic acid, and protein was pelleted by centrifugation for 3 min at 11,000 *g*. The pellets were washed three times with ethanol-ethyl acetate (1:1). The protein was solubilized in 6 M guanidine hydrochloride and 20 mM K-phosphate (pH 2.3). Insoluble material was removed by centrifugation, and maximal absorbance of the supernatant was measured at the wavelength of 360 nm. The carbonyl content was calculated using a molar extinction coefficient of $\varepsilon = 22{,}000$ $M^{-1}cm^{-1}$. All biochemical measurements were performed in triplicate.

Statistics

All data are presented as mean ± SE. To determine the significance of differences between control and experimental muscles, Student's t-test for paired samples was used. A two-way ANOVA was used to investigate the effects of contraction protocol (ISC vs ECC) and time of recovery. If an overall significant *F*-value was obtained, Fisher's least significant different analysis was used to isolate the significant different mean values. The acceptable level of significance was set at $P < 0.05$.

RESULTS

Isometric force output

For control muscles in ISC0 and ECC0, the absolute values of force output were 212 ± 14 and 209 ± 16 kPa, respectively. There were no significant differences among the eight groups (ISC0-6 and ECC0-6) for forces generated in the control muscles. Following 200-repeated ISC, tetanic force declined to 51.0% of the control value (Fig. 1). Full recovery to normal levels was attained after 2 days, the first point of recovery investigated in this study. A much more pronounced alteration was observed with ECC treatment. Force was reduced to 24.4% immediately after ECC. When muscles were allowed to recover for 2 days, force reverted to 52.9% of control. However, significant alterations were not observed thereafter. After 6 days of recovery, force amounted to 56.9% of control. A main effect of contraction protocol was observed (ISC > ECC).

My- protein content and my-ATPase activity

For control muscles in ISC0 and ECC0, the absolute values of the total my-protein content were 9.12 ± 0.56 and 9.93 ± 0.82 mg mL^{-1}, respectively. The corresponding values for my-ATPase activity were 528.8 ± 35.6 and 557.2 ± 38.2 μmol min^{-1} g^{-1} prot, respectively. There were no significant differences among the eight groups (ISC0-6 and ECC0-6) for the content and activity in the control muscles. The total my-protein content and my-ATPase activity were unaltered with ISC treatment (Figs. 2a and b). In ECC-treated muscles, the content was not

changed during 4 days of recovery, but fell to 55.9% after 6 days (Fig. 2a). Similar to what we observed for the my-protein content, my-ATPase activity were unaltered immediately after ECC. However, it reduced to 75.3, 45.3 and 49.3% after 2, 4 and 6 days of recovery, respectively (Fig. 2b). A main effect of contraction protocol was observed both in my-protein content and my-ATPase activity (ISC > ECC).

For control muscles in ECC0, the absolute values of MHC and actin were 20.1 ± 1.7 and 17.4 ± 2.0%, respectively. There were no significant differences among the four groups (ECC0-6) for the values in the control muscles. It is obvious that the alteration in my-ATPase activity shown in Fig. 2b is not due to the reduction in the total my-protein content because the activity was calculated on a my-protein weight basis (see MATERIALS AND METHODS). It seems possible that a loss of a specific protein could contribute to the ECC-induced decay in my-ATPase activity. We explored this possibility using SDS-PAGE (Fig. 3a). The MHC content was found unaltered during 4 days of recovery (Fig. 3b). However, after 6 days, the content was depressed to 63.4% of control. In contrast, no changes were found in the content of actin, the second abundant protein in myofibril.

Calpain activity

For control muscles in ISC0 and ECC0, the absolute values of total calpain activity were 139.4 ± 8.2 and 134.2 ± 4.1 nmol min^{-1} g^{-1} prot, respectively. There were no significant differences among the eight groups (ISC0-6 and ECC0-6) for the activities in the control

muscles. The activities measured by this assay are under the influence of not only calpain but also calpastatin, an endogenous calpain inhibitor (Sultan et al. 2000). The elevations in total calpain activity observed in muscles exposed to ECC are based in the assumption that the relative proportion of calpain present in the supernatants was not different at different time points. A possible underestimation for ECC-treated muscles notwithstanding (see METHODS), elevations in total calpain activity were observed in muscles exposed to ECC (Fig. 4). The activity was unaltered immediately after ECC, but augmented 1.5-fold after 2 days of recovery. Thereafter, it was further increased, reaching a level of approximately 3-fold higher than control after 4 and 6 days.

A zymographical assay developed by Raser et al. (1995) is based on an electrophortic separation and endogenous inhibitors are liberated from calpain upon electrophoresis. Therefore, the assay has the advantages of distinguishing different calpain isoforms and excluding the effect of calpastatin. As shown in Fig. 5a, the obtained supernatant yielded two electrophoretically separated caseinolytic bands. According to Sultan et al. (2000), the faster and slower migrating bands were identified as m- and μ-calpains, respectively. The intensity of the caseinolytic bands was estimated by densitometry (Fig. 5b and c). There were no significant differences among the eight groups (ISC0-6 and ECC0-6) for μ- and m-calpain activities in the control muscles. Similar to what has been shown for total calpain activity, changes were found only in muscles undergoing ECC. m-calpain activity was unaltered immediately after ECC (Fig. 5c). However, an increase in the activity was manifested after 2 days of recovery and reached a peak after 4

days. At this time, the activity amounted to approximately 5-fold higher than control. Thereafter, there was a trend toward a decrease. A main effect of contraction protocol was observed in total and m-calpain activities (ISC < ECC). With regard to μ-calpain, changes were not observed during 4 days of recovery. However, the activity declined to approximately 60% after 6 days (Fig. 5b).

Carbonyl content

For control muscles in ISC0 and ECC0, the absolute values of the carbonyl content were 4.07 ± 0.32 and 4.07 ± 0.38 nmol mg^{-1} prot, respectively. There were no significant differences among the eight groups (ISC0-6 and ECC0-6) for the content in the control muscles. No significant changes in DNPH-reactive carbonyls were found with ISC treatment although there was a trend toward an increase immediately, 2 and 4 days after ISC. (Fig. 6). In ECC-treated muscles, the content was also unaltered during 2 days of recovery. However, muscles which were allowed to recover for 4 and 6 days displayed a 30.6% and 12.9% decrease, respectively. A main effect of contraction protocol was observed (ISC > ECC).

DISCUSSION

In the present study, EDL and TA muscles were used for force output and biochemical assays, respectively. This is because of restrictions on the amount of EDL muscle available. It has previously been shown that EDL and TA muscles are composed exclusively (>95%) of fast-twitch fibers (Pette and Staron 1990; Wada et al. 1992) and exhibit similar functional deficit after ECC *in situ* and comparable recovery patterns (Faulkner et al. 1989). Thus we assume that these two muscles are affected by ECC to a similar extent; this assumption has been adopted in other studies (Ingalls et al. 2004).

The present results confirm previous findings that the muscle's ability to produce force is reduced both immediately and for a prolonged period of time after performing ECC (Ingalls et al. 1998a and 1998b). Early force deficits are accepted to result primarily from an inability of dihydropyridine receptor to stimulate sarcoplasmic reticulum Ca^{2+} release although disturbances in force-generating proteins are also involved (Ingalls et al. 1998b; Zhang et al. 2008). The present investigation provides for the first time evidence that ECC is capable of depressing not only the my-protein content but also the catalytic activity of my-ATPase a few days after ECC. These alterations are specific only for ECC.

In skeletal muscle fiber, my-ATPase accounts for 50-80% of the total ATP consumption during muscle contraction (Baker et al. 1994) and is a major determinant of contractile properties (Bárány 1967; Bottinelli et al. 1994). Rodent adult skeletal muscles are composed of four MHC isoforms: a slow isoform, MHCI, and three fast isoforms, MHCIIa, MHCIId/x and

MHCIIb (Pette and Straton 1990). my-ATPase activity of muscle fibers is purported to be regulated primarily by MHC although some variations exist among the fibers which express the same MHC isoform (Bottinelli et al. 1994). On the average, the four MHC isoforms exhibit increasing my-ATPase activity in the order of MHCI < MHCIIa < MHCIId/x < MHCIIb (Bottinelli et al. 1994). It is generally accepted that the maximal velocity of shortening correlates with my-ATPase activity (Bárány 1967). In addition, a single fiber study by Bottinelli et al. (1994) has shown a positive relationship between maximal isometric force and my-ATPase activity. Taking these findings into account, the observed depression in my-ATPase activity may explain, at least in part, ECC-induced reductions in force and power which can take a number of days to recover.

The mechanisms underlying disruptions in proteins that occur with ECC are of interest, but remain unclear. Immediately after ECC, triad disruption has been shown to occur in conjunction with reductions (~35-50%) in junctophilins present in triad (Takekura et al. 2001; Corona et al. 2010). A stretched sarcomere has also been observed with concomitant decreases in desmin which has a role in stabilizing the sarcomeric structure (Allen 2001), raising the possibility that protein disruptions is induced by mechanical stress and/or strain imposed to the tissue. However, with regard to my-ATPase protein, the possibility appears remote since we were unable to discern alterations in the activity immediately following ECC.

As pointed out by Matsunaga et al. (2001), enhanced protein oxidation seems to play a role in the degradative process of proteins. The fact that contraction leads to elevated production

of reactive oxygen species (ROS) in muscle fiber (Ferreira and Reid 2008) suggests ECC-induced protein disruptions to result from protein oxidation. However, our failure to detect increases in carbonyl groups makes it unlikely that the observed decrease in the my-protein content is linked to protein oxidation.

Unlike our previous studies (Matsunaga et al. 2003; Mishima et al. 2009), the ISC protocol employed in this study did not bring about significant increases of the carbonyl content in the working muscle. The differences between the studies could be explained by differences in exercise protocols and/or assay procedures employed. The current study used a relatively low-frequency contraction protocol (0.25 trains per second), whereas our previous studies (Matsunaga et al. 2003; Mishima et al. 2009) employed a high-frequency contraction protocol (running at high-intensity and 0.5 trains per second). It is conceivable that high-frequency contractions predispose muscle fiber to higher rates of ROS production compared with low-frequency contractions. Alternatively, it is possible that measurements of the carbonyl group content employed in this study are not sensitive enough to detect a small alteration. If this is the case, the present results can reflect the assay variability, small sample size, and consequently likelihood of a Type II error.

The unexpected result was that ECC-treated muscles which were allowed to recover for 4-6 days exhibited substantial decreases in carbonyl (Fig. 6). Little is known about alterations in protein oxidation during recovery after ECC. In one study, detectable changes in carbonyl were not found 2 days after ECC (You et al. 2005). However, there are no published reports of the

impact of ECC beyond 2 days post-ECC. The reason for the diminished carbonyl we observed is unclear. The moderately carbonylated proteins could be dislodged through unknown mechanisms. It should also be noted that although carbonyl formation in protein has been taken as a marker for protein oxidation, its appearance is certainly not specific for oxidative modification (Levine et al. 2000). For example, glycation of proteins may add carbonyl groups into amino acid residues.

The degradative process in muscle proteolysis results from the actions of cytosolic proteases and lysosomal proteases (Mammucari et al. 2007). The principal cytosolic proteases in skeletal muscle are the ubiquitous calpains, μ-calpain and m-calpain, and the muscle-specific isoform, calpain-3. There is evidence to indicate that ECC-induced proteolysis would be mediated by calpains. For instance, treatment with calpain inhibitor minimizes ECC-dependent reduction in dystrophin, a substrate for calpains (Yoshida et al. 1992; Zhang et al. 2008). Taking into account that a number of my-proteins, e.g., titin, desmin, myosin, troponin and tropomyosin are substrates for calpains (Goll et al. 2003) and that no alterations in the my-protein content and calapin activity were found for ISC-treated muscles (Figs. 2a and 4), it may be speculated that calpains proteolyze my-proteins contained in ECC-treated muscles. Decreases in the relative content of MHC in my-proteins shown in Fig. 3 are in agreement with previous observations by Ingalls et al. (1998a) and make it likely that MHC could be more susceptible to calpain than many of the other my-proteins. A ~ 50% reduction in my-ATPase activity observed 6 days after ECC is explained, at least in part, by a ~37% decrease in the MHC content .

Of the three isoforms of calpains, muscle-specific calpain-3 appears to be involved mainly in ECC-induced proteolysis at the early stage of recovery (up to 24 h) after ECC (Murphy et al. 2007). Our analysis using casein zymography indicated pronounced increases in the m-calpain activity at the relatively late stage of recovery (Fig. 5c). Previous studies have been published revealing that m-calpain is involved in myoblast fusion as a part of satellite cell activation (Schollmeyer 1986) and that satellite cell activation is required for force recovery after ECC (Rathbone et al. 2003). It may be speculated that increases in m-calpain is necessary for recovery of my-ATPase function following ECC.

Interestingly, a decay of my-ATPase activity observed 2-4 days after ECC did not coincide with quantitative changes in MHC (Fig. 2). Previous studies (Gagnon et al. 1990; Termin and Pette 1992) have estimated a half-life of MHC as 15-17 days, which makes it unlikely that the depressed activity relates to the MHC transition from fast to slow isoform. As mentioned above, ECC-induced mechanical stress seems to exert little effect on the functional behavior of my-ATPase. It appears that factors other than mechanical stress would result in structural perturbations in the head region of MHC critical for my-ATPase activity, leading to the depressed activity. How ECC impacts on the catalytic activity of my-ATPase remains to be elucidated by further study.

In summary, we show that my-ATPase activity and my-protein content are unaltered immediately after ECC, but decreased during the recovery period up to several days. The decreased my-ATPase activity is accompanied by an elevation of calpain activity. These alterations are specific only for ECC and do not occur with ISC. At the relatively late stage of

recovery, the reduction in my-ATPase activity is related to decreases in the amount occupied by MHC in total my-proteins, whereas at the early stage, the reduction should stem from structural perturbations in the head region of MHC critical for the enzyme function. It is suggested that depressions in my-ATPase activity contribute to ECC-induced decreases in force and power which can take a number of days to recover.

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Figure legends

Fig. 1. Time course of changes in maximal isometric tetanic force after isometric and eccentric contractions. Isometric or eccentric contractions were repeated in the anterior crural muscles of the left hindlimb for 200 cycles by electrical stimulation via the peroneal nerve. The results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. A main effect ($P < 0.05$) of contraction protocol was observed (isometric > eccentric). Values are means ± SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats; [a] $P < 0.05$, vs 0 d.

Fig. 2. Time course of changes in myofibrillar protein content (a) and myofibrillar ATPase activity (b) after isometric and eccentric contractions. The results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. Measures of myofibrillar ATPase activity were not done on muscles that were allowed to recover for 2 days after isometric contraction, since the fresh samples were lost because of wrong treatment. A main effect ($P < 0.05$) of contraction protocol was observed for the content and activity (isometric > eccentric). Values are means ± SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats; [a] $P < 0.05$, vs 0 d; [b] $P < 0.05$, vs 2 d; [c] $P < 0.05$, vs 4 d.

Fig. 3. Electrophoretic separation of myofibrillar proteins (a) and time course of changes in the relative distribution of myosin heavy chain (MHC) and actin (ACT) in total myofibrillar proteins after eccentric contraction. Myofibrillar proteins were separated by polyacrylamide gradient (10-20%) gel electrophoresis. The relative contents of MHC and ACT contained in total myofibrillar proteins were densitometrically determined after electrophoresis. The results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. Values are means±SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats. ECC6, 6 d after eccentric contraction; Cont, control muscle; Exp, experimental muscle.

Fig. 4. Time course of changes in total calpain activity after isometric and eccentric contractions. The activities were determined on muscle homogenates using an assay based on the cleavage of the calpain-specific fluorogenic substrate. The results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. A main effect ($P < 0.05$) of contraction protocol was observed (isometric < eccentric). Values are means±SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats; [a] $P < 0.05$, vs 0 d; [b] $P < 0.05$, vs 2 d.

Fig. 5. Casein zymograms (a) and evaluation of zymographically assessed μ- (b) and m- (c) calpain activity after isometric (ISC) and eccentric (EEC) contractions. μ- and m-calpains contained in muscle homogenates were electrophoretically separated in a casein gel and subsequently incubated in a proteolysis buffer. The intensity of the caseinolytic bands was estimated by densitometry and the results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. A main effect ($P < 0.05$) of contraction protocol was observed in m-calpain activity (isometric < eccentric).Values are means±SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats; [a] $P < 0.05$, vs 0 d; [b] $P < 0.05$, vs 2 d. Cont, control muscle; Exp, experimental muscle; μ, μ-calpain; m, m-calpain.

Fig. 6. Carbonyl content in whole muscle after isometric and eccentric contractions. The carbonyl group content of muscle homogenate was determined by spectrophotometry. The results in the stimulated muscles are expressed as percentages of the values in the unstimulated contralateral (control) muscles. A main effect ($P < 0.05$) of contraction protocol was observed (isometric > eccentric).Values are means±SE (n = 6 for each time point). *$P < 0.05$, vs control muscle within rats; [a] $P < 0.05$, vs 0 d; [b] $P < 0.05$, vs 2 d.

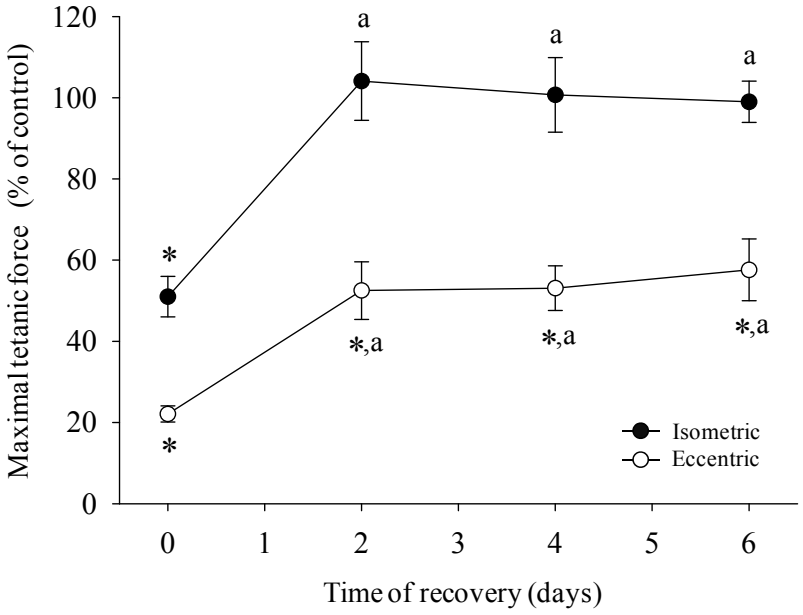
Maximal tetanic force (% of control)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Time of recovery (days)
a
a
a
*
*
*,a
*,a
*,a
Isometric
Eccentric

Fig. 1

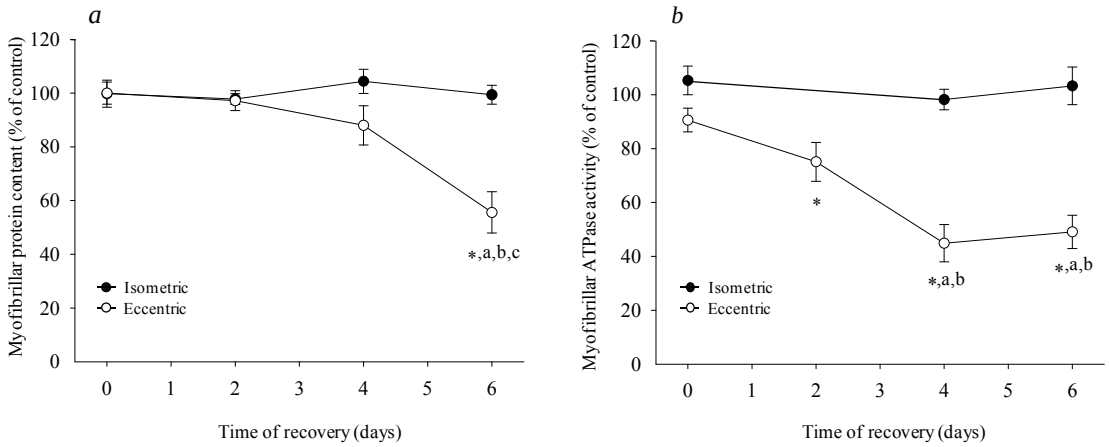
a
Myofibrillar protein content (% of control)
120
100
80
60
40
20
0
0 1 2 3 4 5 6
Time of recovery (days)
Isometric
Eccentric
*,a,b,c
b
Myofibrillar ATPase activity (% of control)
120
100
80
60
40
20
0
0 1 2 3 4 5 6
Time of recovery (days)
Isometric
Eccentric
*
*,a,b
*,a,b

Fig. 2

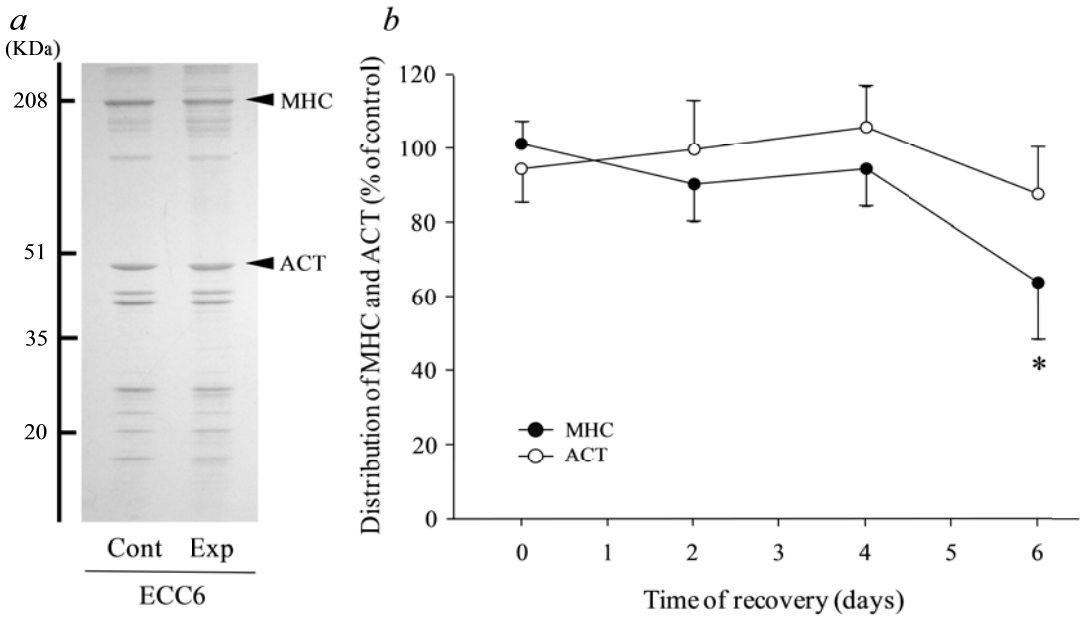
a
(KDa)
208
51
35
20
MHC
ACT
Cont
Exp
ECC6
b
Distribution of MHC and ACT (% of control)
120
100
80
60
40
20
0
MHC
ACT
*
0
1
2
3
4
5
6
Time of recovery (days)

Fig. 3

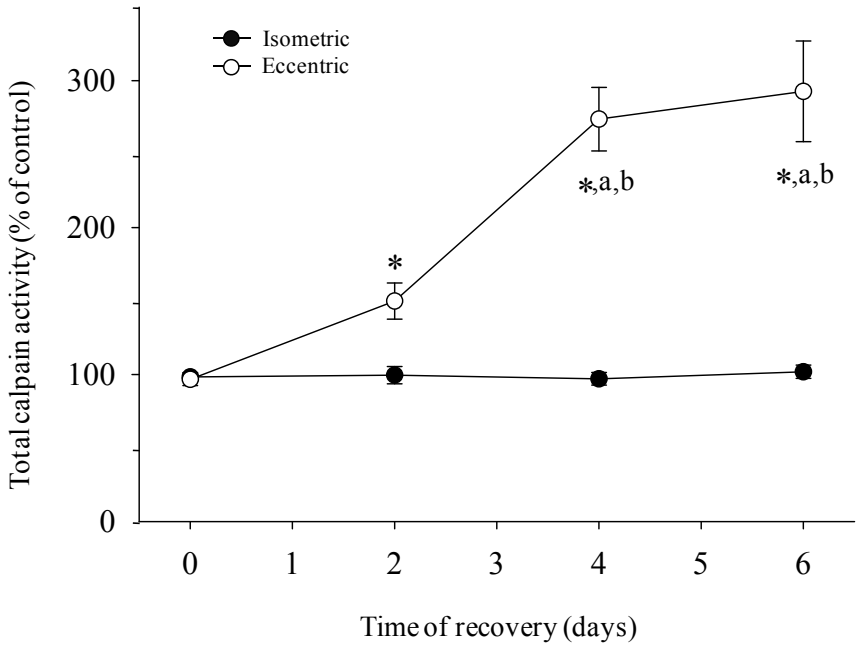
Isometric
Eccentric
Total calpain activity (% of control)
300
200
100
0
*
*,a,b
*,a,b
0
1
2
3
4
5
6
Time of recovery (days)

Fig. 4

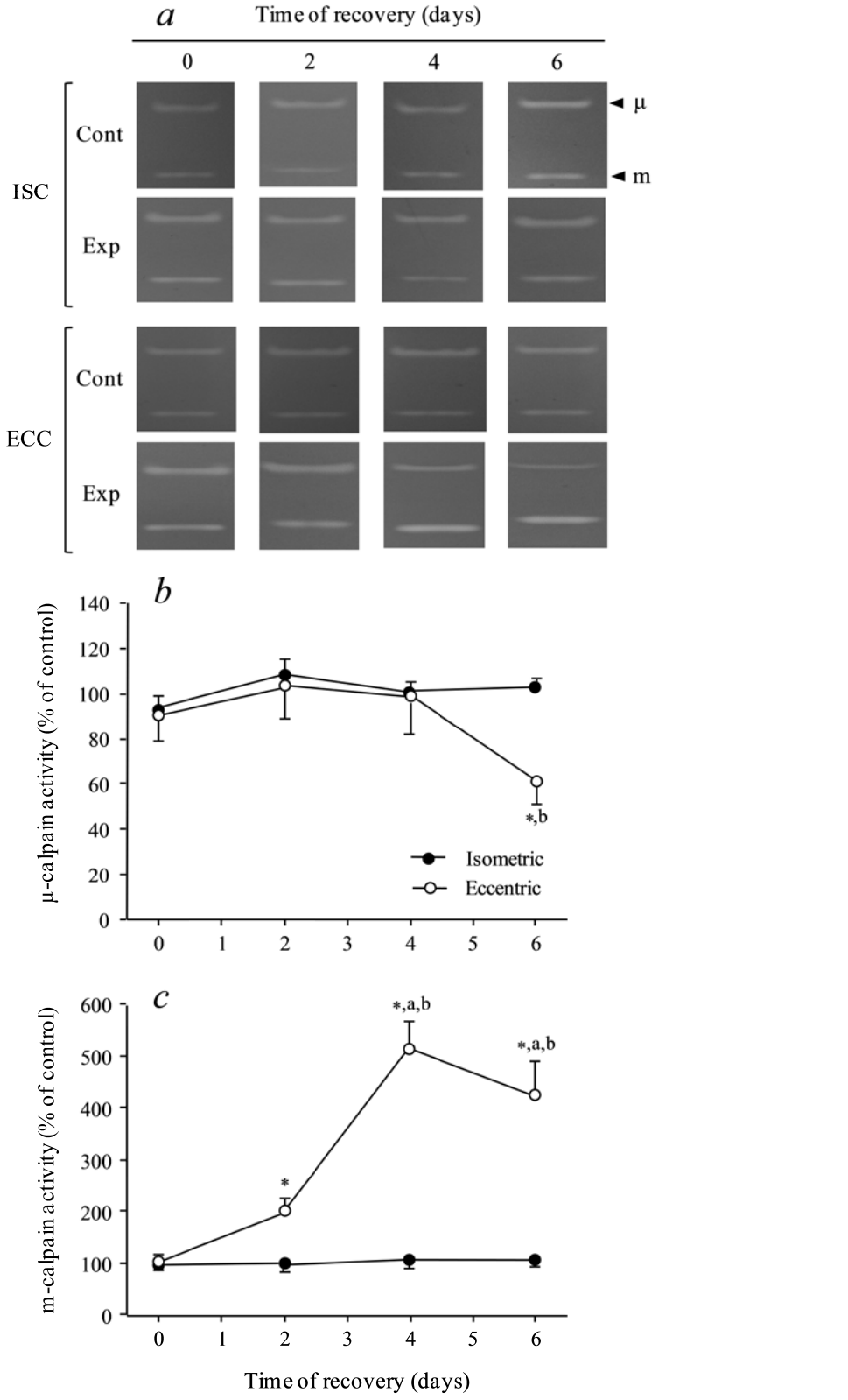
a
Time of recovery (days)
0
2
4
6
ISC
Cont
Exp
ECC
Cont
Exp
μ
m
b
μ-calpain activity (% of control)
140
120
100
80
60
40
20
0
*,b
Isometric
Eccentric
0
1
2
3
4
5
6
c
m-calpain activity (% of control)
600
500
400
300
200
100
0
*
*,a,b
*,a,b
0
1
2
3
4
5
6
Time of recovery (days)

Fig. 5

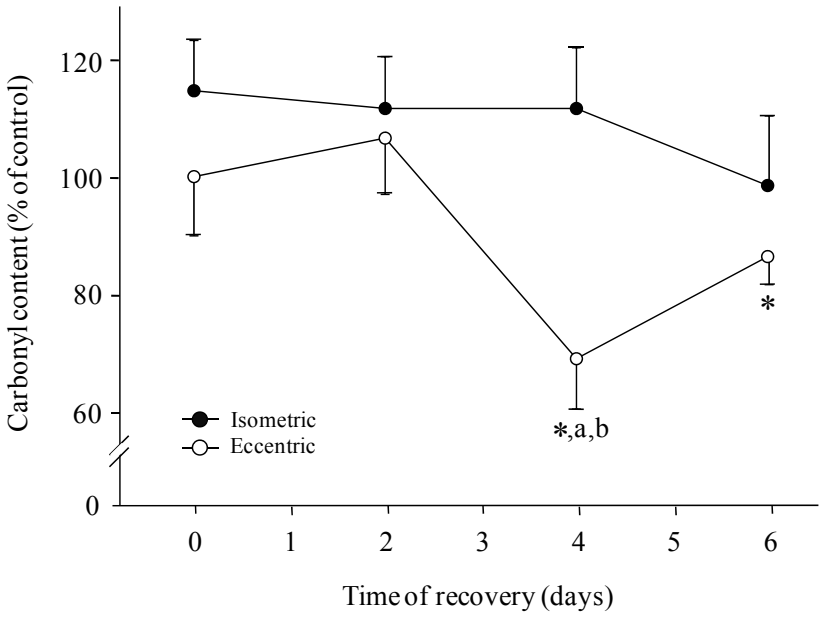
Carbonyl content (% of control)
120
100
80
60
0
Isometric
Eccentric
*,a,b
*
0
1
2
3
4
5
6
Time of recovery (days)

Fig. 6