Intermittent stretch training of rabbit plantarflexor muscles increases soleus mass and serial sarcomere number

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1Human Performance Laboratory, Faculty of Kinesiology, University of Calgary, Calgary, Alberta, Canada; and 2Laboratoire de Physiologie et de Biomécanique de la Locomotion, Institute of Neuroscience, Université catholique de Louvain, Louvain-la-Neuve, Belgium

Submitted 13 June 2014; accepted in final form 26 March 2015

De Jaeger D, Joumaa V, Herzog W. Intermittent stretch training of rabbit plantarflexor muscles increases soleus mass and serial sarcomere number. J Appl Physiol 118: 1467–1473, 2015. First published April 2, 2015; doi:10.1152/japplphysiol.00515.2014.—In humans, enhanced joint range of motion is observed after static stretch training and results either from an increased stretch tolerance or from a change in the biomechanical properties of the muscle-tendon unit. We investigated the effects of an intermittent stretch training on muscle biomechanical and structural variables. The left plantarflexors muscles of seven anesthetized New Zealand (NZ) White rabbits were passively and statically stretched three times a week for 4 wk, while the corresponding right muscles were used as nonstretched contralateral controls. Before and after the stretching protocol, passive torque produced by the left plantarflexor muscles as a function of the ankle angle was measured. The left and right plantarflexor muscles were harvested from dead rabbits and used to quantify possible changes in muscle structure. Significant mass and serial sarcomere number increases were observed in the stretched soleus but not in the plantaris or medial gastrocnemius. This difference in adaptation between the plantarflexors is thought to be the result of their different fiber type composition and pennation angles. Neither titin isoform nor collagen amount was modified in the stretched compared with the control soleus muscle. Passive torque developed during ankle dorsiflexion was not modified after the stretch training on average, but was decreased in five of the seven experimental rabbits. Thus, an intermittent stretching program similar to those used in humans can produce a change in the muscle structure of NZ White rabbits, which was associated in some rabbits with a change in the biomechanical properties of the muscle-tendon unit.

Contrasting results have been obtained when different muscle groups were exposed to the same stretching program. Magnusson et al. (25) did not observe any significant change in hamstring muscle passive stiffness or peak torque as a result of a stretching program consisting of five bouts of a 45-s static stretching period, two times a day for 3 wk. The same stretching program applied by Kubo et al. (21) to ankle plantarflexor muscles resulted in significantly decreased values of passive torque at all ankle angles during dorsiflexion. More recently, Reid and McNair (30) and LaRoche and Connolly (22) did not observe a significant change in the hamstring muscle passive torque or stiffness measured for the same ROM before and after a stretching program. Guissard and Duchateau (16) and Mahieu et al. (26) observed decreased passive torque and passive stiffness, measured at the same ankle dorsiflexion angles before and after a plantarflexor muscle stretching program.

Different results are obtained when different stretching programs are used on the same muscle group. Passive torque and stiffness, measured at the same ankle joint angles before and after a stretching program, were either decreased (16, 21, 26) or unchanged (12, 13) for the ankle plantarflexors, depending on the stretch duration and frequency. Short stretch duration (stretching periods of 15 s) and low frequency (30-50 stretching periods/wk) did not result in changes of passive torques in human plantarflexors (12, 13). When changes in passive force following stretch training are observed, they are thought to result from changes in structure of connective tissues that are arranged in parallel with the muscle fibers (21) or from changes in muscle fascicle lengths resulting from an increase in serial sarcomere number (26).

When studying stretch training in animals, typically continuous stretching to the target muscles is imposed for several days. The effects of these continuous programs on muscle-tendon units are studied by measuring biomechanical parameters, such as the passive stiffness and the passive force-length curve, or by measuring muscle tissue parameters, such as the intramuscular connective tissue amount or organization, the muscle’s mass or length, or its serial sarcomere number. When muscle-tendon biomechanical properties and muscle tissue parameters are collected simultaneously from the same animals, relations between structural and mechanical properties may be derived. An increase in the passive stiffness (measured as the slope of the passive force-length curve) has been associated with an increase in the amount or stiffness of intramuscular connective tissues, while a shift in the passive force-length curve of a muscle has typically been associated with a change in its length, or more precisely a change in serial sarcomere number. After immobilization in a shortened posi-
tion, a muscle’s passive stiffness and relative amount of connective tissue are typically increased and collagen fibers become more acutely aligned relative to the muscle fiber axis (11). In surgical tibial lengthening experiments, passive stiffness and connective tissue content were increased at high rates of distraction for rabbit tibialis anterior muscles. At low and intermediate rates of tibial lengthening, the tibialis anterior passive force-length curve was shifted to increased length, which was associated with an increased serial sarcomere number (34). In mouse soleus, after immobilization in a shortened position, the passive force-length curve was shifted to decreased lengths with a concomitant loss of serial sarcomeres. After immobilization in a lengthened position, there was no clear change in the passive force-length curve although there was a significant increase in the number of serial sarcomeres (38). The addition of sarcomeres was also accompanied by gains in muscle mass (11).

Intermittent stretching of the rat ankle plantarflexor muscles for 40 min every 3 days for 4 wk resulted in an increase in length, serial sarcomere number, and cross-sectional area of the rat soleus (5). The passive torque-angle curve was not measured in this study.

Intermittent stretching of the ankle dorsiflexors appears to be an efficient way to significantly modify the passive dorsiflexion torque-angle curves through an increase in serial sarcomere number (5). However, the reported effects of intermittent stretching on passive muscle force, and the relationship between increases in serial sarcomere number and passive force, have been inconsistent. Therefore, the purpose of this study was to impose an intermittent stretching program to rabbit ankle plantarflexor muscles and measure serial sarcomere numbers in these muscles together with the passive ankle dorsiflexor torque-angle curves before and after the stretch intervention. We took care to select a training program that would be similar to those used in human stretching. As a secondary purpose, we also wanted to quantify possible changes in muscle structure by measuring muscle mass, serial sarcomere number, collagen content, and titin isoforms in the stretched and the nonstretched control soleus, gastrocnemius, and plantaris muscles.

METHODS

Seven skeletally mature female New Zealand (NZ) White rabbits (mass 5.4 ± 0.9 kg) were assigned to a static, intermittent stretching protocol. Their left plantarflexor muscles were passively and statically stretched three times a week for 4 wk. This protocol was approved by the Committee of Animal Care and Ethics at the University of Calgary. To observe the effects of this intermittent stretch training on biomechanical variables, passive torques produced by the left plantarflexor muscles were measured as a function of ankle angle. The experimental rabbits were anesthetized and placed supine in a frame with the left knee joint maintained at 140°. The left ankle was passively dorsiflexed from 70° to 140°, and then back to 70° at 5°/s with a 2-s rest before the next cycle. Five successive cycles were performed. The fifth cycle was used for analysis to ensure consistent preconditioning and to account consistently for viscoelastic stress relaxation.

Joint torques were measured using strain gauges placed in a full Wheatstone bridge on the cam between the servomotor and the footplate. The output signal was sampled at 50 Hz with Windaq data acquisition software. Three mechanical variables served for comparison of the stretch training effect: peak passive torques obtained at maximal dorsiflexion (140°), chord stiffness calculated as the slope between the first and the last point of the passive torque-angle curve, and final stiffness calculated as the slope of the passive torque-angle curve from 130° to 140°. All torque and stiffness values were analyzed using a Wilcoxon signed ranks test (SPSS). Statistical significance was set at $P < 0.05$.

Muscle tissue analysis. Rabbits were tranquilized using 0.3 ml Acepro (25 mg/ml) and then killed by a 1.5-ml injection of Euthanyl into the lateral ear vein. Experimental rabbits were killed at the end of the stretch training, 2 days after the final torque-angle curve measurement. Soleus, gastrocnemius, and plantaris were removed and weighed. Muscles were then longitudinally cut into three sections, one central section that was used for quantifying serial sarcomere numbers and two lateral sections used for determining titin isoforms and collagen content. The two lateral sections were frozen in isopentane that was precooled in liquid nitrogen. The central section was pinned on a cork surface and fixed in 10% formalin for a minimum of 4 wk. The lateral gastrocnemius was not used for serial sarcomere number determination because of its multipennate nature and associated difficulty of identifying repeatable fascicle harvest sites. Because of technical problems (for example, some sample defrosting before analysis), not all samples could be processed and included for final analysis. Left and right muscles were compared using a Wilcoxon signed rank test (SPSS). Statistical significance was set at $P < 0.05$.

**MASS.** Wet mass of the left and right soleus, gastrocnemius, and plantaris was determined by weighing immediately after harvesting. Plantaris and gastrocnemius were weighed with their tendons attached. Left and right muscles were compared for all rabbits ($n = 7$ experimental; $n = 8$ control).

**AVERAGE NUMBER OF SERIAL SARCOMERES.** After fixation, the muscle strips were placed in 30% nitric acid until the connective tissue had been softened enough to allow for teasing of small fascicles from the muscle. Muscle samples were then rinsed in PBS and put into glycerol for at least 5 days. Five full-length fascicles were teased out and carefully placed on cover slips. Fascicle lengths were then measured using video analysis and custom written software. Sarcomere lengths were measured at five points along each fascicle.
The increased serial sarcomere numbers compared with the contralateral control gastrocnemius and plantaris muscles (right leg). No significant differences were observed after the stretching program. Nevertheless, passive peak torque, chord stiffness, and final stiffness were not significantly different from the corresponding values obtained before the stretching program (Table 1). No such difference was observed in the soleus muscles of the untrained control rabbits (P = 0.14, n = 3). Medial gastrocnemius and plantaris muscles showed no significant difference in serial sarcomere numbers between left and right leg muscles, neither for the experimental nor the control rabbits (Table 3).

Because significant increases in mass and sarcomere number were observed in the soleus muscle, further tissue analyses were performed for this muscle only.

**Titin Isoforms**

Soleus titin isoforms were identical for the left (experimental, stretched) and right (control, nonstretched) legs. No difference was observed between the titin isoforms from the experimental and untrained control rabbits (Fig. 2).

**Collagen Content**

No significant differences were observed between the amount of collagen in the left (experimental, stretched) and the right (contralateral control, nonstretched) soleus muscles from the experimental and the control group animals (Table 4).

### RESULTS

**Passive Plantarflexor Torque**

After the 4-wk stretching program, passive peak torque, chord stiffness, and final stiffness were not significantly different from the corresponding values obtained before the stretching program (Table 1). Nevertheless, passive peak torque and stiffness were reduced after training in five of the seven experimental animals (Fig. 1).

**Muscle Mass**

The stretched soleus muscles (left leg) were significantly (P < 0.05) heavier than the contralateral nonstretched control muscles (right leg). No significant differences were observed between the stretched and the corresponding nonstretched contralateral control gastrocnemius and plantaris muscles (Table 2). In the control group rabbits, there were no significant differences between left and right leg muscles.

**Average Serial Sarcomere Number**

The stretched (left) soleus muscle showed a significant increase in serial sarcomere numbers compared with the contralateral nonstretched control (right) soleus (P = 0.03, n = 7). No such difference was observed in the soleus muscles of the untrained control rabbits (P = 0.14, n = 3). Medial gastrocnemius and plantaris muscles showed no significant difference in serial sarcomere numbers between left and right leg muscles, neither for the experimental nor the control rabbits (Table 3).

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### DISCUSSION

A 4-wk static stretch training program applied to the rabbit plantarflexor muscles caused a significant increase in soleus muscle mass and sarcomere number compared with the contralateral control gastrocnemius and plantaris muscles (Table 2). In the control group rabbits, there were no significant differences between left and right leg muscles.

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mass and soleus serial sarcomere number. No training adaptations were observed in plantaris or gastrocnemius. Neither collagen content nor titin isoform was modified in the soleus muscle after the stretch training program. The passive peak torque and the chord and final stiffness, measured at the same ROM before and after the stretch program, were not significantly different even though all passive force measures were decreased in five of the seven experimental rabbits after the stretch program.

Serial Sarcomere Number Adaptation

An increase in serial sarcomere numbers has already been reported for studies using continuous stretch protocols like immobilization or incremental static stretch. After immobilization in a lengthened position for 3 wk, a significant increase (15%) in serial sarcomeres has been observed in mouse soleus muscles (38), and, after 4- to 7-day immobilization period, an 8% increase was observed in rabbit tibialis anterior muscles (35). After 3 wk of immobilization in a shortened position, significant decreases in serial sarcomere numbers have been observed in mouse (decrease of 20%; see Ref. 38) and in rat (decrease of 23%; see Ref. 5) soleus muscles. Significant serial sarcomere number increases were also observed as a result of static stretching that was applied incrementally to rabbit latissimus dorsi muscles (increase of 25% for a final 20% muscle stretch; see Ref. 6), and in rabbit tibialis anterior muscles (increase of 15–20%) after surgical tibial distraction at a low rate (34). These results suggest that serial sarcomere numbers are regulated in a manner to maintain the normal, existing sarcomere lengths for the newly (stretched or shortened) imposed joint configurations and associated muscle lengths.

An intermittent stretching protocol may increase serial sarcomere numbers in skeletal muscles. Immobilization of rat soleus muscles in a lengthened position for 40 min every 3 days for 3 wk (7 stretch periods for a total of 280 min of stretching) significantly increased (4%) serial sarcomere number (5). In our experiment, a significant 9% increase in serial sarcomere number was found after an intermittent stretching protocol imposing 25 bouts of 30 s of static stretching, three times a week for 4 wk (300 stretching periods for a total of 150 min of stretching) in rabbit soleus but not plantaris and medial gastrocnemius. This result suggests that, with an intermittent stretching protocol, stretching frequency may be as strong a regulator of serial sarcomere number as stretch duration.

Muscle sensitivity to stretch. The rabbit soleus consists primarily of slow-twitch, type I fibers (3), whereas the medial gastrocnemius contains only 15% type I fibers (29) and the plantaris just about 1% (2). A higher sensitivity of slow-compared with fast-twitch fibers to immobilization or stretch has been observed before (15, 20). Also, an increase in cross-sectional area has been found in slow but not fast muscle fibers of the rabbit latissimus dorsi after incremental static stretching (6). Differences in neural activation levels, protein turnover rates, and Ankrd2 contents have been suggested as possible explanations for these findings. For example, Cotter et al. (3) proposed that the higher level of neural activation usually received by slow motor units and antigravity muscles, and the greater reduction of this neural activation during immobilization, could be responsible for the greater muscle atrophy.

Table 3. Average number of serial sarcomeres per fascicle

<table>
<thead>
<tr>
<th></th>
<th>Soleus, no. of serial sarcomeres</th>
<th>Plantaris, no. of serial sarcomeres</th>
<th>Gastrocnemius, no. of serial sarcomeres</th>
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</thead>
<tbody>
<tr>
<td>Experimental rabbits (n = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (right) leg</td>
<td>6,376 ± 781</td>
<td>5,309 ± 501</td>
<td>6,730 ± 761</td>
</tr>
<tr>
<td>Stretched (left) leg</td>
<td>6,976 ± 347*</td>
<td>5,325 ± 476</td>
<td>6,640 ± 719</td>
</tr>
<tr>
<td>Control rabbits (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right leg</td>
<td>6,803 ± 620</td>
<td>5,703 ± 398</td>
<td>6,977 ± 419</td>
</tr>
<tr>
<td>Left leg</td>
<td>7,022 ± 880</td>
<td>5,565 ± 609</td>
<td>7,037 ± 241</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of rabbits. *Value of the stretched (left) leg significantly different from that of the control (right) leg.

Table 4. Collagen concentration in soleus muscle

<table>
<thead>
<tr>
<th></th>
<th>Collagen Concentration, mg/g muscle dry mass</th>
<th>Control Rabbits (n = 5)</th>
<th>Experimental Rabbits (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left leg</td>
<td>67 ± 23</td>
<td>51 ± 18</td>
<td></td>
</tr>
<tr>
<td>Right leg</td>
<td>53 ± 15</td>
<td>56 ± 19</td>
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Values are means ± SD; n, no. of rabbits.
observed in these muscles after immobilization. According to others (18), the increased adaptive abilities of slow-twitch fibers could be explained by a higher protein turnover rate in slow-twitch compared with fast-twitch fibers. Another explanation for this higher sensitivity to stretch of the slow-twitch muscles may be related to the higher Ankrd2 content in slow compared with fast fibers. Ankrd2, the ankyrin repeat domain protein, is implicated in muscle signal transduction. McKoy et al. (27) showed that the slow postural mouse soleus muscle contains significantly more Ankrd2 protein than the fast tibialis anterior and extensor digitorum longus muscles.

Therefore, a higher sensitivity to stretch in the slow-twitch soleus than the fast-twitch plantaris and medial gastrocnemius could explain why the serial sarcomere number adaptations occurred in the soleus only.

*Ankle dorsiflexion and muscle/fiber strain.* Besides the potential difference in stretch sensitivity, it is possible that dorsiflexion of the ankle produced different strains in the three plantarflexor muscles. The plantaris and medial gastrocnemius are two-joint muscles while soleus is a one-joint muscle crossing just the ankle (8, 15). In our study, the knee was maintained at 140° during ankle joint stretching. This is a highly extended knee angle for the rabbit, likely inducing tension in plantaris and medial gastrocnemius. Consequently, we consider that the best explanation for our differential results probably does not lie in the single vs. multijoint muscle difference.

Another explanation for the differential adaptations to stretch may be found in the specific muscle architecture of the individual triceps surae muscles. Muscle fiber length and fiber length-to-muscle length ratio are important architectural features for muscle excursion (10). The longer a muscle fiber, the lower the strain applied to this fiber by a defined stretch. Changes in the angle of pennation, which aligns with the longitudinal axis of the muscle upon stretch. As a result, a greater strain may accommodate stretch to a greater degree by changes in fascicle length than plantaris and medial gastrocnemius, which may accommodate stretch to a greater degree by changes in the angle of pennation, which aligns with the longitudinal axis of the muscle upon stretch. As a result, a dorsiflexion stretch at the ankle may produce greater fascicle strains in the soleus compared with the plantaris and medial gastrocnemius.

In conclusion, at least two specific features may explain the observation that only soleus increased its mass and serial sarcomere number following an intermittent passive static stretch training program. First, sensitivity to stretch might be higher in the predominantly slow-twitch soleus muscle than in the predominantly fast-twitch plantaris and medial gastrocnemius muscles. Second, the strain imposed to muscles fibers by ankle dorsiflexion may have been greater in the less pennate soleus than in the more pennate plantaris and medial gastrocnemius. Soleus muscle adaptation was further investigated through collagen and titin analyses.

**Collagen and Titin Adaptation**

We compared titin isoforms between the left (stretched) and right (control) soleus muscles of experimental rabbits and the nonstretched soleus muscles of a control rabbit. No difference was observed as a result of the 4-wk intermittent stretching program. Thus, the reduction in passive stiffness and torque observed in five of the seven experimental rabbits after the stretching program does not appear to be related to a change in titin isoform. Titin is known to be a major contributor to sarcomere passive resistance (19, 24), and it has been suggested that the higher passive stiffness of slow-twitch muscle fibers compared with fast ones could reflect differences in titin isoforms in slow- and fast-twitch fibers (11, 29). Nevertheless, according to Prado et al. (29), the relative contribution of titin to total muscle passive stiffness is low (24%) for the rabbit soleus. Total rabbit soleus passive stiffness is thought to depend primarily on collagen content and orientation.

The amount of collagen in the left (stretched) and right (control) soleus muscles after the stretch training program was the same. In previous studies, increases in collagen content were observed in mouse, rat, and rabbit soleus, tibialis anterior, or latissimus dorsi muscles as a result of different experimental protocols, including immobilization in a shortened position (36, 37, 39), intense or fast stretching in high-rate tibial surgical distractions (33, 34), fast stretching of stimulated muscles (31), and incremental static stretching (6). When surgical tibial distraction was carried out at a slow rate, or when muscles were immobilized in lengthened positions, no change in muscle collagen content was observed (34, 39). From these previous studies and our current results, it appears that moderate stretches applied to muscles do not induce changes in collagen content. Collagen organization, which we did not measure, may have been modified. Indeed, Williams and Goldspink (39) observed that collagen fibers were oriented at a more acute angle 2 wk following immobilization in a shortened position. Coutinho et al. (4) measured the birefringence of intramuscular connective tissues and observed different collagen macromolecular organization after immobilization in a shortened position and after daily bouts of passive stretching applied to previously immobilized muscles. Hence, not only the amount of collagen might be affected by muscle immobilization or stretching, but also its organization, which we did not measure. Because intramuscular collagen is considered to be the major contributor to the extracellular passive resistance to stretch (11, 29), quantitative or qualitative changes affecting intramuscular collagen may cause changes to the passive force-angle relationship (11, 17, 33, 34, 39).

**Passive Torque**

For the same range of motion, the passive ankle torque-dorsiflexion-angle relationship before and after the stretch intervention protocol was the same. This is not necessarily surprising because the passive torque is a global measure resulting from the three plantarflexor muscle-tendon units, among which only the smallest, the soleus muscle, was mod-
ified by the stretching program. Moreover, neither changes in titin isoform nor a decrease in intramuscular collagen content was observed after the stretching program.

Yet, peak passive torque, chord stiffness, and final stiffness were decreased in five of the seven experimental rabbits. Qualitative changes in intramuscular collagen organization, which we did not measure, or the increased serial sarcomere number observed in the soleus muscle may account for these changes in biomechanical properties. An increase in the number of serially arranged sarcomeres has been associated with a shift of the passive force-length curve to increased length in the rabbit tibialis anterior after tibial surgical distraction at low or intermediate rates (34). A corresponding shift in the force-length relationship, however, was not observed in soleus muscles of adult mice after immobilization in a lengthened position that resulted in increased numbers of serial sarcomeres (38).

Differences in adaptations to the stretching program may explain why the passive torque-angle curves were modified differently across the seven experimental animals. Abellaneda et al. (1) showed that, in humans, interindividual differences in the passive torque developed by plantarflexors during ankle dorsiflexion are related to different relative tendon and muscle elongation. We cannot exclude that similar interindividual differences also exist in rabbits and that different adaptations in muscles and tendons may explain the difference we observed between the five rabbits whose passive stiffness and peak torque were decreased after the stretch training, in contrast to the two remaining rabbits. Finally, because most of the plantarflexors are biarticular muscles, the degree of flexion of the knee greatly affects the passive torque developed by these muscles. Although we standardized the rabbit hind limb position during the passive torque measurement, there is always the uncertainty associated with perfectly identical positioning of the hind limbs before and after the stretch intervention program.

In conclusion, a 4-wk intermittent stretching program resembling those used in human training programs was applied to rabbit ankle plantarflexor muscles. Repetitive short bouts of passive static stretching imposed three times a week induced hypertrophy and an increased serial sarcomere number in the soleus. No such adaptations were observed in plantaris and medial gastrocnemius muscles. This difference in adaptation between the rabbit plantarflexors is thought to be the result of their different fiber types and pennation angles. Neither titin isoform nor collagen amount was modified in the stretched compared with the control soleus muscle. Passive torque during ankle dorsiflexion was not modified after the stretch training program on average, but was decreased in five of the seven experimental rabbits.

ACKNOWLEDGMENTS

We thank Dr. Tim Leonard for excellent technical assistance in the stretching protocol.

GRANTS

The financial support of Natural Sciences and Engineering Research Council, Canadian Institutes of Health Research, The Killam Foundation, and the Canada Research Chair Program for Molecular and Cellular Biomechanics is greatly acknowledged.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: D.D.J., V.J., and W.H. conception and design of research; D.D.J. performed experiments; D.D.J. and V.J. analyzed data; D.D.J., V.J., and W.H. interpreted results of experiments; D.D.J. and V.J. prepared figures; D.D.J. drafted manuscript; D.D.J., V.J., and W.H. edited and revised manuscript; D.D.J., V.J., and W.H. approved final version of manuscript.

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