ROS Scavenging Activity and Muscle Damage Prevention in Eccentric Exercise in Rats

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Abstract: Depending on intensity, eccentric exercise is experimentally and clinically documented to have opposing dual effects on skeletal muscle; intense eccentric exercise damages muscle, but daily low-load eccentric exercise prevents damage. To clarify the mechanisms of this dual effect, microscopic damage and oxidative stress were studied in rat quadriceps muscle. Oxidative stress was estimated from an immunostaining of advanced glycation end-products (AGE) and a measurement of muscle tissue preparations, the ability to scavenge reactive oxygen species (ROS). Intense eccentric downhill running (IEE) induced muscle damage that was, microscopically apparent 3 days later. Since AGE-positive cells and decreased ROS scavenging activity were observed earlier (on the day after IEE), cellular damage may be related to ROS production. Intense concentric uphill running (ICE) induced an immediate but transient decrease in ROS scavenging activity, which recovered within a day. Neither AGE-positive cells nor microscopic damage was observed after ICE. Since each contracting muscle fiber develops greater tension during eccentric rather than concentric exercise, the initial trigger of IEE-induced muscle damage may be damage to muscle fibers and connective tissues at the subcellular level. Daily low-load training of eccentric downhill running (LET), but not concentric uphill running, efficiently prevented muscle damage after subsequent IEE. No evident elevation of ROS scavenging activity was evident after LET. We concluded that LET prevents IEE-induced muscle damage not through elevated ROS scavenging activity, but through a suppression of initial subcellular damage that triggers subsequent ROS-producing processes, resulting in cellular delayed damage.

Key words: eccentric exercise, muscle damage, reactive oxygen species, scavenging activity, rats.

In eccentric exercise, the contracting muscle is forcibly lengthened, but in concentric exercise, the muscle is shortened; in isometric exercise, the muscle length is unchanged [1]. Unaccustomed intense eccentric exercise has been found to damage muscle fibers in both animal experiment and human clinical observations [2–4]. Eccentric muscle effort is also known to induce delayed-onset muscle soreness, which is unresponsive to treatment with common analgesic agents. Despite these drawbacks, low-load eccentric exercise is used to prevent muscle injury [5], and clinical studies have found that such low-load eccentric training indeed can protect skeletal muscle from lengthening-related injury induced by intense exercise [6–11]. Therefore, eccentric exercise has a dual effect on muscle; intense eccentric exercise damages muscle, but low-load eccentric exercise protects muscle from this damage. Detailed mechanisms underlying this dual effect have not yet been fully studied experimentally [12].

The involvement of reactive oxygen species (ROS) in exercise-induced injury is increasingly apparent [13, 14]. Aerobic metabolism during exercise and the inflammatory processes in damaged muscle can both produce ROS [14]. Other reports indicate that antioxidant enzyme systems undergo adaptive modulation in response to short- and long-term exercise [15], and that training can strengthen defenses against ROS in muscle [16].

The current study was designed to clarify the mechanisms responsible for the dual effects of eccentric exercise. Defining “training” as low-load daily exercise, we compared cellular muscle damage and estimated oxidative stress in muscle following various sequences and types of exercise and training. Oxidative stress was estimated by using immunostaining against advanced glycation end products (AGEs) and measuring the ability of a muscle tissue preparation to scavenge ROS in vitro. AGEs are reported to reflect oxidative stress in tissues [17], and...
scavenging activity represents the remaining capacity of the tissue for a reduction of ROS.

METHODS

Animals and treatments. The study was approved by the Animal Experiments Committee at the University of Kanazawa. Female Wistar rats (n = 76, Charles River, Japan; 12 weeks old; body weight, approximately 215 to 280 g) were housed in a temperature-controlled room (22 ± 2°C) with 12-h periods of light and darkness. A standard diet (Rat Chow, Oriental Yeast, Japan) and water were provided ad libitum. Compared with male rats, female rats were significantly resistant to exercise stress.

The rats were assigned to seven groups, including a control (CON) group and groups that underwent intense eccentric exercise (IEE), intense concentric exercise (ICE), low-load eccentric training (LET) or low-load concentric training (LCT), and LET plus subsequent IEE (LET+IEE) or LCT plus subsequent IEE (LCT+IEE). They were trained and exercised by running on a treadmill (Treadmill for Rats & Mice, model MK-680; Muromachi Machine, Tokyo, Japan) with variable velocity and inclination. Since the treadmill has a variable setting for uphill slopes only, the whole treadmill was tilted by boards to obtain a downhill slope. Downhill and uphill slopes were used for eccentric and concentric exercise, respectively.

The training (LET and LCT) consisted of treadmill running at 10 m/min on a 15° incline for 30 min every day for 1 week. The exercise (IEE and ICE) consisted of treadmill running at 20 m/min on a 15° incline for 150 min. The intensity of the exercise was determined according to the report of Armstrong et al. [18]. Some electrical stimulation was used to spur the animals, particularly at the beginning of the run, but this was held to a minimum. The rats were anesthetized with pentobarbital sodium (70 mg/ kg i.p.) and killed 0, 1, 3, or 7 days after the termination of the exercise period (Fig. 1). The quadriceps muscles were isolated and freed of connective tissue. The specimens were weighed immediately and stored in a freezer at –80°C.

Histological analysis and AGE immunostaining. Following in situ fixation, a sample of each quadriceps femoris muscle was taken as a complete cross section near the middle of the belly. Cross sections were cut with a cryostat to obtain 10 µm slices. For histological analysis, the slices were stained with hematoxylin and eosin.

To evaluate oxidative stress in muscle, we immunostained the slices against advanced glycation end products (AGE), whose accumulation is considered to be a marker of ROS production [17]. In brief, after being deparaffinized the slices were treated with 0.3% H2O2 in methanol for 30 min at room temperature and with 0.1% trypsin for 15 min at 37°C. The slices were then allowed to react with an anti-AGE monoclonal antibody (Trans Genic, Kumamoto, Japan) for 60 min at room temperature in a humid chamber, followed by incubation with a second antibody (NA9310, Amersham, Tokyo, Japan) for 60 min at room temperature. The slices were then treated with 3,3′-diaminobenzidine (DAB) for 5 min.

ESR measurement of antioxidant capacity against superoxide. An ESR instrument (JES-TE25X; JEOL, Tokyo, Japan), set at frequency, 9.4190 GHz; power, 4.00 mW; field, 334.0 ± 5 mT; sweep time, 1.0 min; modulation, 0.079 mT; and time constant, 0.1 s, was used to measure ROS based on Masuda’s method [19]. Muscle specimens were homogenized with 1.15% KCl homogenate buffer at a 1:10 ratio. The homogenate was diluted 2:1 or 4:1 in 0.2 mM phosphate buffer (pH 7.4). Muscle ROS scavenging activity was determined directly against superoxide anions derived from a xanthine oxidase-hypoxanthine reaction by measuring the inhibition of ESR signals in a mix-
ture of muscle homogenate and the superoxide-generating system. The ESR spectrum allows the original reactive radical to be identified and quantified. The spectrum of the reaction without muscle was recorded as a control, and a standard curve for superoxide dismutase (SOD) activity was constructed based on spectra for 5, 6.25, 10, 12.5, 20, 25, 40, and 50 U/ml SOD. The reaction mixture consisted of 50 µl of homogenate containing 2.5% to 5% muscle tissue, 50 mM phosphoric acid buffer, 2 mM hypoxanthine (6-hydroxypurine), and 20 µl of 9.2 M 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap. The ESR spectrum 45 s after initiation of the reaction by the addition of hypoxanthine was recorded at room temperature (23°C). Signal intensity was normalized as a ratio of the peak located at the lowest magnetic field of the four-line DMPO-OOH signal to the peak from a Mn²⁺ internal standard. Scavenging activity was calculated from SOD activity based on the standard curve [20].

Statistical analysis. Statistical differences between control and exercised groups were analyzed using a one-way analysis of variance (ANOVA) with a Bonferroni post hoc test. Analyses were performed with the Statview software program (Abacus Concepts, Berkeley, CA). Statistical significance was set at $P$ below 0.05.

RESULTS

Histopathology
Muscle damage was not observed in any groups 1 day after IEE (Fig. 2, a, b, and c). Significant polymorphonuclear cell infiltration among vastus intermedius muscle fibers was observed 3 days after exercise in the IEE and LCT+IEE groups (Fig. 2, d and e). No evidence of muscle damage was observed 3 days after exercise in the other groups, including LET+IEE (Fig. 2f). This indicates that IEE specifically induces muscle damage, which becomes microscopically evident 2 or 3 days after IEE, and that this damage is effectively prevented by prior LET.

AGE staining
AGE-positive cells were found in vastus intermedius muscle only in the IEE and LCT+IEE groups 1 and 3 days after IEE (Fig. 3, a and b), and not in the other groups, including the LET+IEE group (Fig. 3c). AGE-positive cells tended to be smaller than AGE-negative cells. This indicates that the muscle-protective effect of LET against subsequent IEE-induced damage is preceded by a suppression of the AGE accumulation reflecting oxidative stress from ROS.

ROS scavenging activity
ROS scavenging activity in quadriceps muscle in the ICE group showed an instantaneous transient decrease, which recovered within a day (Fig. 4). On the other hand, the IEE and LCT+IEE groups showed a delayed decrease in ROS scavenging activity 1 to 3 days after exercise (Figs. 5 and 6). The LET+IEE group showed a recovery of ROS scavenging activity within a day after IEE, as the ICE group did (Fig. 7). Combined with the results of AGE staining, this indicates that LET does not enhance ROS scavenging capacity in muscle, but rather suppresses ROS production during the latent period after IEE before the appearance of cellular muscle damage.
DISCUSSION

Muscle damage induced by intense eccentric exercise

Eccentric exercise induces microscopically evident pathological changes such as fiber necrosis and inflammatory cell infiltration that become apparent a few days later [2, 18]. In the current study, necrotic cells lacking nuclei, accompanied by lymphocytic infiltration, were seen in the quadriceps in the IEE and LCT+IEE groups by 2 to 3 days after IEE, but never within 1 week of observation in the ICE group (Fig. 2). We therefore consider the intensity of the exercise studied to be adequate for an induction of the delayed development of the microscopically evident muscle damage that is specific to eccentric exercise.

Before a development of microscopically evident muscle change, AGE-positive cells and a late decrease in ROS scavenging activity were observed after IEE (Figs. 3 and 5). AGE accumulation has been reported to be an index of ROS accumulation [17], and a decrease in ROS scavenging activity would be a consequence of increased ROS production in muscle. Viewed chronologically, ROS presumably was produced during the development of microscopically evident muscle damage. Pathological changes such as phagocytosis and the invasion and activation of inflammatory cells generally are considered to produce ROS [14]. Therefore delayed ROS production induced by IEE would appear to be a by-product of the inflammatory reactions rather than a direct cause of muscle damage.

We also need to consider which aspect of IEE triggered the subsequent muscle damage process, though ICE did not. A maximally activated isolated muscle fiber has been reported to develop greater tension under eccentric rather than isometric and concentric conditions. Thus the eccentric condition would cause a rightward shift in the length-tension relationship of a maximally activated muscle fiber [2]. This is consistent with reports of in vivo evidence that IEE recruits fewer motor units than ICE does, though ten-
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1. Muscle damage induced by intense eccentric exercise

2. Mechanism of muscle damage prevention by eccentric training

Antioxidant State in Eccentric Exercise

Mechanism of muscle damage prevention by eccentric training

Our results showed that initial LET prevented cellular damage induced by subsequent IEE (Fig. 2), as previously reported [9, 10]. The present study clearly indicated that a concentric exercise of similar intensity has no protective effect against IEE-induced muscle damage (Fig. 7). This indicated that eccentric exercise, at least at low-load, is significantly more effective than concentric exercise in protecting muscle against the damage induced by intense eccentric exercise, and could represent an efficient method for protecting athletes from muscle injury.

The injury-preventing property of LET did not involve enhanced ROS scavenging activity, because no scavenging increase was observed. Instead, the delayed decrease in ROS activity and increase in AGE accumulation from subsequent IEE were suppressed (Figs. 3 and 6). After LET, IEE induced an effect similar to that of ICE: an instantaneous transient decrease in ROS scavenging activity without AGE accumulation or cellular damage (Figs. 2, 3, 4 and 6). We therefore consider the effect of LET to be mediated not through a prevention of ROS accumulation or cellular damage (Figs. 2, 3 and 6). After IEE, ROS production during exercise would be a consequence of inflammatory processes induced by subcellular microscopically inapparent damage to muscle fibers and connective tissues during IEE.

Mechanism of muscle damage prevention by eccentric training

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At present, mechanisms suppressing the initial damage remain unidentified. Some possibilities involve (i) an increase in the number of sarcomeres per unit of muscle length and (ii) modulation of the proprioceptive feedback
system regulating muscle length. In eccentrically trained muscle, Lynn et al. [27] found higher resistance against intense eccentric exercise and more sarcomeres than in concentrically trained muscle. On the other hand, Proske et al. [28] found that motor units in exercised muscle fired more frequently, opposing a given extent of stretch in eccentrically trained muscle. Aiming to develop more efficient methods to prevent muscle injury in athletes, we anticipate further studies concerning mechanisms by which LET protects muscle against subcellular damage to myofibers and connective tissue.

We concluded that daily low-load eccentric training prevented muscle damage induced by intense eccentric exercise through a suppression of the initial subcellular damage, triggering subsequent ROS production, rather than via increased ROS scavenging activity.

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REFERENCES