Slow myosin heavy chain expression in the absence of muscle activity

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1EA300, Université Paris Diderot, Paris; 2Institut National de la Santé et de la Recherche Médicale, U787, Paris; 3Faculté de Médecine, Université Pierre et Marie Curie, Unité Mixte de Recherche S787, Paris; 4Institut de Myologie, Paris; 5Généthon, Évry; and 6Université Paris Descartes, Paris, France

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Agbulut O, Vignaud A, Hourde C, Mouisel E, Fougerousse F, Butler-Browne GS, Ferry A. Slow myosin heavy chain expression in the absence of muscle activity. Am J Physiol Cell Physiol 296: C205–C214, 2009. First published October 22, 2008; doi:10.1152/ajpcell.00408.2008.—Innervation has been generally accepted to be a major factor involved in both triggering and maintaining the expression of slow myosin heavy chain (MHC-1) in skeletal muscle. However, previous findings from our laboratory have suggested that, in the mouse, this is not always the case (30). Based on these results, we hypothesized that neurotomy would not markedly reduced the expression of MHC-1 protein in the mouse soleus muscles. In addition, other cellular, biochemical, and functional parameters were also studied in these denervated soleus muscles to complete our study. Our results show that denervation reduced neither the relative amount of MHC-1 protein, nor the percentage of muscle fibers expressing MHC-1 protein (P > 0.05). The fact that MHC-1 protein did not respond to muscle inactivity was confirmed in three different mouse strains (129/SV, C57BL/6, and CD1). In contrast, all of the other histological, biochemical, and functional muscle parameters were markedly altered by denervation. Cross-sectional area (CSA) of muscle fibers, maximal tetanic isometric force, maximal velocity of shortening, maximal power, and citrate synthase activity were all reduced in denervated muscles compared with innervated muscles (P < 0.05). Contraction and one-half relaxation times of the twitch were also increased by denervation (P < 0.05). Addition of tenotomy to denervation had no further effect on the relative expression of MHC-1 protein (P > 0.05), despite a greater reduction in CSA and citrate synthase activity (P < 0.05). In conclusion, a deficit in neural input leads to marked atrophy and reduction in performance in mouse soleus muscles. However, the maintenance of the relative expression of slow MHC protein is independent of neuromuscular activity in mice.

Skeletal muscle; tenotomy; force; slow myosin heavy chain; power; velocity of shortening; oxidative capacity; atrophy

NEUROMUSCULAR ACTIVITY. INCLUDING both the neural and mechanical aspects, plays an important role in skeletal muscle physiology. It is now well established that increased chronic neuromuscular activity (physical training and electrical stimulation) induces beneficial adaptations in both muscle structure and function (for review, Refs. 23, 46). Moreover, there is a very large body of information showing that reduced neuromuscular activity (i.e., denervation, spinal cord isolation, disuse) causes important impairments in muscle function (for review, Refs. 20, 41). For example, a reduction or the elimination of neural input results in muscle atrophy. Denervated slow muscles produce less maximal force and power, and the kinetics of the twitch is slower. This is accompanied by a reduction in oxidative capacity and a decrease in the relative expression of slow myosin heavy chain (MHC-1) protein (for review, Refs. 41, 53). However, it should be noted that most of this experimental data is derived from experiments on rats.

MHC-1 protein is one of the major contractile proteins and is important for muscle function. A high level of MHC-1 expression in skeletal muscle is associated with a slow contraction speed, increased fatigue resistance, and low-energy expenditure. It has been proposed that the nerve plays an important role in the specification of the slow MHC phenotype via a calcineurin-dependent signaling pathway and the downstream transcription factors, nuclear factor of activated T cells (NFAT) and myocyte-specific enhancer factor 2 (MEF-2) (for review, Refs. 40, 49). This was confirmed by the fact that calcineurin inhibitors decrease the relative expression of MHC-1 protein in the rat soleus muscles (9, 50). Moreover, NFAT and MEF-2 are more active in slow muscle fibers compared with fast muscle fibers that are recruited less and NFAT localization is mainly nuclear in slow muscle fibers. In addition, neuromuscular activity stimulates these processes, whereas denervation has opposite effects (18, 32, 38, 56, 63).

However, this is not always the case, since our laboratory has recently reported that, in mice, the MHC-1 protein expression during soleus muscle regeneration is not always dependent on muscle innervation or calcineurin activity (30). It has also been shown that the slow isoform of sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA-2a), normally expressed in a parallel way with MHC-1, is also known to be independent of muscle innervation (52, 65).

In the present study, we have analyzed the soleus muscles of young adult mice, which were denervated to eliminate neural input. The mouse soleus muscle is a weight-bearing hindlimb muscle that contains a relatively large number of muscle fibers expressing MHC-1 protein (MHC-1+ fibers). We wanted to determine whether denervation would change the relative expression of the MHC-1 protein. Based on our laboratory’s previous findings (30), we hypothesized that neurotomy would not markedly reduced either the relative amount of MHC-1 protein or the percentage of MHC-1+ fibers in these mouse soleus muscles. In addition, functional (isometric and concentric contractile properties) and other cellular (muscle fiber size) and biochemical parameters (enzyme activity of oxidative energy metabolism) were also studied in these soleus muscles to complete our study, since, to our knowledge, both structural and functional changes induced by denervation have only been
examined in fast mouse muscles (47). Such an approach should increase our knowledge of the role of neural input in the maintenance of muscle structure and function, including contractile isoform expression. Importantly, our findings refute the general principle that neural input promotes the relative expression of the slow MHC protein, a key muscle protein. It remains to be determined whether they bring to light only a species difference or suggest a more complex control of this marker of slow phenotype.

MATERIALS AND METHODS

Animals. All procedures were approved by the Department of Veterinary Services of Paris and performed in accordance with national and European legislations on animal experimentation. Unilateral denervation and/or denervation combined with tenotomy was performed in young adult mice (C57BL/6, 129/SV, and CD1 mice, Charles River). The sciatic nerves of the right legs were cut with precaution to prevent reinnervation. Tenotomy was performed by a unilateral section of the Achilles tendon. Denervated or denervated plus tenotomized mice were analyzed either after 1 mo (129/SV and CD1 mice) or at different times (3–77 days, C57BL/6) after denervation. Age-matched intact mice were used as innervated (control) mice. Contralateral muscles (left leg) of denervated mice were not studied to avoid contralateral effects (26). Mice were anesthetized with pento-barbital (60 mg/kg), and soleus muscles were surgically excised. After dissection, animals were killed by rapid neck dislocation.

Isometric and concentric properties. The isometric and concentric contractile properties of soleus muscles were studied in vitro. Measurements were performed as described previously (58). The muscles were dissected free from adjacent connective tissue and soaked in an oxygenated Krebs solution (95% O2 and 5% CO2, pH 7.4) containing the following (in mM): 118 NaCl, 25 NaHCO3, 5 KCl, 1 KH2PO4, 2.5 CaCl2, 1 MgSO4, and 5 glucose, maintained at a temperature of 20°C. Muscles were connected at one end to an electromagnetic puller and at the other end to a force transducer. After equilibration (30 min), electrical stimulation was delivered through electrodes running parallel to the muscle. Isometric contractions were recorded at L0 [which is determined as the length at which maximal isometric tetanic force (P0) is observed]. The L0 was measured with calipers. The following parameters of the twitch were studied: maximum twitch force (Pt), time to peak tension, and one-half relaxation time. Pt was measured (usually frequency of 125 Hz, train of stimulation of 1,500 ms). Specific Pt or P0 was calculated by dividing the force by the estimated cross-sectional area (CSA) of the muscle. Assuming muscles have a cylindrical shape and a density of 1.06 mg/mm3, CSA corresponds to the wet weight of the muscle divided by its fiber length (L0). The L0-to-L0 ratio of 0.70 was used to calculate L0 (39). Maximal velocity of shortening (unloaded velocity of shortening) was investigated using the slack test method, as described by Edman (19). The unloaded velocity of shortening was derived from the slope of the relationship between the extent of shortening and the measured delay of force redevelopment. The force-velocity relationship was determined using the isovelocity method, as described by Maréchal and Beckers-Bleuxx (37). Maximal power (Pmax) was calculated from the force-velocity relationship, and the velocity corresponding to Pmax (optimal velocity) was determined. Specific Pmax (SPmax) was calculated by dividing Pmax by muscle weight. Muscles were weighed and flash frozen either in liquid nitrogen or in isopentane precooled in liquid nitrogen. Samples were stored at −80°C for histological and biochemical analyses.

Histology. Transverse serial sections of soleus muscles (8 μm) were obtained using a cryostat at −25°C. Some of the sections were stained with a hematoxylin and eosin solution, and others were used for immunohistochemistry. For immunohistochemistry, frozen sections were incubated overnight in a blocking solution (BSA 1%, sheep serum 1%, Triton X-100 0.3%). Sections were then incubated with a rabbit antibody directed against laminin (Dako, Z0097) for the determination of muscle fiber CSA, a mouse monoclonal antibody directed against slow MHC (MHC-1, BA-D5, DSMZ), a mouse monoclonal antibody directed against MHC-2a (SC-71, DSMZ), and a mouse monoclonal antibody directed against MHC-2x (6H1, DSHB). Sections were then washed four times in PBS and incubated with a Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch, 111-165-144) or a Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen, A11029). After four washes in PBS, slides were mounted in a mounting solution (mowiol/ hoescht). Images were captured using a digital camera mounted on a bright-field or a fluorescence microscope attached to a computer. Morphometric analyses were made using the software Metavue (Molecular Devices). For muscle-fiber CSA and fiber-type distribution, all of the fibers in each muscle section were analyzed. For CSA, fibers were arranged in several groups according to their size, and each group was expressed as a percentage of the total fiber number.

SDS-PAGE electrophoresis of MHC isoforms. Transverse sections (5–20 μm) of soleus muscles were extracted on ice for 60 min in 50 μl of extracting buffer (pH 6.5) containing 0.3 M NaCl, 0.1 M NaH2PO4, 0.05 M Na2HPO4, 0.01 M Na2P2O7, 1 mM MgCl2, 10 mM EDTA, and 1.4 mM 2-mercaptoethanol. Following centrifugation, the supernatants were diluted 1:1 with glyceral and stored at −20°C. MHCs were separated on 8% polyacrylamide gels, which were made in the Bio-Rad mini-PROTEAN II dual slab gel cell system (0.75-mm thickness), as described previously (3, 30). Electrophoresis was carried out for 31 h at 72 V (constant voltage) at 4°C. Following electrophoresis, gels were silver stained. Gels were then scanned using a video acquisition system. The relative levels of the different MHC isoforms were determined using a densitometric software (Scion Image, NIH).

Western blot analysis for MHC and ubiquitinylation. Western blot analysis was carried out using anti-MHC-1 (clone BA-D5 DMSZ), anti-MHC-2a (clone SC-71, DSMZ), and anti-ubiquitin (Santa Cruz Biotechnology) antibodies, as described previously (30). Antibody reacting bands were visualized following development with peroxi-dase-labeled secondary antibodies (Pierce Biotechnology) and a chemiluminescent detection system (ECL Plus, GE Healthcare). The levels of the different bands were determined using a densitometric software (Scion Image, NIH).

Enzyme activity measurements. Frozen cryostat sections were dropped into 100 μl of ice-cold extraction buffer to 15 mM sodium phosphate buffer, pH 7.2, containing 4 mM magnesium acetate and a proteinase inhibitor, aprotinin, as indicated by the manufacturer (Boehringer Mannheim, Meylan, France). Following 1 h of centrifu-gation in the cold at 1,500 g, pellets were recovered for citrate synthase (CS) enzymatic activity measurements with 100 ml CS buffer [5 mM HEPES, pH 8.7, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl2, Triton X-100 (0.1%)], followed by incubation for 60 min at 4°C to ensure complete enzyme extraction from mitochondria. All assays were performed in 96-well plates, with a final volume of 200 μl. Protein concentrations were determined using a commercial kit (protein assay system kit 600–0005, Bio-Rad). Determination of CS activity was assayed, according to previously described methods (58). Each measurement was carried out at least in duplicate.

Detection of oxidized protein. Oxidized proteins were detected by analyzing protein carbonyls using the Oxyblot Kit (Chemicon), according to the manufacturer’s instructions after separation of the different MHC isoforms, using a high-resolution gel electrophoresis technique (3, 30). In brief, denatured protein samples were derivatized to 2,4-dinitrophenyl hydrazine (2,4-DNPH) by reaction with 2,4-DNPH and separated by electrophoresis. 2,4-Dinitrophenol (DNP)- derivatized proteins were detected by using a polyclonal anti-DNP moity. A protein carbonyl detection procedure without the derivati-zation step was used to evaluate the selectivity of carbonyl measure-ments (negative controls). Secondary anti-rabbit horseradish peroxi-
dase-labeled antibodies were used for detection. Antibody binding was revealed using the enhanced chemiluminescence detection system (ECL Plus, GE Healthcare). The intensity of the different bands was quantified using a densitometric software (Scion Image, NIH).

Relative quantification of gene expression by real-time RT-PCR. Total RNA was extracted from soleus muscle using RNeasy Mini Kit (Qiagen), and the first-strand cDNA was synthesized using random hexamers, according to the manufacturer’s instructions (Roche Diagnostics). PCR analysis was then carried out with SYBR Green PCR technology using Light Cycler 480 system (Roche Diagnostics). The reaction was carried out in a 12-μl reaction volume containing 6 μl of SYBR Green Master Mix, 1,000 nM each for the forward and reverse primer, and 5 μl of diluted cDNA. The appropriate cDNA dilution was determined from the calibration curves established for each primer pair. The thermal profile for SYBR Green real-time RT-PCR was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. Primer sequences used in this study are available on request. GAPDH was used as the reference transcript. Results from three independent RT-PCR experiments are expressed as the ratio between denervated and control samples.

Statistical analysis. Data were analyzed using GraphPad Prism 4.0b software. Innervated (control) and denervated mice were compared using analysis of variance. P < 0.05 was taken as significant.

RESULTS

Denervation of the mouse soleus muscle does not reduce MHC-1 protein expression, despite important structural, functional, and biochemical modifications. Soleus muscles from 129/SV mice were studied 1 mo after denervation. At this time, a marked muscle fiber atrophy was observed, indicating that the neurotomy was effective. The denervated 129/SV mice exhibited a greater percentage of small-diameter fibers and a lower percentage of large-diameter fibers, compared with innervated mice (Fig. 1, P < 0.05). In the denervated muscles, both slow (MHC-1+ fibers) and fast (MHC-1−) muscle fibers had smaller CSA (reduction, respectively, by 47 or 37%, Fig. 1, P < 0.05).

When the slow MHC-1 phenotype was examined, interestingly we found that it was not modified by denervation. Histological analyses showed that the percentage of muscle fibers expressing MHC-1 protein in denervated and innervated mice was almost identical in the 129/SV mice (Fig. 2; P > 0.05). Using the high-resolution gel electrophoresis technique, we found that the relative amount (percentage of total) of MHC-1 and MHC-2a/2x proteins was not modified by denervation in 129/SV mice soleus muscle (Fig. 2). In addition, Western blotting analyses showed that the relative amounts (arbitrary unit/protein) of MHC-1 and MHC-2a proteins were not modified by denervation (Fig. 2). In contrast, our real-time RT-PCR analysis demonstrated that the amount of MHC-1 mRNA was lower in denervated muscles (P < 0.05), whereas MHC-2a, MHC-2x, and MHC-2b mRNA remained unchanged (Fig. 2), indicating that the MHC transcript level did not reflect what was observed at the protein level. In agreement with the lack of effect of denervation on MHC-1 protein, we found that NFATc1 and one of the kinases that dephosphorylates NFAT (glycogen synthetase kinase-3β) were also not modified by denervation (Fig. 3). Moreover, this absence of an effect of denervation on MHC-1 protein was also consistent with the fact that MEF-2 and the class II histone deacetylase that repressed MEF-2 transcriptional activity were not modified in these denervated mouse muscles (Fig. 3).

Fig. 1. Cross-sectional area (CSA) of soleus muscle fibers in 129/SV mice. CSA is shown of soleus muscle fibers expressing myosin heavy chain-1 protein (MHC-1+) or not MHC-1 protein (MHC-1−). *Denervated significantly different from (control) 129/SV mice (P < 0.05). aDenervated + tenotomized significantly different from denervated muscles (P < 0.05). n = 7/group.

In contrast to MHC-1 protein, oxidative capacity, an another important marker of slow muscle fiber phenotype, was significantly lower in the denervated muscles (Fig. 4). CS activity was decreased by denervation (Fig. 4; P < 0.05). The amount of CS transcripts was also reduced (Fig. 4; P < 0.05). It has been previously demonstrated that peroxisome proliferator activated receptors (PPAR) and PPAR-γ coactivator-1α (PGC-1α) are transcriptional regulators that play important roles in oxidative and free fatty acid metabolism (7, 33, 34, 64). Consistent with the reduction in the oxidative capacity of the muscle, we also found that PGC-1α and PPAR-α were decreased by denervation (Fig. 4; P < 0.05).

We also studied the contractile properties of these muscles to determine whether the cellular and molecular changes induced by the denervation would be associated with specific functional consequences in the skeletal muscle. We found that muscle performance was markedly decreased by denervation in the 129/SV mice. The denervated soleus muscles had lower absolute and specific P0 compared with innervated mouse muscles (Fig. 5; P < 0.05). Moreover, the denervated muscles also exhibited significant changes in twitch parameters such as decreased maximal twitch isometric force and increased contraction times and one-half relaxation times (P < 0.05). Fur-
Furthermore, maximal velocity of shortening, velocity of shortening corresponding to $P_{\text{max}}$, and absolute $P_{\text{max}}$ and $sP_{\text{max}}$ values were lower in the denervated muscles (Fig. 5; $P < 0.05$).

The lack of effect of denervation on MHC-1 protein accumulation is independent of mouse strain. To determine whether the lack of effect of denervation on the MHC-1 protein expression in 129/SV mice was due to this particular mouse strain, we have also examined CD1 and C57/BL6 denervated soleus muscles. As for the 129/SV mice, 1 mo after surgical operation, muscle fibers from denervated CD1 mice were atrophied, and CS activity in homogenates was reduced by denervation (Fig. 6; $P < 0.05$). The percentage of MHC-1+ muscle fibers remained unchanged in denervated CD1 mice (Fig. 7). Moreover, using high-resolution gel electrophoresis technique, we show that the relative expression of both MHC-1 and MHC-2a/2x proteins remained unchanged by denervation in 129/SV mice soleus muscle (Fig. 7). Immunostaining with MHC-2x antibody indicates that the MHC-2a/2x band in these gels corresponded almost exclusively to MHC-2a, since very few muscle fibers reacted with MHC-2x antibody (2.8–5.5% in both denervated and innervated muscles).

Next, we examined the denervated soleus muscles in C57BL/6 mice up to 77 days after denervation to determine whether long-term denervation decreases the MHC-1 protein expression. High-resolution gel electrophoresis analysis showed no reduction in the relative amount of MHC-1 protein in C57BL/6 during the 77-day period after denervation (Fig. 8). Analysis of the time course reveals that the relative expression of MHC-1 in fact increased, possibly due to the fact that the postnatal development was not yet finished at the time of denervation (2). To determine whether MHC phenotype remained unchanged in the mouse muscles, the time course of a denervated fast muscle was also studied. In contrast to postnatal development (2), denervation of the extensor digitorum longus markedly reduced the relative expression of MHC-2b (Fig. 8), indicating that the absence of denervation on MHC phenotype was specific to muscles with a predominance of slow-muscle fibers. Therefore, our results indicate that the lack of effect of denervation on the MHC phenotype of soleus muscles is independent of both mouse strain and the duration of the denervation and is confined to muscles expressing MHC-1 protein.

MHC-1 protein expression remains unchanged when denervation is combined with tenotomy. In an additional study, we have further reduced neuromuscular activity to evaluate its impact on MHC-1 protein phenotype. Denervated muscles from 129/SV and CD1 mouse were also tenotomized (to reduce passive tension). Removal of neural input and mechanical load worsens both muscle fiber atrophy (Figs. 1 and 6) and reduction in CS activity (Fig. 6) compared with denervation alone ($P < 0.05$). However, tenotomy combined with denervation had no further effect on the percentage of MHC-1+ fibers (Figs. 2 and 7) compared with denervation alone. Moreover, high-resolution gel electrophoresis shows that the relative amount of MHC-1 protein was not changed by tenotomy combined with denervation in 129/SV and CD1 mice (Figs. 2 and 7). In the case of 129/SV mice, it should be noted that the soleus muscles of denervated/tenotomized mice exhibited decreased relative amounts of MHC-1 protein compared with denervated mice (Fig. 2). Therefore, in contrast to other structural and cellular muscle parameters, the relative expression of MHC-1 protein seems not to be controlled by neural input...
combined with passive stretch in the mouse strains we have studied.

**Denervation causes posttranslational modification of the MHC protein.** Our results demonstrate that, after denervation, the mouse soleus muscles do not present any modification in the accumulation of MHC protein, despite important structural, functional, and biochemical modifications. To identify the underlying molecular factors, which cause the reduced muscle performance, we have examined both MHC carbonylation and MHC ubiquitinylation in the denervated CD1 mouse soleus muscles. First, we evaluated the accumulation of oxidized MHC proteins in the soleus muscles from both denervated and innervated CD1 mouse using the oxyblot kit. Our results showed that, compared with controls, oxidized MHC-1 and MHC-2a/2x proteins were more than four times higher in denervated muscles (Fig. 9). This accumulation of oxidized MHC isoforms is slightly higher in denervated plus tenotomized soleus muscle than denervated muscle. In addition, our Western blot analysis demonstrated that ubiquitinylated MHC isoforms is four times higher in denervated animals. Ubiquitinylation occurred only with respect to MHC2a/2x and not with respect to MHC-1 in denervated muscles and denervated plus tenotomized muscles.

**DISCUSSION**

In the present study, we have analyzed the effect of denervation on the soleus muscles of young adult mice, which contains both slow- and fast-muscle fibers. We originally reported that neural input and passive load were not required for a normal expression of MHC-1 protein in the mouse soleus muscles. In contrast, reduction in neuromuscular activity leads to marked functional and structural impairments in mouse soleus muscles.

Neural input is not essential for the maintenance of MHC-1 protein expression. In the present study, we have clearly confirmed our hypothesis, since we have demonstrated using histological and biochemical analyses (gel electrophoresis and immunoblotting) that the relative expression of MHC-1 protein in soleus muscles is not altered by denervation in 129/SV, CD1, and C57BL/6 mice. We have confirmed that the variations observed at the level of the mRNAs should be interpreted with caution, since they do not reliably predict the changes.
observed at the protein level (47). A reduced MHC-1 ubiquitinylation (degradation) could explain the discrepancy between MHC-1 protein and mRNA levels, which we have observed in the denervated muscles. Our results are in agreement with those of a previous study, suggesting that denervation has very little impact on the accumulation of MHC-1 protein in mouse soleus muscles (60). Moreover, we have found that denervation combined with tenotomy, which, in addition, suppresses passive muscle tension, had no further effects on the percentage of MHC-1 fibers, despite a greater muscle fiber atrophy and a reduction in oxidative capacity. Other models of reduced neuromuscular activity (such as hindlimb suspension and microgravity) induced no change in the slow phenotype in the soleus muscles of C57BL/6 and CD1 mice (24, 51, 59).

It has been well accepted as a general dogma that innervation via the calcineurin signaling pathway, NFAT and MEF-2, plays an important role in the specification of the slow MHC phenotype (for review see Refs. 40, 49). However, our results do not support this dogma in mice, since denervation did not affect either the MHC-1 protein, or the NFATc1 and MEF-2 transcription factors. Our observation was, in fact, in agreement with several previous studies, which have indicated that calcineurin activation or inhibition has little or no impact at all on the slow phenotype in the soleus muscles of mice (27, 30, 42, 43, 54). For instance, in a study that showed a significant effect of calcineurin inhibition, the percentage of slow-muscle fibers in the mouse soleus muscles showed only a very small decrease (65 to 56%) after several weeks of treatment with 50 mg/kg cyclosporin A, a drug known to inhibit calcineurin activity (27). In another study, genetic activation of calcineurin did not increase the percentage of slow-muscle fibers in mouse soleus muscle (54).

We have suggested previously that factors other than the neural input and calcineurin pathway may contribute to the establishment of the slow MHC phenotype in the regenerating soleus muscle in mice (30). One possibility is that the relative expression of MHC-1 protein in the mouse soleus muscles could be determined by some intrinsic properties of the muscle fibers, such as the type of muscle progenitor cells, which would be in agreement with the study of Rosenblatt et al. (48). It should be noted that human myoblasts, when differentiated in culture, are also able to express slow MHC in the absence of innervation (44). In this case, the slow muscle fibers in mouse soleus muscles would be committed during postnatal development (2) in a nerve-independent manner, in contrast to rat soleus muscles (1). The reason why mouse slow MHC protein phenotype is resistant to decreased neuromuscular activity could be that most of the slow soleus fibers come from primary myogenesis. It has been reported that muscle fibers derived from primary myotubes are less affected than those derived from secondary myotubes by mechanical loading (66). A second possibility that has recently emerged is that microRNA encoded by MHC-1 gene (miR-208b) should play an essential role in mouse soleus muscle, by repressing fast MHC protein (57). It should be noted that the invariance of mouse MHC protein phenotype with reduced neuromuscular activity could be that most of the slow soleus fibers come from primary myogenesis. It has been reported that muscle fibers derived from primary myotubes are less affected than those derived from secondary myotubes by mechanical loading (66).
slower MHC phenotype in the denervated EDL muscle (the present study, Ref. 47).

Altered structural and biochemical muscle parameters in denervated mouse soleus muscle. In contrast to the relatively high level of accumulation of the MHC-1 protein, analysis of contractile properties indicated that denervation triggered a clear deterioration in soleus muscle performance. Denervated muscles exhibited a 67% reduction in absolute $P_o$ compared with innervated muscles. This decrease in force can be explained by both a lower muscle mass ($-37\%$) and a decreased specific force ($-50\%$). Both MHC-1 fiber and MHC-1 fibers (expressing MHC-2a protein) were equally atrophied in denervated mice and, therefore, contributed to muscle atrophy. The cause of this muscle atrophy might be the result of an upregulation of the ubiquitin-proteasome system and autophagy involved in protein degradation (36, 47), together with a reduced protein synthesis.

Moreover, $P_{max}$ was reduced by denervation ($-81\%$) due to the decrease in maximal force production, maximal velocity of shortening, and optimal velocity. The reduced $P_{max}$ and velocity cannot be explained by an increased MHC-1 protein expression (unchanged MHC-1 protein expression). Usually, $P_{max}$ and velocity of shortening are related to myosin ATPase activity and MHC protein expression (10). It should be noted that the aging process and spinal cord injury also modify maximal velocity of shortening without inducing any corresponding change in MHC-1 protein expression (15, 35). Furthermore, the twitch kinetics were slower in denervated muscles (contraction and one-half relaxation times increased), indicating changes in the properties of the sarcoplasmic reticulum (41, 45).

To our knowledge, most of these observations concerning the effects of denervation on the physiological properties of mouse soleus muscles are very novel observations and have not

Fig. 6. CSA of soleus muscle fibers and citrate synthase activity in CD1 mice. *Denervated significantly different from (control) 129/SV mice ($P < 0.05$).

*Denervated + tenotomized significantly different from denervated mice ($P < 0.05$). $n = 7$/group.

Fig. 7. Expression of MHC-1 protein in soleus muscles from CD1 mice. Relative number of muscle fibers expressing MHC-1 protein (immunohistology) and relative amount of MHC-1 protein (electrophoresis) are shown. $n = 7$.

Fig. 8. MHC protein expression changes with time in soleus (A) and extensor digitorum longus (B) muscles following denervation in C57BL/6 mice. $n = 1$/day (d).

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been previously reported. We do, however, confirm the previous findings of Webster and Bressler (61), who showed that denervation reduced the specific maximal tetanic force and slowed the twitch kinetics of mouse soleus muscles. A possible additional explanation for this decreased muscle performance in the denervated soleus muscle of mice is posttranslational modifications of proteins involved in the excitation-contraction-relaxation process. Our results showing much greater amounts of oxidized and ubiquitylated MHC support this hypothesis. These are also in agreement with a recent study showing that intrinsic modifications of the myosin protein contributed to impaired skeletal muscle function in chronic heart failure (12).

In contrast to MHC-1 protein, the strong reduction in CS activity indicates that the maintenance of oxidative capacity, another conventional marker of slow-muscle fibers (shared with fibers expressing MHC-2a protein), is neural activity and load dependent. The reduced mitochondrial function is explained by the decreased levels of PGC-1α and PPAR-α in denervated muscles, which have been demonstrated to be important regulators of oxidative capacity (7, 33, 34, 64).

It should be noted that neurotomy has distinct effects on mixed and fast mouse muscles. MHC protein expression in fast muscle was sensitive to denervation (see above). Moreover, denervation in mouse fast muscles does not decrease specific maximal force, equally atrophies all muscle fiber types, and markedly reduces oxidative capacity (47).

The effect of denervation in different species. To determine whether the influence of innervation and muscle activity on MHC-1 expression is species specific, we have compared the results obtained in different studies (Table 1). Table 1 indicates that the effects of denervation were globally similar between species (5, 6, 11, 13, 14, 21, 22, 28, 31, 35, 45, 55, 60). However, there are two notable exceptions. First, MHC-1 protein expression is not consistently reduced in denervated mouse and rabbit muscles (the present study, Refs. 6, 13, 14, 60). It is very unlikely that this difference is explained by the fact that the rate at which mouse MHC-1 protein adapted to denervation is much slower than for rats (for example), as has been reported for humans compared with smaller animals (53). A possibility is that MHC-2a/x is degraded at a higher rate than MHC-1 in denervated mouse muscle compared with rat denervated muscle (the present study shows that MHC-1 ubiquitylation is low in mouse). Second, it appears that the muscle performance of slow rabbit muscles (soleus or semimembranosus proprius) is less affected by denervation compared with other species (13). Therefore, it is possible that the slow-muscle fiber response to neural influence varies with both species and the function of the muscle.

Denervation and other neurological diseases. It would be interesting to compare our results in the denervated mice with those of studies using different animal models of neuromuscular diseases affecting primarily motoneurons and motor synapses. Clearly, neurotomy exerts different effects than these diseases. Reduced absolute maximal force, muscle atrophy, but increased proportion of slow muscle fibers, are observed in the soleus muscles of mice lacking the ε-subunit of the ACh receptor (29, 62). Absolute maximal force was reduced, but $P_{\text{max}}$, specific maximal tetanic force, and twitch kinetics were unchanged, and the relative expression of MHC-1 protein was reduced in soleus muscles from the mice lacking acetylcholinesterase (58). Expression of MHC-1 protein was not altered, but specific maximal force and mitochondrial enzymes were unchanged in the soleus muscles of mice lacking neurotrophin-4 and exhibiting neuromuscular junction dysfunctions (8). In a mouse model of amyotrophic lateral sclerosis (expression of a mutant SOD1 gene), a decrease in twitch kinetics was observed in the soleus of presymptomatic mice, but there is a shift toward a fast type of muscle phenotype, without modification in maximal force and muscle atrophy (4, 16, 17, 25).

Conclusion. Our mouse findings refute the general principle that muscle activity promotes the relative expression of the slow MHC protein, a key muscle protein. Neural input and passive muscle tension do not control the maintenance of a cardinal aspect of the slow phenotype, MHC-1 protein accumulation. These results raise the question of the suitability of the mouse model for studies on the impact of the neural activity. However, almost all aspects of muscle performance were severely impaired by denervation: atrophic muscles produce less force and power and are mechanically slower (twitch kinetics and maximal velocity of shortening). They provide important information to improve our understanding of neuromuscular disorders and therapy. For example, a knowledge that could be the basis for a new therapy is that MHC-1 protein, which is an important component of normal muscles, can be expressed without the control of nerve. This underlines the importance of studying, increasing our understanding of the factors and signaling pathways involved in the control of muscle proteins such as these.

Table 1. Effects of denervation on slow muscle fibers in different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Slow Fibers MHC-1 Protein Expression, %</th>
<th>Twitch Kinetic</th>
<th>Velocity of Shortening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>= (55)</td>
<td>= (31)</td>
<td>= (31)</td>
</tr>
<tr>
<td>Human</td>
<td>= (35)</td>
<td>= (60)</td>
<td>= (61)</td>
</tr>
<tr>
<td>Mouse</td>
<td>= (present study)</td>
<td>= (present study)</td>
<td>= (present study)</td>
</tr>
<tr>
<td></td>
<td>= (60)</td>
<td>= (13)</td>
<td>= (13)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>= (14)</td>
<td>= (13)</td>
<td>= (13)</td>
</tr>
<tr>
<td></td>
<td>= (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>= (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>= (22)</td>
<td>= (31)</td>
<td>= (31)</td>
</tr>
<tr>
<td></td>
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<td>= (11)</td>
<td>= (45)</td>
</tr>
<tr>
<td></td>
<td>= (28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>= (5)</td>
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</tbody>
</table>

MHC, myosin heavy chain. Nos. in parentheses are ref. nos. –, Decrease; =, unchanged.
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