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Acute change of titin at mid-sarcomere remains despite 8 wk of plyometric training

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Macaluso F, Isaacs AW, Di Felice V, Myburgh KH. Acute change of titin at mid-sarcomere remains despite 8 wk of plyometric training. J Appl Physiol 116: 1512–1519, 2014. First published January 23, 2014; doi:10.1152/japplphysiol.00420.2013.—The purpose of this study was to investigate skeletal muscle changes induced by an acute bout of plyometric exercise (PlyEx) both before and after PlyEx training, to understand if titin is affected differently after PlyEx training. Healthy untrained individuals (N = 11) completed the 1stPlyEx (10 × 10 squat-jumps, 1-min rest). Thereafter, six subjects completed 8 wk of PlyEx, while five controls abstained from any jumping activity. Seven days after the last training session, all subjects completed the 2ndPlyEx. Blood samples were collected before and 6 and 1, 2, 3, and 4 days after each acute bout of PlyEx, and muscle biopsies 4 days before and 3 days after each acute bout of PlyEx. The 1stPlyEx induced an increase in circulating myoglobin concentration. Muscle sample analysis revealed Z-disk streaming, a stretch or a fragmentation of titin (immunogold), and increased calpain-3 autolysis. After training, 2ndPlyEx did not induce Z-disk streaming or calpain-3 activation. The previously observed post-1stPlyEx positional change of the titin COOH terminus was still present pre-2ndPlyEx, in all trained and all control subjects. Only two controls presented with Z-disk streaming after 2ndPlyEx, while calpain-3 activation was absent in all controls. Eccentric explosive exercise induced a stretch or fragmentation of titin, which presented as a positional change of the COOH terminus. Calpain-3 activation does not occur when titin is already stretched before explosive jumping. Enzymatic digestion results in titin fragmentation, but since an increase in calpain-3 autolysis was visible only after the 1stPlyEx acute bout, fragmentation cannot explain the prolonged positional change.

Z-disk streaming; calpain; eccentric exercise; acute bouts

LOWER BODY PLYOMETRIC EXERCISE (PlyEx) involves performing repeated consecutive jumping-type exercises with one’s own body weight against gravity. The stretch-shortening cycle is activated with each jump to produce maximal force in the shortest possible amount of time, placing significant strain on sarcomeric and cytoskeletal proteins and musculo-tendinous junctions (32). During the stretch-shortening cycle, Z-band proteins, such as desmin, and intermediate filaments, such as titin, are subject to strain, or with excessive stress become disrupted (18, 28). Recently, our research group reported histological evidence that a single bout of PlyEx preferentially damaged type II muscle fibers (30). Electron microscopy analysis indicated that >80% of fast-twitch fibers presented with damaged sarcomeres distinguishable as mild, moderate, or severe. Z-disk disruption is considered severe when streaming is severe, involving complete disruption of multiple adjacent Z-disks and sarcomeres. At the level of the sarcomere, the protein that confers elastic properties to skeletal muscle is titin, a giant structural protein (3.0–3.7 MDa) (15, 31, 33).

Considering the regularity in the structure of the sarcomere, titin has been proposed to act as a molecular “ruler.” It displays multiple binding sites for proteins spanning from Z disk to M line (14). Within the Z disk, the NH2 terminus of titin is bound with the mechanically stable Z-disk complex of titin/telethonin (4, 41), implying that titin is preferentially unfolded first. In vivo, albeit in the heart, it has been shown that titin does not act only as an “extensible spring,” but its function is influenced by many other cytoskeletal proteins to which it is bound (40), as well as signaling proteins that have binding sites located on titin (34). Indeed, as the sarcomere stretches, induced changes in these proteins, a cascade of molecular events follows, hence titin can be considered as a “stretch sensor.” In vivo, albeit in the heart, it has been shown that calcium/calmodulin kinase II-dependent phosphorylation of the PEVK region and the N2B region result in lower passive force (17). At the level of the M line, atomic force microscopy experiments conducted on the COOH terminus of titin showed relatively low unbinding forces (30 pN) for the titin/obscurin complex (localized in the M line) compared with the mechanically stable Z-disk complex of titin/telethonin (4, 41), implying that this region is preferentially unfolded first.
Unfolding at this region, without Z-disk disruption, could imply mobility that is not involved in the spring property, but rather the stretch-sensing function. These findings have directed more attention to regions closer to the COOH terminus rather than the NH2 terminus of titin.

In vivo, acute and adaptive responses to externally applied loads have been studied using various exercise models. In a critical review, Markovic and Mikulic (32) reported that plyometric training increases athletic performance, especially vertical jump ability. It is well known that α-actinin influences skeletal muscle mechanics during exercise (48), especially eccentric exercise, and this has been elucidated in both rat models and human subjects (12, 43). Acute or adaptive responses of titin have received less attention thus far. In human studies, a decrease in titin protein content in skeletal muscle has been shown after (1–24 h) an acute bout of eccentric exercise (46, 49), interpreted as an injury response rather than a functionally important response. Indeed, the high-tensile forces caused by eccentric exercise may result in fragmentation of titin observed in focal areas of damage. Other factors can result in sarcomere destruction, and these include enzymatic digestion, such as occurs after calpain-3 autolysis. Trappe et al. (46) reported that a few (2–8) days after exercise, the myofibers still revealed areas lacking titin. However, other areas stained strongly for titin, desmin, and actin. These data obtained using multiple immunofluorescence staining in longitudinal sections suggested myofibrillar remodeling (49). The authors hypothesized that this was an adaptive response rather than simply regeneration of the damaged area (49). Other long-term adaptation mechanisms may include titin gene transcription and alternative splicing, leading to translation of different titin isoforms that may be longer or shorter (26). This may alter titin-derived stiffness or change force production, as discussed by other authors (39).

In vivo, it is not possible to test titin stiffness in human subjects, but eccentric training in rodents resulted in an adaptation that enhanced tension during passive lengthening of whole muscle and increased force production during active lengthening contraction, which was interpreted as evidence of the “stiffer spring” concept (43). In vitro experiments conducted on the stretch of a single titin molecule has lead to another hypothesis, based on the observation that, unlike a true spring, the unraveled domains were not easily reversible on removal of the extending force (11, 19, 44). These authors interpreted their results as suggesting that, at high forces, which may occur during extreme stretch, titin’s domains unravel and maintain the longer resting length (11, 19, 44). It is unclear whether or not such a change would occur in vivo within the range of thigh musculature stretch induced during plyometric jumping, or whether it could be considered a positive adaptation that might reduce risk of damage from subsequent similar insults, or both.

It is well known that there is more muscle damage in response to an acute bout of eccentric exercise in a previously unexposed subject, than after an acute bout following eccentric training. We hypothesize that plyometric training will reduce evidence of sarcomere damage, and that this will be related to structural adaptations in titin. Biopsies were taken before and after an acute bout of PlyEx and both before and after PlyEx training, to understand if titin stretch occurs without sarcomere destruction after PlyEx training. Due to the relatively low forces required to extend the region of titin closer to the COOH terminus and the potential for mobility, we chose to investigate changes in the physical characteristics at this end of the giant titin molecule using electron microscopy.

MATERIALS AND METHODS

Subjects. Twelve healthy untrained individuals (age: 21.5 ± 1.7 yr; height: 173.6 ± 10.7 cm; weight: 68.5 ± 18.4 kg) volunteered for the research study after being informed of the experimental procedures and associated risk before providing written, informed consent. The Health Research Ethics Committee of Stellenbosch University approved the study, which was conducted according to international and locally accepted ethical guidelines for research. All participants were healthy, untrained individuals and had no muscle or lower limb injury 6 mo before the start of the study. None of the participants was on chronic corticosteroid-containing medications, including inhaled forms. The participants were asked and frequently reminded to abstain from any unaccustomed or strenuous physical activity, and to refrain from taking anti-inflammatory medication 2 wk before and for the duration of the study.

Experimental design. Participants completed two acute bouts of PlyEx (1stPlyEx and 2ndPlyEx) separated by 8 wk. Muscle biopsies were obtained 4 days before and 3 days after both acute bouts of PlyEx. Blood samples were collected immediately before and 6 h after the two acute bouts of PlyEx interventions, as well as on days 1, 2, 3, and 4. During the 8 wk that separated the two acute bouts of PlyEx, participants were randomly assigned to a training or control group. One of the subjects assigned to the training group was removed from the study because he showed a high level of myoglobin (Mb) in serum. The trained group (Try, n = 6) performed plyometric jump training for 8 wk during which the total numbers of jumps per session was increased progressively, while the control group (CNT, n = 5) abstained from any physical activity for 8 wk. Individual characteristics of Try and CNT groups were as follows: age, 21.4 ± 1.3 and 21.4 ± 1.7 yr; height, 176.6 ± 13.0 and 169.3 ± 10.2 cm; and weight, 75.4 ± 27.9 and 63.0 ± 6.8 kg, respectively. Four days after the last training session, all subjects completed the 2ndPlyEx.

PlyEx protocol. Participants performed a brief 10-min warm-up, consisting of backward and forward running before the start of the exercise intervention. The exercise intervention consisted of 10 sets of 10 maximal squat jumps separated by 1-min rest intervals between sets. Before exercise commenced, each participant first completed three maximal vertical jumps of which the maximum height that the top of the head reached was taken as the highest point of the jump. The participants then had to maintain a target height equivalent to 95% of the highest point of the jump during each jump. During sets, participants were allowed to move around freely and do general stretching for no longer than 30 s. All participants had to complete 100 jumps, jumps where participants could not maintain their target jump height, trunk position, or 90° knee joint angle were not considered. Participants were stopped and given 1-min rest period before having to complete that set (30).

Training and rest protocols. Subjects of Try group performed 5 × 5 and 7 × 7 maximal squat jumps during the first 2 wk and second 2 wk, respectively, after which the full 10 × 10 squat jumps were completed during the remaining 4 wk. A new target height was established every 2 wk of training according to each participant’s individual jump improvements. The subjects of the CNT group were convened in the laboratory each week to ensure that they were abstaining from any physical activity. All the subjects performed three maximum jumps at the beginning of each week (data not shown).

Blood collection and analysis. Participants were instructed to lie down in the supine position for 5 min before blood was drawn from a superficial forearm vein. Serum Mb concentration was determined by a commercial pathology laboratory (PathCare, Stellenbosch Medi
Muscle biopsy and sample preparation. A total of four biopsies were obtained from each subject during the study. Muscle biopsies were obtained from the vastus lateralis muscle in a similar position for all participants using the suction-assisted technique (21, 29). The biopsy was split into two parts: one was embedded in tissue-freezing medium and frozen in isopentane (cooled in liquid nitrogen) to maintain structure before subsequent biochemical analysis of several sections from each biopsy, and the second part (1 x 3 mm) was fixed in 4% paraformaldehyde, after which samples were dehydrated with graded ethanol (70, 95, 100%). These samples were then embedded by placing into LR-white resin (Electron Microscopy Sciences, Hatfield, PA) with two passages of 1 h, then pure resin (24 h) and one more passage of 1 h before inclusion at 50°C for 48 h.

Western blotting. The mini electrophoresis system (Bio-Rad Laboratories, Hercules, CA) was used for protein separation. The concentration of the separating gel was 8%. The running conditions for the minigel apparatus were 100 V for 10 min (staking gel) and 200 V for 45 min (resolving gel) at constant voltage and at room temperature. Proteins run on the minigel were transferred to PVDF membrane (Immobilon, Millipore) for 1 h 15 min at constant 0.5 A and 15 V, using the mini transblot system (Bio-Rad Laboratories). Following the transfer, membranes were incubated and blocked in blocking solution containing 5% dry milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h 30 min at room temperature. Calpain-3 protein levels were detected by immunoblotting using antibody NCL-CALP-12A2 (calpain 3; dilution 1:1,000; mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed in TBS-T and incubated for 1 h with a secondary antibody: horse-radish peroxidase-linked donkey anti-mouse (DAKO; dilution 2:5,000). The membranes were incubated with luminescence reagent (ECL Plus, Amersham, GE Healthcare, Biosciences), exposed to X-ray film (Hyperfilm, Amersham, GE Healthcare, Biosciences), according to manufacturer instructions, developed, and scanned. Antibodies were then stripped from membranes in 0.2 M NaOH for 5 min and reprobed with β-actin (dilution 1:1,000; rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), which served as loading control. Densitometry analysis was performed with specialized software, ImageJ 1.41 (National Institutes of Health, http://rsb.info.nih.gov/ij). Ultrastructural analysis. Ultrathin longitudinal sections were cut at 60–70 nm (gold color of section) and placed on nickel grids on 150 mesh (formavvar-coated). Sections were then washed with 0.1% TBS-T for 6 x 12 min, stained using uranyl acetate (5 min), dried with filter paper, and stained with lead citrate for 2 min in the dark. Finally the sections were washed with distilled water for 2 x 5 min, dried with filter paper once more, and then ready to be viewed under a transmission electron microscope (Jeol-Jem 1011 TEM, Leica Microsystems Nussloch). Quantitative analysis was performed in 15 sarcomeres for each muscle biopsy. The number of colloidal gold particles was counted in a specific area of the sarcomere, divided by the total colloidal gold particles counted in the whole sarcomere, and expressed as a percentage. The sarcomere was virtually divided into five areas: M line, H zone, A band, I band, and M/M (space between two adjacent parallel M lines; area indicated with a circle in Fig. 3B). The distances of each colloidal gold particle from the M line were measured. Immunogold quantitative analysis was performed by FM with sample code blinded.

Statistical analysis. Data were assessed for normality by inspecting normal probability plots. A logarithmic transformation (log10) was applied to nonnormally distributed data of Mb before analysis. Changes in serum Mb and titin immunogold localization over time in both groups were analyzed by two-way mixed-model repeated-measures analysis of variance. If a significant difference was detected, this was further evaluated by the post hoc Fisher least significant difference test. Significance was accepted at an α of P < 0.05.
Z-disk disruption/damage following eccentric exercise are illustrated in Fig. 2.

**Titin localization by immunogold.** Analysis of baseline (before the 1stPlyEx) muscle biopsies showed that colloidal gold particles binding titin were almost exclusively localized along the M line of the sarcomere, where the COOH terminus of titin resides. The muscle biopsies after the 1stPlyEx showed a different localization of colloidal gold particles binding titin: 1) in the H zone (mainly) and also around the A band of the sarcomere; and 2) in the space between adjacent parallel M lines (Fig. 3). Qualitative analysis of muscle biopsies taken before and after performing the 2ndPlyEx showed that, at both time points, colloidal gold particles binding the COOH terminus of titin were localized around the H zone and A band of the sarcomere and in the space between adjacent parallel M lines. No differences were observed between CNT and Try groups for each time points. Quantitative analysis of titin localization is reported in Table 1.

**Calpain-3 activation.** Autolysis of calpain-3 resulted in products of ∼55–60 kDa. The total of all calpain-3 autolyzed products (normalized by β-actin) was not significantly different comparing before and after the 1stPlyEx and the 2ndPlyEx in both the groups. But there was a significant increase in the 56-kDa product observed only after the 1stPlyEx in Try and CNT group (Fig. 4).

**DISCUSSION**

The highly ordered assembly of sarcomeres requires titin, a molecular ruler protein, to form a giant scaffold that interacts with almost all other sarcomeric proteins. Therefore, it is not surprising that titin depletion or truncation, in prenatal life, disrupts sarcomere assembly (5). In postnatal life, the titin scaffold is thought to be protective during sarcomere stretch by resisting excessive strain, as well as returning the sarcomere to the proper resting length during relaxation, i.e., behaving as a molecular spring. During eccentric exercise, mechanical stress induces ultrastructural damage at the level of the Z disk, suggesting that the titin spring is fallible. However, the role of titin in the second bout effect and in the adaptation to eccentric exercise training is less clear. The present study was designed to investigate the effects of high levels of force on sarcomere integrity at time points separated by 8 wk, with specific attention to Z-disk streaming, the M line, and titin.

The main findings were that, 3 days after the first acute bout of eccentric exercise, titin protein still exhibited a positional change: either stretched or fragmented. Furthermore, it remained in this form for at least 8 wk. Titin remained stretched or fragmented irrespective of whether the subjects were sedentary or participated in plyometric jump training sessions over the 8 wk. Although damaged sarcomeres were observed in all subjects after the 1stPlyEx, none was evident after the second acute bout 8 wk later in the Try group. Z-disk damage was observed only in two subjects of the CNT group after the 2ndPlyEx acute bout. We propose that these data argue against fragmentation and regeneration of titin to its original sarcomeric conformation, but rather that an acute change in titin was maintained for at least several weeks.

The literature reports an increase in titin expression in animal skeletal muscle following an acute bout of eccentric exercise (27), as well as after a specific training protocol (3, 25), despite the fact that different laboratory techniques were used by the different studies to investigate this change. Data from human studies are inconsistent with reports of decreased titin mRNA content early after eccentric exercise (20), no change (24), or an immediate decrease in titin expression after an acute bout of eccentric exercise (46, 49) followed by a subsequent increase in titin expression over the following 7–8 days. The immunohistochemical images in the latter study presented with strongly stained areas for titin, suggesting that a response of increased titin protein may be a stimulus for formation of new sarcomeres (49). At that time, this was the first interpretation of a titin response that differed from the traditional view of titin as a spring, which would return to its original shape and length after acute stretch, and that repair following fragmentation simply replaces the damaged titin.

Since then, a new view has been proposed, also suggesting that titin does not operate only as a spring, but rather as a filamentous protein that can unfold and refold and behave as a stress sensor. When titin’s immunoglobulin domains are unfolded under conditions of extreme stretch, they provide a reservoir of extra length, which Erickson (11) described as making titin somewhat more similar to a leash than a true spring that would retract during relaxation. Based on in vitro

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**Fig. 2.** Electron micrographs of longitudinal skeletal muscle sections obtained from a single biopsy taken 3 days after the 1stPlyEx intervention. Different areas in this biopsy contained normal sarcomeres with regular alignment and regular banding patterns [Z disk (Z); M line (m); A]; and disrupted sarcomeres showing different grade of damage: 1 = wavy appearance of the Z disk; 2 = mild Z-disk streaming; 3 = disintegration of the Z disk (B).
mechanics and energetics, the author(s) suggested that there was no obvious way for the protein to refold its extended domain passively, but that titin could be renatured slowly by other means, perhaps by chaperones (11). Neither of these proposed theories could be excluded or categorically favored in our study, but we can conclude without any doubt that the titin scaffold was altered, even when the sarcomere did not present the signs of damage, namely Z-disk streaming.

The electron microscopy analysis of the present study showed that all of the subjects presented with ultrastructural damage at the level of the Z disk in focal areas, and that a change in the localization of colloidal gold particles binding titin occurred in these focal areas 3 days after the 1stPlyEx. In fact, in the baseline muscle biopsies, all of the sarcomeres showed a straight Z-disk arrangement and regular pattern with the localization of the COOH terminus of titin along the M line, while after the 1stPlyEx the COOH terminus of titin was localized around the A band and H zone of the sarcomere and in the space between adjacent parallel M lines. The first ultrastructural localization appeared to indicate that titin had been fragmented and colloidal gold particles bound to the COOH terminus of titin were present in the A-band area rather than the usual position at the M line.

Colloidal gold particles were also visible in the H zone of the sarcomere, suggesting, in our opinion, that titin had been stretched to this point, not far from the original resting position. This may support the results of da Silva Lopes et al. (7) on cardiac titin, which revealed that titin travels efficiently within the myofiber, consistent with a model where titin could detach from either the M band or Z disk and freely move in either direction before it reintegrates into the sarcomere. In vitro atomic force microscopy experiments conducted on the titin/
obscerin-like-1 complex showed relatively low unbinding forces (30 pN) for the titin/obscerin complex (localized in the M line) compared with the mechanically stable Z-disk complex of titin/telethonin (4, 41), implying that mobility is more likely at that end. Considering this theory of mobility and our data, we suggest that, during plyometric jumping, titin was stretched, and its domains had not refolded “correctly,” resulting in the localization of colloidal gold particles between two adjacent parallel M lines, or in the H zone of the sarcomere.

The findings of the first part of the study confirmed that the titin scaffold had been compromised during in vivo dynamic eccentric contraction. The longitudinal part of the study addressed the issue of potential adaptation in response to repeated exposure over 8 wk. Quantitative analysis conducted with electron microscopy and immunogold staining before the 2ndPlyEx showed that titin had still not refolded “correctly” or repaired in either the Try or CNT groups, since titin remained localized mainly in the A band, and also in the H zone and in the space between adjacent parallel M lines of the sarcomere. These results in the Try group suggest that the plyometric training continually stressed the sarcomere, thereby not allowing sufficient time for titin’s M line immunoglobulin domains to refold and repair, and that this may have resulted in the absence of further Z-disk streaming from the acute 2ndPlyEx. However, since the CNT group also still demonstrated the positional change in the titin COOH terminus that was evident after only one bout of plyometric jumping, we propose that the failure of titin to return to its original position after 8 wk of

<table>
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<th>Sarcomeric Zone</th>
<th>1stPlyEx Before</th>
<th>1stPlyEx After</th>
<th>2ndPlyEx Before</th>
<th>2ndPlyEx After</th>
<th>Control Group Before</th>
<th>Control Group After</th>
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<tbody>
<tr>
<td>M line, %</td>
<td>69 ± 10</td>
<td>11 ± 6*</td>
<td>13 ± 9*</td>
<td>12 ± 5*</td>
<td>70 ± 20</td>
<td>10 ± 7*</td>
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<tr>
<td>H zone, %</td>
<td>15 ± 6</td>
<td>47 ± 10*</td>
<td>42 ± 12*</td>
<td>43 ± 10*</td>
<td>14 ± 6</td>
<td>48 ± 13*</td>
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<td>A band, %</td>
<td>5 ± 1</td>
<td>22 ± 7*</td>
<td>22 ± 5*</td>
<td>23 ± 9*</td>
<td>9 ± 3</td>
<td>23 ± 5*</td>
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<tr>
<td>M/M line, %</td>
<td>2 ± 1</td>
<td>14 ± 5*</td>
<td>15 ± 6*</td>
<td>15 ± 5*</td>
<td>2 ± 1</td>
<td>11 ± 3*</td>
</tr>
<tr>
<td>I band, %</td>
<td>2 ± 1</td>
<td>5 ± 1</td>
<td>8 ± 5</td>
<td>7 ± 5</td>
<td>2 ± 1</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Distance, nm</td>
<td>54.8 ± 11.1</td>
<td>188.0 ± 164.0*</td>
<td>179.3 ± 122.9*</td>
<td>210.7 ± 157.9*</td>
<td>52.3 ± 12.8</td>
<td>191.0 ± 150.9*</td>
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Values are means ± SE. 1stPlyEx and 2ndPlyEx, first and second acute bout of plyometric exercise, respectively. Percentage is shown of the colloidal gold particles binding titin in sarcomere within specific areas: M line, H zone, A band, I band, and M/M (space between two adjacent parallel M lines; indicated with a circle in Fig. 3B). Distance from M line, averaged distance measured from the colloidal gold particles and M line. *Significantly different at \( P < 0.05 \) compared with respective control (before 1stPlyEx).
eccentric training was not due to insufficient repair between training sessions. It is possible that cytoskeletal protein structural rearrangement forms part of the explanation for the prolonged nature of the second-bout effect (>8 wk) (6).

In stretch-induced damage, other sarcomeric proteins clearly undergo fragmentation, resulting in Z-disk “streaming” (23). It has been suggested that this is due to significant proteolysis promoted by calpains, in particular calpain-3 (10). Calpain-3 is a protein homologous to the ubiquitous calpain family members, μ-calpain and m-calpain, and is involved in sarcomeric remodeling (10, 22). Calpain-3 becomes proteolytically active once the insertion sequences IS1 and IS2 have been excised by autolysis. It has been demonstrated previously that calpain-3 remains in its non-autolyzed state after intensive eccentric-concentric exercise, such as sprinting and even after less highly intensive endurance running in humans (38), but is autolyzed 24 h after eccentric knee extensor exercise (36), thus providing evidence that isolated, maximal eccentric exercise represents a particular exercise-induced circumstance that results in the in vivo activation of calpain-3. Two mechanisms have been proposed to cause calpain-3 autolysis in healthy adult muscle after eccentric exercise: 1) a prolonged increase in resting cytoplasmic Ca^{2+} concentration ([Ca^{2+}]), because calpain-3 is a muscle-specific [Ca^{2+}]-dependent cysteine protease; and 2) titin stretch, since calpain-3 has been shown to bind titin at the N2A region and M line, and it has been proposed that the IS2 sequence will be excised more easily with titin stretch (35, 37). Studies by the Murphy research group conducted on isolated fibers provided sufficient evidence that endogenous calpain-3 activation was primarily governed by cytoplasmic [Ca^{2+}] and was not dependent on stretch in their in vitro model (35, 37). The present study results following the 1stPlyEx indicated a temporal association between an increase in the 56-kDa autolysed calpain-3, Z-line damage, titin stretch, and sarcomere streaming, which conceivably is associated with increased calcium from disrupted sarcoplasmic reticulum. The muscle biopsies taken after the 2ndPlyEx had levels of autolyzed calpain-3 that were not different from those seen before the 1stPlyEx when muscle was in an undamaged condition. The acute 2ndPlyEx bout did not result in Z-disk disruption and sarcomere streaming in the Try group and most of the CNT subjects, suggesting that elevated free calcium was unlikely to be present. These data are consistent with those of Beaton et al. (2), who previously showed that administration of a calcium channel blocker reduced Z-band streaming. Taken together, the evidence points to a stronger role of free calcium activation of calpain than stretch activation.

In the present study, Mb was selected as an early indicator of muscle damage. The Mb kinetic curve indicated that Mb concentration peaked 6 h after both the acute bouts of PlyEx and thereafter tended to return to baseline values. Although a similar trend over time was observed after the 1stPlyEx and 2ndPlyEx, the peak elevation at 6 h was substantially lower after the 2ndPlyEx. This was the case for subjects of both groups. Although this reduction after 2ndPlyEx may have been partly due to the new state of titin or the lack of significant calpain-3 autolysis, the peak still indicated transient muscle damage in both groups after the second acute bout of PlyEx. Some other component(s) of muscle damage, possibly membrane damage, did not undergo a lasting adaptation, and the small molecule was still able to leak from the cytosol to the circulation.

In conclusion, an acute bout of explosive eccentric exercise induces Z-disk streaming in persons unaccustomed to eccentric exercise, who, if trained for 8 wk, will not present with damaged Z disks. Moreover, under conditions of muscle stretch, such as during plyometric jumps, titin undergoes a positional change of the COOH terminus, either due to unfolding or prolonged fragmentation. This state remains for several weeks, thus possibly explaining (at least in part) the prolonged second-bout effect. Furthermore, this is associated with lack of calpain-3 activation in individuals who had trained for 8 wk doing regular plyometric jumping, as well as in those who did not do any further jumping until the second acute bout. Future studies need to address if eccentric exercise affects the localization of other titin domains.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: F.M., A.W.I., and K.H.M. conception and design of research; F.M., A.W.I., and V.D.F. performed experiments; F.M., A.W.I., and V.D.F. analyzed data; F.M., A.W.I., V.D.F., and K.H.M. interpreted results of experiments; F.M., A.W.I., and K.H.M. drafted manuscript; F.M. and K.H.M. edited and revised manuscript; F.M., V.D.F., and K.H.M. approved final version of manuscript; A.W.I. prepared figures.

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