Differential serial sarcomere number adaptations in knee extensor muscles of rats is contraction type dependent

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Butterfield, Timothy A., Timothy R. Leonard, and Walter Herzog. Differential serial sarcomere number adaptations in knee extensor muscles of rats is contraction type dependent. J Appl Physiol 99: 1352–1358, 2005. First published June 9, 2005; doi:10.1152/japplphysiol.00481.2005.—Sarcomerogenesis, or the addition of sarcomeres in series within a fiber, has a profound impact on the performance of a muscle by increasing its contractile velocity and power. Sarcomerogenesis may provide a beneficial adaptation to prevent injury when a muscle consistently works at long lengths, accounting for the repeated-bout effect. The association between eccentric exercise, sarcomerogenesis and the repeated-bout effect has been proposed to depend on damage, where regeneration allows sarcomeres to work at shorter lengths for a given muscle-tendon unit length. To gain additional insight into this phenomenon, we measured fiber dynamics directly in the vastus lateralis (VL) muscle of rats during uphill and downhill walking, and we measured serial sarcomere number in the VL and vastus intermedius (VI) after chronic training on either a decline or incline grade. We found that the knee extensor muscles of uphill walking rats undergo repeated active concentric contractions, and therefore they suffer no contraction-induced injury. Conversely, the knee extensor muscles during downhill walking undergo repeated active eccentric contractions. Serial sarcomere numbers change differently for the uphill and downhill exercise groups, and for the VL and VI muscles. Short muscle lengths for uphill concentric-biased contractions result in a loss of serial sarcomeres, and long muscle lengths for downhill eccentric-biased contractions result in a gain of serial sarcomeres.

Sarcomerogenesis, or the addition of sarcomeres in series within a fiber, has been studied extensively with in vitro (10, 22, 23) and in vivo (2, 5, 6, 8, 9, 17, 19, 24–26, 30, 35, 42–46) models, and ultimately it has a profound impact on both static and dynamic muscle properties. Rack and Westbury (38) were among the first to show the relationship between sarcomere length and joint angle, and the association with the force-length relationship (FLR) (18). It has been suggested that the primary role of sarcomerogenesis is to maintain a close relationship between joint angle and sarcomere length within muscle fibers, irrespective of fiber length itself (46). Because fiber lengths may vary within a muscle, directing sarcomerogenesis at the cellular level allows the maintenance of consistent sarcomere lengths within fibers. Increasing serial sarcomere number would be of benefit in a static contractile situation, improving muscle function by altering the force-length relationship.

After sarcomerogenesis, the FLR is broader by an amount proportional to the number of extra sarcomeres compared with control muscles (32, 36), thus allowing the muscle to produce greater force at short as well as long lengths (46). Dynamically, the performance of a muscle could be improved through increasing its contractile velocity (27, 40, 44, 49) and muscle power (16). Thus sarcomerogenesis may provide a beneficial adaptation to prevent injury when a muscle consistently works at long lengths (11, 36, 44), supporting the original hypothesis of sarcomerogenesis accounting for the repeated bout effect by Fridén et al. (12), whereby skeletal muscle becomes “immune” to the injurious and painful effects of eccentric exercise after a few eccentrically biased training sessions.

The association between eccentric exercise and sarcomerogenesis has been proposed to depend on damage (36). Fiber integrity is compromised as sarcomeres are actively lengthened beyond filament overlap, resulting in necrosis and regeneration (34, 36). The subsequent regeneration, accompanied by sarcomerogenesis, allows sarcomeres to work at shorter lengths compared with lengths preceding sarcomerogenesis, for a given muscle-tendon unit length (37). Indirect evidence for this hypothesis has come from studies in humans, where the FLR is shifted to a longer muscle length after repeated eccentric exercise of hamstring muscles compared with the contralateral control limb without eccentric exercise (3). Direct evidence has been found in animal models where serial sarcomere number was increased after controlled eccentric exercise bouts in rabbit dorsiflexor muscles (24). Recently, it has been shown that rats trained to walk downhill have a greater serial sarcomere number in the vastus intermedius (a knee extensor muscle) than rats trained to walk uphill (31, 32), implicating eccentric exercise-induced injury as the stimulus for sarcomerogenesis.

The assumption that the knee extensor muscles in rats walk eccentrically in downhill walking is a logical one, because evidence has suggested that eccentrically biased exercise results in severe muscle injury and necrosis within the knee extensor muscles of rats after downhill walking (1, 41). However, uphill walking results in significantly less injury to the knee extensor muscles (1), arguably from the concentric-biased contractions required to produce positive work by the antigravity muscles of the hindlimb (41). However, there are published reports suggesting, rather surprisingly, that the knee extensor muscles of rats seem to undergo lengthening contractions during stance and activation, regardless of the walking speed (13) or surface grade (14), suggesting eccentric contractions of the knee extensor muscles during downhill and uphill walking. Assuming that these results published by Gillis and Biewener (14) are correct, and that eccentric exercise results in muscle injury acutely, and sarcomerogenesis chronically, then one

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would expect the knee extensor muscles to undergo a similar increase in serial sarcomere number, regardless of surface grade used during exercise.

Therefore, the purpose of this paper was to elucidate the relationships between contraction type, exercise duration, and serial sarcomere number adaptation in the knee extensor muscles of rats. Because of the challenges presented with instrumenting the vastus intermedius (VI) muscle, we chose to directly measure fiber dynamics within the vastus lateralis (VL) muscles during uphill and downhill walking, which allows for direct comparison to the findings of Gillis and Biewener (14). After chronic exercise, serial sarcomere numbers were measured in the VL and the VI, which permitted direct comparison of our results to the findings of Lynn and Morgan (31).

METHODS

Forty-eight male Long-Evans rats (571.8 ± 7.7g) were obtained (Charles River Laboratories, St. Constant, PQ, Canada) and studied with the approval of the Animal Care Committee of the University of Calgary. Animals were housed locally in accordance with Canadian Council on Animal Care guidelines. All animals were housed in pairs and allowed normal activities in a cage, and they received standard rat chow and water ad libitum. When rats reached the age of 150 days, they were divided into one of six experimental groups, defined as protocols I–VI.

Protocol I. This protocol was designed to directly measure fiber dynamics in the VL during incline and decline walking. It would have been desirable to make the same measurements in the VI, but that was not possible, given the location and architecture of the muscle. Eight rats were randomly selected, and they were initially anesthetized using 5.0% isoflurane and 1,000 ml/min of O2 in an induction box. Rats were randomly selected, and they were initially anesthetized using isoflurane, 400 ml/min O2. The left lateral lumbar and hindlimb muscles were then removed from the box, placed on their right side on a heated pad, and maintained under anesthesia using a face mask (2.5% isoflurane, 400 ml/min O2). The left lateral lumbar and hindlimb region was shaved, and an incision was made along the length of the femur, exposing the iliotibial band (ITB), gluteal muscle, and hamstring muscle of the left hindlimb. The facial layers were carefully dissected, and with the knee flexed, the anterior portion of the ITB was carefully reflected posterior, exposing the VL muscle. The fascia was removed, and a superficial fascicle was identified using surface microstimulation (7). A 32-gauge needle tipped with methylene blue dye was used to mark the proximal and distal ends of the fascicle, and a small incision was made to insert a 1-mm piezoelectric crystal at these locations (Sonometric, London, ON, Canada). The crystals were then secured using small 5-0 silk sutures. Once the crystals were securely implanted, barbed, indwelling electromyograph (EMG) fine-wire electrodes constructed of Teflon-coated 10 strand stainless steel wire (AS-631, Cooner Wire, Chatsworth, CA) were introduced into the muscle belly, in line with the muscle fibers and between the sonomicrometry crystals, using a small, curved surgical needle. A common ground electrode was sutured to the fascia overlaying the lateral aspect of the patellar tendon, and all leads were routed to the posterior-superior aspect of the incision. The fascia and skin were closed using 4-0 Vicryl suture. All animals received an injection of 0.03 ml of Torbugesic (Ayerst Veterinary Laboratories, Montreal, PQ, Canada) for pain. Animals were then removed from anesthesia and allowed to recover.

Rats were placed in the first lane of a three-lane treadmill (EXER 3/6 treadmill, Columbus Instruments, Columbus, OH), which was initially set to either an incline or decline of 16°. The rats were first introduced to slow walking speeds of ~10 m/min, and this was slowly increased to the target speed of 16 m/min. Rats were closely monitored to ensure they did not limp after the surgery. Because of the slow introduction to the treadmill, as well as the short walking duration required in this protocol, no means of encouragement were needed for the rats to walk. EMG and sonomicrometry signals were collected at 498 samples/s. A video camera positioned at the side of the treadmill was used to record the kinematics of the walking rats at 60 frames/s. The camera output was fed through a time code generator (model 9300, Datam, Anaheim, CA) to allow for synchronization of the visual data with the fiber length changes recorded in Sonosoft (Sonometric, London, ON, Canada). In addition, a custom-built sync pulse generator was used to synchronize the visual data with the fiber and EMG data, by emitting a voltage spike to the data acquisition software as well as illuminating a small diode in front of the video camera. After the recording of at least 20 steps from each rat for each grade, rats were again anesthetized via the induction box, and they were subsequently decapitated. Visual inspection postmortem revealed that all crystal and EMG wires were intact during exercise and that they were placed appropriately.

Protocols II and III. Sixteen rats were randomly assigned to one of two groups, to assess the effects of uphill or downhill walking, at either a 16° incline or 16° decline, for 5 days. On the first day, the rats were introduced to the treadmill at ~10 m/min and then to increasing in speed every minute. For some rats (particularly the uphill walking group) encouragement was required, but it was kept to a minimum. On the first day, the electrical grid at the base of the treadmill was activated, and a blast of compressed air was simultaneously aimed at their tails when they received a shock. On subsequent days, only the blast of air was required. After 5 min, the rats were allowed 90 s of rest, before training for another 5 min, at a speed of 16 m/min. This was followed by another 90-s rest period, before the rats performed a final 5-min exercise bout. In all, the rats trained for 15 min on day 1. This was increased by 5 min each successive day for 5 training days in total. Thus, on the last day, the rats trained for 35 min. Seventy-two hours after the last exercise bout, the rats were anesthetized in the induction box as described above and decapitated. The hindlimbs were immediately skinned and dissected free from the pelvic girdle at the acetabulum, with care being taken not to damage the one-joint knee extensor muscles. The hindlimbs were flexed and secured in 10% neutral buffered formalin for at least 4 wk.

Protocols IV and V. These protocols were identical to protocols II and III; however, the training durations was doubled, to assess the potential effects of time on sarcomere number adaptations. The rats were trained for 15 min on day 1, and the time increased by 5 min every day until the daily duration was 35 min, achieved on the fifth day. For the remaining 5 days, the rats trained for 35 min. Methods of encouragement identical to those of protocols II and III were used. At 72 h after the last exercise bout, rats were euthanized, and the tissue was harvested as described above.

Protocol VI. Eight rats served as sedentary controls. These rats were left in their cages and not disturbed. The rats were euthanized on the same day as protocols II and III, and their tissue was harvested and fixed as described above.

Analysis of in vivo fiber dynamics. Visual inspection of the fiber length data, in conjunction with video analysis, allowed us to select 5–10 step cycles that were consistent and resulted in fiber length changes that were consistently reproducible between cycles. Video data were matched to sonomicrometry data via the visual and digital sync pulse respectively. When a consistent walking pattern was observed, the time of the previous sync pulse was located and designated as time 0. Similarly, the corresponding digital sync pulse recorded in sonomicrometry was also designated as time 0. In this manner, although video and digital inputs were recorded at different sampling rates, the occurrences of touchdown and toe-off during step cycles could be determined from the time following the designated sync pulse. For the sequential steps selected, data were analyzed for the stance phase (from touchdown to toe-off of the left hindlimb) and swing phase (toe-off to subsequent touchdown of the left hindlimb). EMG data were only used to show the period of activation during each cycle, to permit the qualification of active lengthening of the fibers, or
eccentric contractions. Fiber length changes during uphill and downhill walking were calculated as the percent difference in fiber length at toe-off (FL-to) compared with the fiber length at touchdown (FL-td), or \[ \frac{(L_{f-to} - L_{f-td})}{L_{f-td}} \times 100 \]. Mean fiber length changes were found for each stance phase in sequence and also for all steps in uphill and downhill walking rats. Negative fiber strains indicate fiber shortening during the stance phase, and positive strains indicate fiber lengthening.

**Sarcomere number calculation.** Hindlimbs were removed from the fixative, and the bones and muscles of the shank were carefully removed from the hindlimb and discarded. Subsequently, all muscles were removed from the femur, except the VL and the VI. The vastus

![Fig. 1](image1.png) Fiber length changes and electromyograph (EMG) recordings in the vastus lateralis muscle for 8 consecutive steps (from left to right) during in vivo uphill walking in 1 representative rat. Solid lines, stance phase; dotted lines, swing phase. Note that during the stance phase, active fibers undergo an overall shortening from touchdown to toe-off, indicating concentric contractions in every step shown.

![Fig. 2](image2.png) Fiber length changes and EMG recordings in the vastus lateralis muscle for 7 consecutive steps (from left to right) during in vivo downhill walking in 1 representative rat. Solid lines, stance phase; dotted lines, swing phase. Note that during the stance phase, active fibers undergo an overall lengthening from touchdown to toe-off, indicating eccentric contractions in every step shown.

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lateralis was carefully dissected free of its attachments, leaving the vastus intermedius attached to the femur. Each muscle was placed in a separate dish containing 30% nitric acid for at least 6 h to partially digest the connective tissue. The muscle tissue was then transferred to saline for 12 h and subsequently placed in glycerol. Six small fascicles from each muscle were carefully teased out and placed on glass slides. Fascicle length was used as representative fiber length in this study, as fascicles spanned the entire distance from origin to insertion of the muscles. The length of each fascicle was measured using commercially available imaging software (Matrox Inspector, Matrox Systems, Dorval, PQ, Canada). Sarcomere lengths were measured at six regions along the length of the fiber using laser diffraction (beam diameter 0.8 mm, wavelength 633 nm). Sarcomere number per fiber was obtained by dividing fiber length by average sarcomere length.

Statistical analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS, Chicago, IL). Fiber strains and sarcomere numbers were analyzed using a two-way analysis of variance, to assess the effects of surface grade and training duration on fiber strain and sarcomere number. Multivariate analysis of variance with a Dunnett’s post hoc analysis was used to assess the differences in sarcomere number between groups at 5 and 10 days of training. All outlying values were included in the analysis. Significance was set at $P \leq 0.05$. All data are reported as means $\pm$ SE.

RESULTS

Fiber length changes, VL. Comparison of video for instrumented and noninstrumented walking rats confirmed that the rats did not limp after surgery. Fiber strain magnitudes in the VL during the stance phase did not significantly change with repetition in either the uphill or downhill walking group ($P = 0.596$), indicating that there were no effects of time on fiber strain magnitudes. Simply stated, fiber strains did not change systematically over the series of step cycles selected for analysis, further confirming the lack of serious injury, pain, or fatigue that could confound the results. Visual inspection of the data indicated that the VL muscle was activated while the fibers shortened during the stance phase of uphill walking (Fig. 1). During uphill walking, fibers actively shortened ($-1.2 \pm 0.1$ mm) for the duration of the stance phase, and this resulted in fiber strains that ranged from $-0.9\%$ to $-13.1\%$ during touchdown to toe-off. Mean fiber strain across all step cycles ($n = 46$) was $-6.4 \pm 0.4\%$, indicating that fibers underwent concentric contractions during uphill walking.

During downhill walking, the VL was activated during the stance phase (Fig. 2). Changes in fiber length ranged from $-0.04$ mm to $2.3$ mm, as fibers lengthened for 57 of 58 step cycles analyzed, resulting in an average fiber lengthening of $+1.1 \pm 0.1$ mm. Therefore, the VL fibers underwent eccentric (lengthening) contractions during downhill walking, actively lengthening the fibers by an average of 5.7 $\pm 0.5\%$.

Serial sarcomere adaptations, VI. Chronic exposure to uphill or downhill walking resulted in a significant interaction of time and surface grade on serial sarcomere number in the VI ($P < 0.001$), illustrating that sarcomere number could not be predicted by time or surface grade alone. After 5 days of uphill or downhill walking, there was no significant effect of surface grade on serial sarcomere number ($P = 0.092$). However, after 10 days of exercise, the VI from the uphill group had significantly fewer sarcomeres in series than both the control ($P = 0.05$) and downhill group ($P = 0.006$, Fig. 3A). In addition, the VI from the downhill walking group had significantly more sarcomeres in series compared with the control group ($P = 0.049$). Thus the significant interaction effect of time and surface grade resulted in a differential adaptation, because eccentric exercise resulted in a gain of serial sarcomeres, and concentric exercise resulted in a significant loss, compared with the control group, after 10 days of chronic exposure (Table 1).
Table 1. Serial sarcomere numbers for the vastus intermedius and vastus lateralis muscles after 5 or 10 days of uphill (concentric-biased contractions) or downhill (eccentric-biased contractions) walking

<table>
<thead>
<tr>
<th>Vastus Intermedius</th>
<th>Vastus Lateralis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Days</td>
<td>10 Days</td>
</tr>
<tr>
<td>Uphill</td>
<td>3,963±54</td>
</tr>
<tr>
<td>Downhill</td>
<td>4,092±53</td>
</tr>
<tr>
<td>Control</td>
<td>3,934±55</td>
</tr>
</tbody>
</table>

Values are means ± SE.

**Serial sarcomere adaptations, VL.** Similarly, for the VL, serial sarcomere number adaptation could not be predicted by one variable alone, because we observed a significant interaction effect of time and surface grade (\(P = 0.003\); Fig. 3B). After 5 days of exercise, serial sarcomere numbers were not significantly different between any of the three exercise groups (\(P = 0.885\)). However, after 10 days of exercise, the VL of the uphill walking group had significantly fewer sarcomeres in series than the control group (\(P = 0.027\)), but there was no significant difference compared with the downhill walking group (\(P = 0.891\)). Serial sarcomere number within the vastus lateralis muscles of the downhill walking rats was not significantly different from that of the control group (\(P = 0.096\)). Therefore, exposure to 10 days of exercise resulted in a significant reduction in serial sarcomere number after uphill walking, but it had no significant effect on serial sarcomere number in the VL for the downhill walking group (Table 1).

**DISCUSSION**

**Fiber dynamics.** Severe injury to all four knee extensor muscles has previously been shown after one 90-min bout of unconstrained downhill walking in rats (41). Because of the severity of muscle injury, the authors surmised that the knee extensor muscles underwent eccentric contractions during downhill walking, and they compared this with the lack of muscle injury in a group of uphill walking rats, assumed to have undergone repeated concentric contractions. Since then, it has become well accepted that eccentric exercise, compared with concentric exercise, is associated with a greater degree of muscle injury (15, 28, 29, 33). Although the assumption that the knee extensor muscles of rats undergo eccentric contractions during downhill walking and concentric contractions during uphill walking seemed plausible, this had not been confirmed by the authors. At the time, no fiber length measurements of the rat knee extensors were available for in vivo protocols.

Here, we have shown that the fibers of the vastus lateralis in uphill walking rats performed active concentric contractions and positive work during stance of every step analyzed. Conversely, the knee extensors underwent active eccentric contractions during the stance phase of downhill walking. Thus our results confirmed the assumption made by Schwane and Armstrong (41), and other researchers who performed similar work without fiber length measurements (1, 21, 47). Furthermore, these observations also agree with the qualitative observation of knee extension during stance of uphill walking and knee flexion during stance of downhill walking rats from the video records.

However, our results are in direct contrast to the first-ever measurements of rat knee extensor fiber length changes during uphill walking made by Gillis and Biewener (14). These authors found that the VL fibers were actively stretched (eccentric contraction) during the stance phase of uphill walking (Fig. 4). Although Gillis and Biewener commented that their result was somewhat of a surprise, it also did not fit the indirect evidence of other studies on muscle injury (1, 21, 41, 47) and adaptation (31, 32, 41) of the rat knee extensors and direct fiber length measurements in other species during uphill walking (39).

In view of the importance of these results and no other published research on fiber length changes in the rat knee extensor muscles, two of the present authors performed all analyses independently and obtained the same results: VL fibers shorten actively during uphill walking, and they lengthen actively during downhill walking for the surface grades and speeds observed here. Therefore, we conclude that the results by Gillis and Biewener (14) on fiber length changes in the VL of uphill walking rats are likely incorrect. Our results also receive indirect support from the injury and adaptation litera-
ture. For example, there is evidence that rat knee extensors experience a significant protective adaptation after one 30-min bout of downhill walking (41). Thus exposure to the damaging effects of eccentric contractions provides protection from future bouts of eccentric exercise, often referred to as the repeated-bout effect. This training effect, however, has not been found in uphill walking (31, 32) or in human muscles exposed to a training regimen of concentric exercise before one injurious eccentric exposure (48). Although others have shown a beneficial training effect after level walking in rats, these benefits were reported after 17–20 days of exercise (21, 47). Level walking results in both eccentric as well as concentric contractions (13, 41). Thus, if eccentric exercise is indeed the stimulus for the repeated-bout effect adaptation to occur, then a concentrated downhill regimen, composed of exclusively eccentric contractions, would be expected to show a beneficial adaptation sooner than level walking, comprised of concentric and eccentric contractions.

Serial sarcomere number adaptations. The lack of serial sarcomere adaptation within the knee extensor muscles after 5 days of exercise in the present study conflicts with previous reports of significant differences in serial sarcomere numbers between uphill and downhill walking rats of identical duration (32). In our results presented here, the lack of significance between the three groups after 5 days of exercise does not rule out a small decrease in serial sarcomere number in the uphill group or a small increase in the downhill group. This may provide a small protective effect for the downhill group early in the training regimen, which may explain the early adaptations shown previously (41).

Although previous studies have shown both knee extensor muscles to be injured during downhill walking (41), fiber dynamics for the vastus intermedius have never been measured directly. Implicitly, it has been assumed that fiber strains of all four knee extensor muscles are similar to those measured in the vastus lateralis (14). This may not be an appropriate assumption, because the VI has a greater angle of pennation and shorter fibers than the VL. In addition, the fiber types between these two muscles differ, and the slow-twitch VI may be selectively recruited during unconstrained downhill locomotion. Because of these structural and morphological differences, it is quite possible that the magnitudes of fiber strain in the VI and VL are different too, which might explain the different amount of injury previously reported (41), as well as the different serial sarcomere number adaptations observed in this study. Thus the short fibers of the VI may be more susceptible to muscle injury during downhill walking, because short fibers have been proposed to be more susceptible to muscle injury than long fibers for a given excursion of the muscle (28, 29). This may be due to the shorter fibers working on the descending limb of the FLR, undergoing a greater magnitude of strain, and resulting in a more rapid and greater magnitude of sarcomerogenesis.

Although uphill walking rats show minimal (if any) muscle injury, the uphill walking rats had significantly fewer serial sarcomere numbers in the VI and VL compared with the muscles of control rats. Evidence for serial sarcomere loss can be assessed indirectly from FLRs after long-term concentric or eccentric exercise (4, 20, 31). Unfortunately, there is only one study in which a control group was used to directly measure serial sarcomere numbers compared with uphill and downhill walking rats (31). In our study, serial sarcomere numbers in the uphill walking rats adapted rapidly and similarly in both knee extensor muscles tested. This suggests that this adaptation is concentric contraction type specific, may not depend on muscle injury, and may be a more rapid adaptive mechanism than the increase in serial sarcomere number seen in the downhill group.

In summary, the differential adaptations that occur in knee extensor muscles after uphill and downhill walking in rats appear to be related to contraction type. Knee extensor muscles of uphill walking rats undergo repeated concentric contractions, there is likely no contraction induced injury, and VI and VL lose serial sarcomeres after 10 days of exercise. Conversely, the knee extensor muscles during downhill walking undergo repeated eccentric contractions, there is likely some injury, and the VI gains serial sarcomeres after 10 days of exercise compared with controls and uphill walking rats. However, there is no adaptation in the VL. Although the dynamics of the individual knee extensor muscles are assumed to be similar, the adaptations vary between the VI and VL probably because of the different architecture of the muscles and fiber lengths, which may influence the magnitude of fiber strain and/or injury. This idea needs to be carefully addressed in future work.

GRANTS

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