Effects of hindlimb unweighting and aging on rat semimembranosus muscle and myosin

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Zhong, Sheng, Dawn A. Lowe, and LaDora V. Thompson. Effects of hindlimb unweighting and aging on rat semimembranosus muscle and myosin. J Appl Physiol 101: 873–880, 2006. First published May 11, 2006; doi:10.1152/japplphysiol.00526.2005.—We tested the hypothesis that lower specific force (force/cross-sectional area) generated by type II fibers from hindlimb-unweighted rats resulted from structural changes in myosin (i.e., a change in the ratio of myosin cross bridges in the weak- and strong-binding state during contraction). In addition, we determined whether those changes were age dependent. Permeabilized semimembranosus muscle fibers from young adult and aged rats, some of which were hindlimb unweighted for 3 wk, were studied for Ca2+-activated force generation and maximal unloaded shortening velocity. Fibers were also spin labeled specifically at myosin Cys707 to assess the structural distribution of myosin during maximal isometric contraction using electron paramagnetic resonance spectroscopy. Myosin heavy chain isoform (MHC) expression and the ratio of MHC to actin were evaluated in each fiber. Fibers from the unweighted rats generated 34% less specific force than fibers from weight-bearing rats (P < 0.001), independent of age. Electron paramagnetic resonance analyses showed that the fraction of myosin heads in the strong-binding structural state during contraction was 11% lower in fibers from the unweighted rats (P = 0.019), independent of age. More fibers from unweighted rats coexpressed MHC IIB-IIX compared with fibers from weight-bearing rats (P = 0.049). Unweighting induced a slowing of maximal unloaded shortening velocity and an increase in the ratio of MHC to actin in fibers from young rats only. These data indicate that altered myosin structural distribution during contraction and a preferential loss of actin contribute to unweighting-induced muscle weakness. Furthermore, the age of the rat has an influence on some parameters of changes in muscle contractility that are induced by unweighting.

Skeletal muscle function diminishes following periods of unloading, denervation, and immobilization (5, 11, 23, 36). To investigate underlying mechanisms associated with the unloading-induced losses of muscle contractility, the hindlimb-unweighted (HU) rodent model is often used (1, 32, 38). Thus other physiological mechanisms, such as intrinsic changes to the muscles fibers, may be contributing to the deleterious changes in skeletal muscle function with unweighting. Because permeabilized fibers do not have intact membranes, force generation reflects the interactions of myosin and actin, exclusive of other factors in excitation-contraction (e.g., sarcoplasmic reticulum calcium release). Therefore, it appears that reductions in force-generating capacity induced by unweighting are a result of intrinsic changes (e.g., quality) of the contractile proteins, regulatory proteins, and or changes in the ultrastructure of the sarcomere.

In two previous studies, our laboratory demonstrated that changes in the catalytic domain of myosin provide a molecular explanation for muscle weakness with aging (22, 23). Electron paramagnetic resonance (EPR) spectroscopy was used to determine the relative changes in the distribution between two structural states of myosin: strong binding (force generating) and weak binding (no force generation). We showed that the fraction of myosin heads in the strong-binding (force-generating) structural state during a maximal isometric contraction was 30% lower in fibers from aged rats, which was comparable to the 27% age-related decrement in specific force (22). Thus it is plausible that changes in the structural distribution of myosin may explain the muscle weakness or the reduction in specific force associated with unweighting as well. Therefore, in the present study, we used EPR to probe the underlying molecular mechanism of skeletal muscle weakness associated with unweighting of the semimembranosus muscle from young and old rats. We hypothesized that the reduced force-generating ability of fibers from HU rats was due to a decreased population of myosin heads in the strong-binding (force-generating) structural state during muscle contraction relative to fibers from weight-bearing rats. We found a significant decrease in strong-binding myosin with unweighting but not enough to fully account for all of the specific force loss. Riley and coworkers (28–31) have shown that unweighting causes a preferential loss of thin filament proteins, so we also measured the content of actin and myosin heavy chain (MHC) in fibers to determine whether a loss in one contractile protein, relative to another, contributed to the loss of specific force.

Because type II fibers show age-related changes in contractility to a greater extent than type I fibers and the semimembranosus muscle is composed primarily of type II fibers, in the
present study we also hypothesized that unweighting-induced decrements in myosin function and structural distribution would be greater in the older age group (3, 16a, 21, 36). Our previous studies on age-related changes in myosin were conducted using the Fischer 344 × Brown Norway F1 hybrid rat model. Thus a secondary purpose of the present study was to confirm the age-related changes in myosin function and structural distribution in fibers from the semimembranosus muscles using the Fischer 344 rat model. The Fischer 344 rat model represents a second type of animal model that is commonly used to study aging in rats (20).

METHODS

Animals and unweighting protocol. Fisher 344 male rats, aged 8–12 mo and 24–25 mo, were randomly assigned to a control, weight-bearing group (n = 8 and 7, respectively) or an unweighted group (n = 6 and 4, respectively). An additional group of seven weight-bearing, 28-mo-old rats was also studied. All rats were obtained from the aging colony maintained by the Minneapolis Veterans Affairs. Unweighting was accomplished using a harness attached to the proximal two-thirds of the rat’s tail (1). After 3 wk of unweighting, or normal cage activity for the control groups, rats were anesthetized with pentobarbital sodium (55 mg/kg ip), and semimembranosus muscles were dissected and weighed. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with guidelines established by the American Physiological Society.

Tissue preparation. Semimembranosus muscles were placed in relaxing buffer [7 mM EGTA, 0.016 mM CaCl2, 5.6 mM MgCl2, 80 mM KCl, 20 mM imidazole (pH 7.0), 145 mM creatine phosphate, 4.8 mM ATP] on ice. Bundles of fibers ~8 mm long and ~1 mm in diameter were formed for single-fiber contractile analyses. These bundles were tied to pieces of capillary tubes and stored in 50% glycerol-50% relaxing buffer for up to 5 wk at 20°C (37). Additional bundles of fibers were permeabilized and stored at ~20°C in fresh glycerol buffer containing 0.1 mM DTT for EPR experiments, as described previously (22).

Single-fiber analyses. Individual fiber segments (~2 mm long) from permeabilized bundles were isolated and studied at 25°C, as described in detail previously (37). Fiber segments were mounted in relaxing buffer, sarcomere lengths were set to 2.4 μm, diameter was measured at three places along the length of the fiber, and then maximal isometric force was determined. Maximal unloaded shortening velocity (V0) was determined by the slack test. In brief, at peak isometric force, fibers were rapidly shortened (slacked) by 10–20% of fiber length such that force dropped to zero. The time between zero force and force redevelopment was measured. This procedure was done five to seven times at different slack distances. The slack distances were then regressed against the corresponding times of force redevelopment, and the slope of that line (mm/s) is reported as V0, after normalizing to fiber length (fiber length per second). The correlation coefficient for the straight line fit was >0.98. Four to fifteen fibers from each muscle were studied, and their values (force, specific force, V0) were averaged to represent fiber contractility for that rat.

Following contractility measurements, each fiber was solubilized in 20 μl of sample buffer [24 mM EDTA, 60 mM Tris (pH 6.8), 1% SDS, 5% β-mercaptoethanol, 15% glycerol, 2 mg/ml bromophenol blue] and stored at ~80°C. MHC isoform expression was determined by gel electrophoresis and silver staining (21). To determine the ratio of MHC to actin (MHC/actin), ~1 nl of fiber volume was calculated and then loaded onto a second gel (3.5% acrylamide stacking-12% acrylamide separating). Stained gels were scanned on a flatbed scanner, and image analysis software (Sigma-Scan, Jandel Scientific) was used to quantify the relative levels of MHC (all isoforms) and actin in each fiber. A total of 308 single, permeabilized fibers were assessed for force, V0, MHC isoform expression, and MHC/actin content.

EPR spectroscopy. Permeabilized fiber bundles were further dissected and prepared for EPR experiments by spin labeling specifically at Cys507 (SHI) with 0.5 mM 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperdinioxy spin label (IASL; Sigma; Ref. 25). Briefly, SHI is a reactive sulfhydryl group that is located in the catalytic domain of the myosin head, near the “converter” region that is thought to couple the active site to the large structural changes that generate force (22). IASL is site specific and is a reporter of changes in ATP-dependent weak and strong structural states (22). The IASL-labeled fibers were prepared for spectroscopy by attaching 7.0 silk suture to each end of the bundle and pulling the bundle into a glass capillary tube and placing it in a TE102 cavity (4102ST/8838; Bruker Instruments, Billerica, MA) perpendicular to the magnetic field. One end of the fiber bundle was attached to a force transducer (SensoNor Ackers 801 strain gauge; Aksjeskaptet, Norway), and the other end was stabilized to hold the fibers isometrically. The capillary tube inside the cavity was in line with tubing and a peristaltic pump, such that buffers were flowed over the fibers at a rate of 2 ml/min at 22°C. Force was monitored throughout EPR spectra collection. Low-field EPR spectra were collected (3,425 G central peak, 38 G sweep width, 5.0 G peak-to-peak modulation amplitude, and 16 mW microwave power) under conditions of rigor, relaxation, and contraction on an E500 EleXsys spectrometer (Bruker Instruments). Spectra obtained during maximal isometric contraction were analyzed as a linear combination of the spectra obtained during rigor and relaxation, as described previously (22, 23, 26). Briefly, for each bundle, the spectrum obtained during maximal isometric contraction (VCon) was analyzed as a linear combination of the spectra obtained during rigor and relaxation using VCon = xVRig + (1 − x)VRel, where VRig (rigor) corresponds to all heads in the strong-binding structural state (x = 1), and VRel (relaxation) corresponds to all heads in the weak-binding structural state (x = 0), as shown previously and in Fig. 5 (22, 23, 26). Thus for the contraction spectrum, x was solved at each of the 1,024 field positions to determine the fraction of myosin heads in the strong-binding structural state, using x = (VCon − VRel)/(VRig − VRel). Two to three bundles from each semimembranosus muscle were analyzed, and their values were averaged to represent strong-binding myosin fraction for that rat.

Statistical analyses. The effects of age (6 mo and 24–25 mo) and 3 wk of hindlimb (un)weighting on muscle mass, fiber contractility, and myosin parameters were evaluated by using a two-way ANOVA. When a significant interaction between age and (un)weighting was found, pairwise comparisons were made with Tukey tests. A one-way ANOVA with Tukey post hoc tests was used to further evaluate muscle mass, fiber contractility, and myosin parameters from the three age groups of control, weight-bearing rats. All statistical analyses were performed using SigmaStat version 2.03 (Systat Software). Values in the RESULTS are means ± SE. Differences were considered significant when P < 0.05. Least square means were used to calculate the percent differences reported in the RESULTS.

RESULTS

Effects of HU (two-way ANOVA). Unweighting affected semimembranosus muscle mass, but the effect depended on age (Fig. 1; age × HU, P = 0.018). Among the 24- to 25-mo-old rats, muscle mass was 31% lower in the unweighted rats than in controls (P < 0.05); however, muscle mass was not different between 6-mo-old unweighted and control rats. Body weight of the control rats was 430 ± 14 and 492 ± 29 g in 6-mo and 24- to 25-mo groups, respectively. Following unweighting, the 6-mo-old rats weighed 377 ± 19 g, and the 24- to 25-mo-old rats weighed 379 ± 12 g (P < 0.05). When semimembranosus muscle mass was normalized to body mass,
no significant effect of unweighting was detected (independent of age, \( P = 0.630 \)).

Overall, HU significantly affected fiber contractility. Absolute force generation was lower in fibers from unweighted rats, independent of age (Fig. 2). Similarly, unweighting resulted in lower specific force, independent of age (Fig. 3). Specific force was 34% lower in fibers from the unweighted rats compared with those from control rats. Depending on age, \( V_o \) was affected by unweighting (Fig. 4). Fibers from the 6-mo-old unweighted rats were significantly slower than fibers from 6-mo-old control rats and were also slower than fibers from the 24- to 25-mo-old unweighted rats (\( P < 0.05 \)).

There was a significant main effect of unweighting on the fraction of strong-binding myosin in fibers during contraction, independent of age (Figs. 5 and 6). HU for 3 wk reduced the fraction of strong-binding myosin from 0.340 ± 0.015 to 0.296 ± 0.010 in the 6-mo-old rats and from 0.299 ± 0.011 to 0.276 ± 0.011 in the 24- to 25-mo-old rats, an overall reduction of 11%. These data indicate that part of the reduced force-generating capacity of fibers following unweighting was an alteration in the structural distribution of myosin during contraction.

The ratio of MHC/actin in fibers that were assessed for contractility was affected by unweighting, depending on age (Fig. 7). Fibers from the 6-mo-old unweighted rats had ~25% greater content of MHC, relative to actin, compared with fibers from 6-mo-old control rats and also compared with fibers from 24- to 25-mo-old control rats and from the 24- to 25-mo-old unweighted rats (\( P < 0.05 \)).

MHC isoform expression was determined on all fibers that were assessed for force, specific force, and \( V_o \). Only 2 of 308 fibers expressed MHC I, and those fibers were removed from the database. The percentage of fibers that expressed MHC isoforms IIB, IIX, IIX/IIA, or IIA in each semimembranosus muscle studied was not affected by unweighting (\( P \geq 0.063 \); Fig. 8). However, the percentage of fibers that coexpressed MHC IIB/IIX in unweighted rats was twofold greater than that in controls (\( P = 0.049 \), independent of age (interaction, \( P = 0.862 \)).

**Effects of age (one-way ANOVA).** Semimembranosus muscle mass was significantly affected by aging when all three age groups were taken into account. The 28-mo-old rats had smaller muscles than the 24- to 25-mo-old rats (Fig. 1). Semimembranosus muscle mass normalized to body mass was not different between age groups (\( P = 0.873 \)). Force generation was affected by age (Figs. 2 and 3). Specific force was 25 and 28% lower in fibers from 28-mo-old rats relative to fibers from 6- and 24- to 25-mo-old rats, respectively. \( V_o \) was insignificantly affected by age (Fig. 4). There was a significant overall effect of aging on the fraction of strong-binding myosin during contraction (Fig. 6). The fraction of strong-binding myosin was 21% lower in fibers from 28-mo-old rats than in fibers from 6-mo-old rats. The content of MHC/actin in fibers from 6-mo-old control rats and also compared with fibers from 24- to 25-mo-old control rats.
was not affected by aging (Fig. 7). MHC isoform expression was not affected by aging when all three age groups were considered ($P \geq 0.062$). However, when only the 6-mo-old and 24- to 25-mo-old rats were considered (two-way ANOVA), the percentage of fibers that coexpressed MHC IIB/IIX was 2.6-fold greater in the older rats ($P = 0.012$; Fig. 8).

**DISCUSSION**

The first purpose of this study was to determine the extent to which altered myosin structural distribution during contraction contributed to the decline in specific force following 3 wk of HU and to investigate if age affected those maladaptations. Our findings suggest that intrinsic characteristics related to fiber cross-bridge mechanics were altered with unweighting as single-fiber force-generating capacity and myosin structure were detrimentally altered, independent of rat age. Unweighting also induced a slowing of $V_o$ in type II fibers and an increase in the ratio of MHC/actin in those fibers from young but not older rats.

**HU model and the semimembranosus muscle.** Unloading of skeletal muscle from spaceflight, immobilization, and HU results in dramatic and rapid muscle atrophy and loss of strength (7–9, 11, 36). To study the effects of mechanical unloading in rodents and underlying cellular mechanisms for the observed muscle dysfunction, the HU model has been used effectively (43). HU results in atrophy and strength loss in muscles and fibers composed of MHC type I (7, 27). Those changes occur rapidly and are observed within very short periods of unloading. An extensive amount of literature is available on the responses of type I fibers to unweighting, but less information is reported on type II fibers. Short-term unloading (7–14 days) does not appear to alter the contractile function of type IIA fibers from gastrocnemius, plantaris, or extensor digitorum longus muscles of adult rats, but, in bedrest and spaceflight studies of longer duration (37 and 84 days), type II fibers from vastus lateralis muscles have altered contractile function (8, 9, 12, 17, 30, 40–42). For example, 37 and 84 days of bed rest result in type II fiber atrophy, decline in force (peak and specific force), and slower velocities (17, 40).

The extent of contractile function adaptation in the type II fibers appears to be variable. The variability exists because the reported studies examine different subjects (e.g., humans, rodents, nonhuman primates), muscles (e.g., vastus lateralis, gastrocnemius), modes of unweighting, and length of unweighting. This study clearly demonstrates that type II fibers from semimembranosus muscle are sensitive to the removal of weight bearing for 3 wk.

Other factors besides fiber type have been attributed to a muscle’s (in)sensitivity to unloading, including its in vivo...
function (e.g., extension vs. flexion), locomotor involvement (postural vs. nonpostural), and position during unweighting (shortened vs. normal length). The semimembranosus muscle is a nonpostural knee flexor/hip extensor involved in locomotion, and previous studies demonstrate that the postural-extensor muscles are more sensitive to unloading compared with the nonpostural muscles (11). This bias may be due to a fundamental alteration in motor control with unloading (11). Positioning of the limb and the subsequent working position of the muscle may play a role in the muscle’s sensitivity with unloading. For example, Riley and coworkers (28) showed a greater reduction in specific force in fibers from unloaded-shortened muscle (soleus) compared with fibers from unloaded-nonshortened (adductor longus) muscles. The hip joint of a rat did not significantly change from standing to the HU position, and the length of adductor longus muscle did not change (28). From those data, we might contend that the semimembranosus muscle is maintained at its normal in vivo length during unweighting as well.

Effects of unweighting on fiber force generation. First, Fig. 6 highlights that the fraction of myosin heads bound to actin during contraction in fibers from 6-mo control animals is 34%. Although the Huxley cross-bridge model predicts that the fraction of myosin heads strongly bound to actin should be ~80%, more recent investigations have shown that 80% is an overestimation (13, 16, 19, 26). Using technology that can directly measure stiffness and the fraction of strong-binding myosin heads in contracting fibers (EPR spectroscopy), the fraction of myosin heads bound to actin during contraction is in the range of 30–40% (13, 19, 26).

In the present study, permeabilized type II fibers from the semimembranosus muscle of unweighted rats generated ~35% less force than fibers from weight-bearing rats, irrespective of age. We hypothesized that this reduced force-generating capacity was due to a decreased population of myosin heads in the strong-binding (force-generating) structural state during muscle contraction. Results of EPR spectroscopy substantiated this hypothesis: we found that strong-binding myosin was 11% lower in fibers from unloaded rats. Thus structural changes in myosin explain about one-third (11 of the 34%) of the force loss due to unweighting. Previously, we found that myosin structural changes accounted for essentially all of age-related losses in force generation, but in the case of unweighting, other factors must contribute to the force loss (22, 23, 35). Likely candidates include alterations in calcium kinetics and/or a decrease in force per cross bridge. Based on our statistical analysis, the underlying mechanisms contributing to the HU-induced decline in force production are likely similar between young and old rats, since the decline is independent of the age of the animal.

Effects of unweighting on fiber-shortening velocity. The shift in MHC isoform expression may have contributed to the overall decrease in shortening velocity we observed, but, when we compared fibers that only expressed IIB MHC, a 48% decrement in \( V_0 \) with unweighting persisted. Thus we conclude that factors in addition to MHC must contribute to unloading-related reductions in contractile velocities.

\( V_0 \) is thought to be limited by the rate of actomyosin cross-bridge detachment, a process that has been shown to vary with MHC and myosin light chain (MLC) isoform expression,
troponin C (TnC) regulation, and the physical properties of the myofilament geometry. For example, MLC composition is believed to play a role in modulating shortening velocity in moderate to fast contracting fibers, presumably by altering ATPase hydrolysis (6, 14, 34). Thus changes in the essential and regulatory MLC warrant investigation. The reduction in \( V_o \) may be related to a partial loss of TnC, since partial extraction of TnC causes a slowing of shortening velocity (14, 25).

Lastly, in type I fibers from unloaded conditions, a reduction in myofilament packing and thin filament density correlates with increases in \( V_o \), but this correlation is not strong in type IIa fibers from this same research group (29, 31). The role of myofilament geometry in type II fibers requires further investigations, since myosin content, packing densities, and extent of short thin filaments appear to have fiber-type-specific properties (28–31). Presently, it is not clear which of these processes or combination of processes is responsible for the depressed \( V_o \).

Interaction between age and HU. We found three dependent variables, muscle mass, MHC/actin, and \( V_o \), had a differential response to unweighting in old rats vs. young rats (significant interaction of age and unweighting). First, unweighting resulted in semimembranosus muscle atrophy of the older rats but not the younger rats. Since the semimembranosus muscle is composed primarily of type IIB and IIX fibers and these fiber types preferentially show age-related deterioration, it is likely that the decline in muscle mass may be due to selective fiber atrophy and fiber loss in the older rats with unweighting (16a, 21, 36).

Second, unweighting resulted in increases in MHC/actin of the younger rats but not the older rats. An increase in MHC/actin suggests preferential loss of actin compared with myosin with unweighting. Riley et al. (11, 29, 30) have shown that actin protein is preferentially lost compared with myosin with 17 days of spaceflight and bed rest from the soleus muscle. Our results in the young animals are consistent with this preferential loss of actin. Because protein degradation is critical in skeletal muscle adaptation with unweighting, the differential response between the age groups points to possible age-related changes in this cellular process. The proteasome is the main protease for degrading proteins, and we recently reported significant age-related changes in proteasome structure, function, and oxidation state (10, 15). These age-related changes could inhibit removal of specific proteins, such as myosin and actin, in the older rat, providing a possible mechanism for the lack of unweighting-induced change in MHC/actin (10, 15).

In contrast to protein degradation, in unweighted aged rats, there is also a reduced ability of fast muscles to upregulate transcription factors that are necessary for muscle gene expression (2). Thus it is likely that the increase in MHC/actin of the younger rats may be due to efficient degradation and synthesis processes with unweighting, which are less efficient in the older rats.

Fig. 7. Ratio of myosin heavy chain (MHC) to actin protein content in fibers assessed for contractility from 6-, 24- to 25-, and 28-mo-old rats. Values are means ± SE. Within fibers from 6-mo-old rats, unweighting resulted in a greater MHC-to-actin ratio relative to fibers from control rats or from 24- to 25-mo-old HU rats. *Significantly different than 6-mo-old control group. #Significantly different than 6-mo-old HU group.

Fig. 8. Percentage of fibers assessed per semimembranosus muscle from 6-, 24- to 25-, and 28-mo-old rats that expressed and coexpressed the various MHC isoforms. Values are means ± SE. Only the coexpression of IIB/IIX MHC was affected by unweighting and aging, with both conditions causing an increase. Con, control rats.

2-WAY ANOVA

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1-WAY ANOVA

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Lastly, we found the unweighting resulted in a decrease in $V_o$ in the younger rats, but not the older rats. As noted above, the underlying mechanism(s) responsible for the reduction in $V_o$ is not clear and warrants investigation. Because $V_o$ is regulated by the MLCs, it is possible that the reduction in $V_o$ is due to removal of MLC3f in HU young rats, which is impaired in the older rats. Overall, we propose that unweighting-induced decrements in contractility in the older could be impacted by an accumulation of modified proteins (i.e., myosin, MLC) because of increased protein oxidation, slowed removal of proteins due to decreased proteasome function, and slowed transcriptional activity required for making new proteins (i.e., unmodified myosin, MLC).

Effects of age. A second purpose of the present study was to confirm our previous observations on age-related decrements of myosin in semimembranosus muscle fibers from Fischer 344 × Brown Norway rats (22, 23). In the present study, we used Fischer 344 rats and found that type II fibers from 28-mo-old rats generated 25% less specific force and had 21% fewer strong-binding myosin heads during contraction relative to fibers from 6-mo-old Fischer 344 rats. These data confirm our earlier reports that a major mechanism of age-related loss of $V_o$ is not clear and warrants investigation. Because $V_o$ is set by the underlying mechanism(s) responsible for the reduction in $V_o$, however, this is consistent with our laboratory’s previous study in which we reported a statistically significant 20% age-related slowing of $V_o$ in type II semimembranosus fibers (21).

Summary. Unweighting the hindlimbs of young and old rats for 3 wk induced a decline in specific force of type II fibers that was, in part, due to myosin structural alterations. Our data indicate that other mechanisms are involved in the decline in contractility, and these mechanisms may be age dependent. In the unweighted young rat, preferential loss of actin in type II fibers occurred, but this did not occur in unweighted older rats and did not affect shortening velocity.

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