Age effect on expression of myosin heavy and light chain isoforms in suspended rat soleus muscle

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Saitoh, Ayako, Tadashi Okimoto, Hiroshi Nakano, Masanobu Wada, and Shigeru Katsuta. Age effect on expression of myosin heavy and light chain isoforms in suspended rat soleus muscle. J. Appl. Physiol. 86(5): 1483–1489, 1999.—This study was designed to test the hypothesis that myosin heavy (MHC) and light chain (MLC) plasticity resulting from hindlimb suspension (HS) is an age-dependent process. By using an electrophoretic technique, the distribution of MHC and MLC isoforms was quantitatively evaluated in the soleus muscles from 3- or 12-wk-old rats after 1–3 wk of HS treatment was maintained. In normal 12- and 15-wk-old rats, the soleus muscles contained a predominance of MHC I (94%) with small amounts of MHCIIa, but not MHCIIId or MHCIIib. The suspended muscles of adult rats were characterized by the appearance of MHCIIId and MHCIIib, the latter reaching ~6% after 3 wk of HS treatment. In contrast to changes in MHC, HS did not induce a transition in the MLC pattern in the soleus muscles from adult rats. Compared with adult rats, in juveniles HS had a much more pronounced effect on the shift toward faster MHC and MLC isoform expression. The soleus muscles of 6-wk-old rats after 3 wk of HS were composed of 37.0% MHCI, 19.1% MHCIIa, 23.7% MHCIIb, and 20.2% MHCIIc. Changes in MLC isoforms consisted of an increase in MLC1f and MLC2f concomitant with a decrease in MLC2s. These results indicate the existence of a differential effect of HS on MHC and MLC transitions that appears to be age dependent. They also suggest that the suspended soleus muscles from young rats may acquire the intrinsic contractile properties that are intermediate between those in the normal soleus and typical fast-twitch skeletal muscles.

Development; hindlimb suspension; transition

Skeletal muscle fibers can be envisaged as dynamic structures because they are capable of changing certain properties in response to altered functional demands. Generally, increased contractile activities, e.g., chronic stimulation, the removal of a synergist muscle, or heavy exercise training, are responsible for muscular hypertrophy, an increase in maximal tension, and/or a reduction in contraction speed (19, 22, 27). In contrast, hindlimb suspension (HS), introduced by Morey (18) as a model for weightlessness during spaceflight, leads to muscle atrophy and causes a shift toward faster contractile characteristics, especially in the muscles with postural functions such as the soleus muscle (18).

Myosin, the major myofibrillar protein, consists of two heavy (MHC) and four light (MLC) chains. Both subunits exist as multiple isoforms. The skeletal muscles of adult rodents contain four major MHC isoforms, i.e., one "slow," MHC-I, and three "fast," MHCIIa, MHCIIib, and MHCIIc. They also contain five major MLC isoforms, i.e., two slow, MLC1s and MLC2s, and three fast, MLC1f, MLC2f, and MLC3f. Muscle fiber types that are categorized on the basis of histochemical staining for myofibrillar ATPase (mATPase) differ in their MHC composition. Fibers comprising MHC-I, MHCIIa, MHCIIb, and MHCIIc are referred to as type I, IIA, IID/X, and IIB, respectively. The importance of both MHC and MLC isoforms with regard to contractile properties has been demonstrated (25). Although, on average, the maximum velocity of shortening (Vmax) increases in the order type I << type IIA < type IID/X < type IIB, a large scattering of Vmax exists within each fiber type (10). This scattering is thought to relate to the significant role of MLC isoforms in the contractile properties of muscle fiber (5, 13).

In accordance with previous observations on the relationship between the contractile properties of muscle fibers and myosin within them, shifts toward faster contractile characteristics brought about in the suspended soleus muscle are accompanied by an increase in the amount of fast-type MHC isoforms (15). On the other hand, the distribution of MLC isoforms seems to be unaltered, or less affected, by HS compared with that of MHC. McDonald et al. (14), for instance, investigated single fibers of adult rat soleus muscles using one-dimensional electrophoresis and found no change in MLC composition after 3 wk of HS.

In the rat soleus muscle, differentiation of muscle fibers is barely evident at birth but proceeds rapidly in the following 2 mo. It has been shown that the changes in the contractile properties of the soleus muscle after HS are more pronounced in young rats than in fully grown rats (1), suggesting that the introduction of HS at a time when the animals were rapidly growing might have a greater effect on myosin expression. The present study was undertaken in an attempt to elucidate a possible age and/or developmental stage dependence of HS with regard to MHC and MLC isoforms. Experi-

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ments conducted in the soleus muscles in 6- and 15-wk-old rats subjected to HS for 3 wk have revealed that, compared with adult animals, the muscles of juveniles exposed to this intervention not only contain smaller amounts of MHC, but also express large amounts of MHCd and MHCt, which are absent in normal soleus muscles. In addition, HS imposed on juveniles produces a significant elevation in the relative distribution of fast MLC isoforms.

MATERIALS AND METHODS

Animals. Forty-four 3- (young; Y)- and forty-six 12-wk-old (adult; A) female Wistar rats (number at the beginning of the experiments) were used for this study. They were assigned to two groups: hindlimb-suspended (HS-Y and HS-A) or non–hindlimb-suspended (non-HS-Y and non-HS-A) rats. All animals were housed in the same environment (12:12-h light-dark daily cycle, food and water provided ad libitum). The experiments received authorization from the Experimental Animal Care Committee of the University of Tsukuba.

Suspension procedure. The animals designated for HS were prepared by a noninvasive tail traction procedure, in which approximately one half of the tail remains uncovered, thereby allowing normal thermoregulatory processes to occur. The portion of exposed tail maintained normal color, indicating that blood flow was not compromised. A wire was fastened to the tail with adhesive tape and connected to another wire via a fish swivel. The other end of the wire was attached to the top of the cage, and the suspension height was adjusted so that only the front legs were able to make contact with the floor. In this apparatus, the rats could rotate a full 360°, allowing them access to food and water without contact of the hindlimb with the cage floor or walls. The animals were checked daily for signs of tail lesions, discoloration, or undue discomfort. The hindlimbs of the HS animals were elevated for 1 (HS-Y4, n = 9; HS-A13, n = 9), 2 (HS-Y5, n = 9; HS-A14, n = 10), or 3 (HS-Y6, n = 6; HS-A15, n = 9) wk.

Tissue preparation. The rats in the non-HS group were separated into two groups. The animals from one group were killed at the age of 3 (non-HS-Y3; n = 10) and 12 (non-HS-A3; n = 9) wk, i.e., at the beginning of HS. The remaining animals were killed at the age of 6 (non-HS-Y6; n = 10) and 15 (non-HS-A5; n = 9) wk, i.e., at the termination of experimental treatment. The animals were weighed and given an anesthetic overdose of diethyl ether. The soleus muscles were removed, trimmed clean of visible fat and connective tissues, and stored at −80°C until used for analyses. Small muscle pieces were homogenized in an all-glass homogenizer in a 40-fold volume of a solution consisting of 5 M urea, 2 M thiourea, 10 mM sodium pyrophosphate, and 0.1% (vol/vol) 2-mercaptoethanol; 30–50 µl of this homogenate were used for MLC analysis by using two-dimensional electrophoresis.

MHC electrophoresis. Muscle homogenates were diluted 75-fold with a solution composed of 62.5 mM Tris·HCl (pH 6.8), 2% (mass/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.02% (mass/vol) bromophenol blue. Then, 20 µl were applied to the gel for electrophoretic separation of MHC isoforms. Electrophoresis in the presence of SDS was carried out by using a 0.75-mm-thick 7.5% separating gel and a 3.5% stacking gel. Electrophoresis lasted 36 h at a constant voltage of 200 V in a cold room (4°C). After electrophoresis, gels were silver-stained by using a Wako Silver Stain Kit (Wako). The percent distribution of the various MHC isoforms was estimated by densitometric evaluation (CS-9000, Shimadzu).

Two-dimensional electrophoresis. Electrophoresis in the first dimension was performed on a rectangular gel (1 × 2 × 60 mm) according to OF arrell (20) by using 1.6 (pH 5.0–8.0) and 0.5% (pH 3.5–10.0) ampholines (Pharmacia) in 4.2% (mass/vol) polyacrylamide (Atto AE-6050A apparatus, Atto). Electrophoresis was first run for 20 min at 100 V and then for another 90 min at 300 V. Separation in the second dimension was carried out for 90 min at 200 V in a minigel chamber on a 1-mm-thick 15% separating gel and a 3% stacking gel (33). The gels were stained with 0.25% (mass/vol) Coomassie brilliant blue R250 in 45% (vol/vol) methanol and 10% (vol/vol) acetic acid. After destaining, the spots corresponding to MLC were excised, placed into centrifuge tubes containing 1.5 ml 25% (vol/vol) pyridine, and incubated overnight. This allowed elution of the bound dye to determine relative protein amounts (8). The eluted dye was measured spectrophotometrically at 605 nm.

Statistical analyses. A three-way variance analysis was performed to evaluate the influence of age, HS, and duration of HS. If an overall significant F-value was obtained, a Scheffé’s post hoc analysis was used to isolate the significantly different means. All comparisons were performed at a 95% confidence level. All data shown are presented as means ± SD.

RESULTS

Body weight. As is commonly reported in studies on rat growth (7), the control animals in each age group in the present study exhibited markedly different growth rates, with young rats gaining 3.5 g of body weight on a daily basis, whereas adults grew at 0.2 g/day. With HS, a significant decrease in body weight gain was observed in juveniles (Table 1). After 3 wk of HS, the body weight in the HS-Y group was 14% lower than that found in the age-matched control group (non-HS-Y). In adult animals, a significant reduction in body weight was brought about at an early stage of HS. Compared with that in non-HS-A, animals, it decreased by 13% during the first week of HS. Therefore, body weight of the rats in HS-A groups was not recovered (Table 1).

Table 1. Muscle wet weight of soleus muscles from and body weight of young and adult, normal, and tail-suspended rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BW, g</th>
<th>MW, mg</th>
<th>M/W/BW × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-HS-Y3</td>
<td>10</td>
<td>53.3±7.2</td>
<td>20.3±3.7</td>
<td>37.8±3.0</td>
</tr>
<tr>
<td>HS-Y4</td>
<td>9</td>
<td>55.9±9.7</td>
<td>17.5±4.8</td>
<td>31.8±7.7</td>
</tr>
<tr>
<td>HS-Y5</td>
<td>9</td>
<td>85.3±12.8†</td>
<td>14.4±2.6</td>
<td>17.2±4.3‡</td>
</tr>
<tr>
<td>HS-Y6</td>
<td>10</td>
<td>108.7±15.3*‡</td>
<td>16.1±3.6‡</td>
<td>15.0±1.4†‡</td>
</tr>
<tr>
<td>Non-HS-Y6</td>
<td>9</td>
<td>125.9±12.8*</td>
<td>53.5±8.4*‡</td>
<td>41.3±4.6</td>
</tr>
<tr>
<td>Non-HS-A12</td>
<td>9</td>
<td>266.9±20.8</td>
<td>106.1±9.5</td>
<td>39.9±5.4</td>
</tr>
<tr>
<td>HS-A13</td>
<td>9</td>
<td>231.1±15.3*</td>
<td>85.5±9.4*‡</td>
<td>36.4±5.4</td>
</tr>
<tr>
<td>HS-A14</td>
<td>10</td>
<td>237.6±16.8</td>
<td>77.0±12.0*</td>
<td>28.2±4.1†‡</td>
</tr>
<tr>
<td>Non-HS-A15</td>
<td>9</td>
<td>236.8±24.0†</td>
<td>57.0±9.4*‡</td>
<td>24.8±5.0*‡</td>
</tr>
<tr>
<td>HS-A15</td>
<td>9</td>
<td>271.1±24.4</td>
<td>108.4±12.8</td>
<td>40.1±3.6</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, No. of rats; BW, body weight; MW, muscle wet weight; HS, hindlimb suspension; Y, young; A, adult; substrates refer to weeks of HS for suspended rats or week of euthanasia for nonsuspended (non-HS) rats. Comparisons were not performed between non-HS-Y and HS-Y and HS-Y groups and between non-HS-A and HS-A13 and HS-A14 groups. *Significantly different from non-HS-Y or non-HS-A (P < 0.05). †Significantly different from non-HS-Y6 or non-HS-A15 (P < 0.05). ‡Significantly different from HS-Y or HS-A13 (P < 0.05). §Significantly different from HS-Y5 or HS-A14 (P < 0.05).
Muscle weight. Similar to body weight, normal growth caused a remarkable gain of soleus wet weight in juveniles. It was augmented 2.6-fold for 3 wk (Table 1). HS of young animals led to remarkable retardation of normal growth during the same period of development. The muscle weight tended to decrease during 3 wk of HS, although its reduction from 20.3 ± 3.7 to 16.1 ± 3.6 mg was nonsignificant. Increased body weight and unaltered muscle weight resulted in a persistent decline in the ratio of muscle weight to body weight. A conspicuous decrease to 36% in the values of the age-matched control group (non-HS-Y₆) was found after 3 wk of HS.

In adult rats, there were significant decreases in muscle weight during HS. Soleus muscle weight in the HS-A₁₃, HS-A₁₄, and HS-A₁₅ groups was 80.6, 72.6, and 53.7% of that in non-HS-A₁₂ animals, respectively (Table 1). The muscles in HS-A₁₅ rats weighed 47.4% less than those in their age-matched control group (non-HS-A₁₅). The ratio of muscle weight to body weight was progressively reduced during HS. As a consequence, the ratio after 3 wk of HS amounted to only 61.8% of the age-matched control value.

MHC. Independent studies by Schiaffino et al. (24) and Bär and Pette (3) in rat skeletal muscle were able to detect new MHC isoforms, designated as MHC₁id and MHC₁if, respectively. Electrophoretic analyses by Termin et al. (30) provided evidence that MHC₁id may be identical to MHC₁i. In the present study, the four MHC isoforms detected in rat skeletal muscles were referred to as MHC₁ia, MHC₁id, MHC₁ib, and MHC₁i, in the order of increasing electrophoretic mobilities according to the nomenclature system of Termin et al. (Fig. 1). As illustrated in Fig. 2, the soleus muscles in 3-wk-old animals expressed 62.2 ± 4.3% MHC₁, 37.8 ± 4.3% MHC₁ia, but not MHC₁id or MHC₁ib. A gradual increase in MHC₁i, with a concomitant decrease in MHC₁id, occurred in the developing soleus between the third and sixth postnatal week. However, the increase in MHC₁ from 62.2 ± 4.3 to 68.3 ± 5.2% was not significantly different from nonsuspended muscles of 6-wk-old rats (P = 0.075). The muscles from the rats subjected to 1 wk of HS exhibited a reduction in MHC₁ and the appearance of MHC₁id and MHC₁ib. The decline in MHC₁ and the increase in MHC₁id reached a plateau after 2 wk of HS. On the other hand, a persistent increase in MHC₁ib was found during the experimental period. The muscles in HS-Y₆ were composed of 37.0 ± 1.9% MHC₁i, 19.1 ± 5.4% MHC₁ia, 23.7 ± 2.0% MHC₁id, and 20.2 ± 2.6% MHC₁ib.

The effects of HS on the expression of MHC isoforms in the adult rat soleus are shown in Fig. 3. The distribution of MHC was less affected in adult than in juvenile rats. No change in the relative amounts of MHC₁ and MHC₁ia isoforms was found in HS groups. Appreciable amounts of MHC₁ib and MHC₁id were detected during 3 wk of HS. The muscles in HS-A₁₅ consisted of 89.7 ± 6.1% MHC₁ib, 4.6 ± 4.1% MHC₁ia, 5.6 ± 2.5% MHC₁id, and 0.1 ± 0.2% MHC₁ia. Compared with the age-matched control group (non-HS-A₁₅), only the change in MHC₁id was significant.

MLC. Two-dimensional electrophoresis of extracts from juvenile soleus muscles revealed the expression of five MLC isoforms (Figs. 4 and 5). Similar to MHC, significant changes in MLC isoforms were not found in the developing soleus between the third and sixth postnatal week (Fig. 5). HS induced progressive and marked increases in MLC₁f and MLC₂f, with a concomi-
The relative amounts of MLC1f and MLC2f increased about twofold after 3 wk of HS. The soleus muscles in HS-Y 6 were composed of 38.0 ± 4.2% MLC1s, 26.3 ± 5.6% MLC2s, 15.1 ± 2.2% MLC1f, 18.9 ± 6.1% MLC2f, and 1.7 ± 0.6% MLC3f, whereas non-HS-Y 6 muscles contained 41.4 ± 5.6% MLC1s, 48.4 ± 6.9% MLC2s, 4.1 ± 2.0% MLC1f, 5.0 ± 3.0% MLC2f, and 1.1 ± 1.8% MLC3f. As seen in Fig. 5, the percent distribution of MLC2f, MLC1f, and MLC2f isoforms in HS-Y 6 was significantly different compared with both non-HS-Y 3 and non-HS-Y 6.

Measurements of MLC in non-HS soleus from adult animals gave results similar to those obtained for MHC; no conspicuous change was found for any MLC isoforms between non-HS-A 12 and non-HS-A 15 (Fig. 6). In terms of the effect of HS, despite a significant rise in fast MHC, MLC isoforms remained unaltered during the observation period of the experiment. MLC3f was not detected in any suspended or control muscles (data not shown).

**DISCUSSION**

The primary purpose of the present study was to examine the age dependence of HS-induced muscle phenotype plasticity with regard to MHC and MLC isoform expression. It has been widely accepted that HS enables a transition from slower to faster MHC in rat soleus muscle (32). However, this intervention does not appear to be a powerful stimulus for the upregulation of fast-type MHC isoforms in adult rats, because the distribution of MHC isoforms or fiber types in fully grown animals is reported to be only slightly affected or unaltered. Histochemical analyses by Leterme et al. (12), who explored the effect of HS on the soleus muscles of adult rats, revealed a significant shift in type I fibers from 91.2 to 73.4%. In studies of similar design, Asmussen and Soukup (2), McNulty et al. (16), and Simard et al. (26) found no change in muscle fiber composition. Assuming that there is a general correspondence between mATPase histochemistry and MHC isoforms expressed (30), the present results showing that the relative amount of MHC 1 in adult rats was unaltered by 3 wk of HS are in accordance with the findings from the latter studies (2, 16, 26) (Fig. 3).

It seems that a shortcoming of histochemical discrimination of fiber types lies in its low sensitivity for detecting MHC isoforms. Some fibers composed of more than one MHC isoform exist, in addition to the majority of fibers expressing only one isoform. As previously shown, an increase in the number of such hybrid fibers is brought about in the muscles in which fiber-type transformation is proceeding (29). Using immunohistochemical and electrophoretic techniques, Talmadge et al. (28) found that the whole muscle of the rat soleus exposed to 2 wk of HS contained slight but significant MHC1Id content (5%) and that MHC1Id always coexisted with another MHC isoform in single fibers. Because of the above-mentioned low sensitivity of the mATPase-based
histochemical technique, such hybrid fibers must have been identified as type IIA or type I. The expression of MHCIIId in the suspended soleus muscle may, therefore, have escaped detection in previous studies using histochemical analyses. The present study found that the suspended soleus muscles in adult animals exhibited the expression of MHCIIb as well as MHCIId (Figs. 1 and 3), extending the findings of Talmadge et al. to another fast MHC isoform.

A major finding in the present study is that, in addition to decreased MHC I and MHCIIa, the suspended soleus muscles in juveniles are composed of large amounts of MHCIId (23.7%) and MHCIIb (20.2%) isoforms, which are absent in the normal soleus. MHC transitions in rodents are shown to occur in a sequential manner, following the order of MHCIIb †MHCIId †MHCIId †MHCI. According to this sequence of MHC transition, the changes illustrated in Fig. 2 may result from a progression in MHC isoform expression from MHCI †MHCIId †MHCIId †MHCI. However, the possibility cannot be ruled out that some fibers have the capacity to adapt in a manner different from the commonly accepted sequence because a small but appreciable number of muscle fibers in the suspended soleus muscle consist of both MHC I and MHCIIid isoforms.

The fact that slow-twitch soleus muscles subjected to HS express large amounts of MHCIIid and MHCIIb is of interest with regard to its functional significance. A recent single-fiber study has indicated that type II fibers consisting of fast MHC isoforms exhibit up to threefold higher $V_{\text{max}}$ than do type I fibers containing MHC I (6). As can be seen in Fig. 2, total fast MHC (IIa, IIId, and IIb) from 3-wk-suspended muscles in juveniles amounted to 63.0%. On the basis of the findings of positive correlations between $V_{\text{max}}$ and mATPase activity (4) and between mATPase activity and the relative distribution of fast-twitch fibers comprised in muscles (31), we calculated the $V_{\text{max}}$ of the soleus muscles in young animals used in this study. The results derived from this calculation indicate that 3-wk-suspended soleus muscles in juveniles are characterized by a 1.62-fold higher $V_{\text{max}}$ compared with those in the age-matched control animals.

In the present study, we adopted a two-dimensional electrophoretic technique to evaluate the change in MLC pattern because the muscle homogenate contains some protein, the molecular weight of which is similar to MLC. To our knowledge, this is the first study in which the increases in MLC1f and MLC2f in juvenile soleus muscle subjected to HS have been quantitatively measured by this technique (Fig. 5). Recently, it was demonstrated that $V_{\text{max}}$ in mammalian skeletal muscles correlates with not only MHC but also MLC isoforms (6, 11, 13). Larsson and Moss (11), for example, observed a lower $V_{\text{max}}$ in type IIA fibers expressing slow regulatory

![Fig. 5. Evaluation of electrophoretically determined changes in MLC isoforms of hindlimb-suspended (●) and nonsuspended (□) soleus muscles from young rats. Percent distribution of MLC isoforms is expressed as means ± SD. Comparisons were not performed between nonsuspended muscles of 6-wk-old rats and suspended muscles of 4- and 5-wk-old rats. * †Significantly different from nonsuspended muscles of 3- and 6-wk-old rats, respectively (P < 0.05). ‡Significantly different from suspended muscles of 4-wk-old rats (P < 0.05). §Significantly different from suspended muscles of 5-wk-old rats (P < 0.05).](http://jap.physiology.org/doi/abs/10.1152/japplphysiol.00672.2016)
MLC than in the fibers lacking this MLC. In view of these findings, in addition to the increase in fast MHC, the rise in fast MLC observed in the suspended juvenile soleus muscle also appears to contribute to the shift toward faster contractile characteristics.

It is important to point out that disproportionate increases in fast MHC and MLC occurred in the suspended soleus muscles from young rats. Thus the soleus muscles from HS-Y6 consisted of 63.0% fast MHC (IIa, IId, and Iibi) in total, whereas the same muscles were comprised of 35.7% fast MHC (I, 2f, and 3f). These observations raise the possibility that there exists an appreciable proportion of fibers characterized by isomyosins composed of fast MHC and slow MLC. At the least, MHCIIa, for example, has been shown to be capable of associating with slow as well as fast MLC isoforms (9). Further studies with native electrophoresis are needed to elucidate how many isomyosins exist in single fibers in the suspended soleus muscle.

The mechanisms that could produce differences in responses depending on age are unclear. The differentiation of the innervation pattern in rat soleus muscle is complete at the age of 16 days (17). Therefore, it is unlikely that HS that began from the age of 21 days would affect the process of transition from polyneuronal to single innervation. It has been widely recognized that the activity pattern of motoneurons plays an important role in the expression of myosin (21). Riley et al. (23), studying the impact of HS on soleus electromyographic activity in adult rats, found that such activity shifted from tonic to phasic. The change in the activity pattern of motoneurons innervating the soleus muscle may account, at least in part, for the increase in MHC IIa and MHC Iib observed in adult rats. One hypothesis explaining pronounced changes in the expression of myosin that occurred in juveniles is that motoneurons in young rats may be more responsive to altered functional demand than those in adult animals.

In conclusion, the alterations in the expression of MHC and MLC isoforms in response to HS in adult and juvenile rats have been compared. The soleus muscles in young rats were much more markedly affected than were those of adults. After 3 wk of HS, the muscles in juveniles contained 63.0% fast MHC and 35.7% fast MLC in total, whereas adult muscles were composed of 10.3% fast MHC and 7.4% fast MLC. These results indicate that there is a differential effect of HS on MHC and MLC transitions that appears to be age dependent. They also suggest that the suspended soleus muscles from young rats may acquire the intrinsic contractile properties that are intermediate between the normal soleus and typical fast-twitch skeletal muscles.

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