Muscle weakness in a mouse model of nemaline myopathy can be reversed with exercise and reveals a novel myofiber repair mechanism

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Patients with the inherited muscle disease nemaline myopathy experience prolonged muscle weakness following periods of immobility. We have examined endurance exercise as a means of improving recovery following muscle inactivity in our α-tropomyosinslow(Met9Arg)-transgenic mouse model of nemaline myopathy. Physical inactivity, mimicked using a hindlimb immobilization protocol, resulted in fiber atrophy and severe muscle weakness. Following immobilization, the nemaline mice (NM) were weaker than WT mice but regained whole-body strength with exercise training. The disuse-induced weakness and the regain of strength with exercise in NM were associated with the respective formation and resolution of nemaline rods, suggesting a role for rods in muscle weakness. Muscles from NM did not show the typical features of muscle repair during chronic stretch-immobilization of the soleus muscle (regeneration occurred with relative lack of centralized nuclei). This indicates that the normal process of regeneration may be altered in nemaline myopathy and may contribute to poor recovery. In conclusion, endurance exercise can alleviate disuse-induced weakness in NM. The altered myofiber repair process in the nemaline mice may be a response to primary myofibrillar damage that occurs in nemaline myopathy and is distinct from the classical repair in muscular dystrophy resulting from plasma membrane defects.

INTRODUCTION

Nemaline myopathy is a neuromuscular disorder characterized by muscle weakness and the presence of electron dense structures, called nemaline rods, within the muscle fibers and more rarely in the nuclei (1,2). Nemaline rods are thought to be accumulations of sarcomeric proteins, principally α-actinin, formed through an unchecked expansion of the Z-line (3). The clinical presentation of the disease is variable and has been classified into five main sub-types on the basis of the pattern of weakness and age of onset: severe congenital, typical, intermediate congenital, mild form of childhood-onset and adult-onset (2). Mutations in five genes encoding proteins that form or associate with the thin filament of the sarcomere have been identified as causing nemaline myopathy: β-tropomyosin, α-tropomyosin slow (α-Tmslow), nebulin, α-skeletal actin and troponin T slow (4).

A feature of nemaline myopathy that distinguishes it from the muscular dystrophies is the absence of pathological signs of myonecrosis and regeneration (central-nucleated fibers) (5), the principal characteristics of dystrophic muscle. Muscular dystrophies result from mutations in proteins (e.g. dystrophin and sarcoglycans) thought to be involved in maintaining the functional integrity of sarcolemma (6). This leads to increased susceptibility to contraction-induced injury and sarcolemmal damage resulting in necrosis (6). In contrast, membrane damage and muscle degeneration is not a feature of nemaline myopathy (5), presumably because the primary defect is in the sarcomeric thin filament. However, a recent array analysis on muscle from human nemaline patients showed increased
expression of genes associated with activated satellite cells and immature fibers (NCAM1, CDK1 and PAX7) (7). The significance of these observations is yet to be established, but it raises the possibility that in nemaline muscle a novel regenerative process may be occurring that does not display the traditional features of muscle repair.

We have generated a transgenic mouse model of nemaline myopathy by expressing the dominant-negative α-Tm<sub>slow</sub> (Met9Arg) mutant (human TPM3 gene) in skeletal muscle (8). This was the first nemaline-associated mutation to be identified and results in a childhood-onset form of the disease (9). This mouse model has all features of the human disease including the presence of nemaline rods in skeletal muscle and an increase in slow/oxidative fibers. As has been observed in muscles of a human nemaline patient (10), in the mouse model the number of rod-containing fibers between muscles varied significantly. However, the number of rod-containing fibers in individual muscles was consistent within litters of α-Tm<sub>slow</sub>(Met9Arg) mice and through generations. The increase in number of slow/oxidative fibers was present at 1 month of age and was maintained through adulthood, indicating disruption of the early postnatal maturation of the different fiber types.

The hypotonia and muscle weakness in patients with nemaline myopathy often lead to extended periods of immobility (2). Lack of activity exacerbates the problem as muscle disuse in itself leads to myofiber atrophy and loss of muscle strength (11), and the nemaline patients appear to be more susceptible to the effects of inactivity (12). We have used our α-Tm<sub>slow</sub>(Met9Arg) mouse model to examine the following questions. (i) Does expression of a nemaline myopathy mutation exacerbate disuse-induced muscle atrophy and muscle weakness? (ii) Can endurance exercise improve recovery of muscle strength following immobilization in nemaline mice? (iii) Does a nemaline mutation impact on the process of recovery following immobilization? We show that the nemaline mice are more sensitive to the effects of chronic inactivity and that endurance exercise was required for recovery of whole-body strength. Increased nemaline rod density in the extensor digitorum longus (EDL) muscle correlated with muscle weakness raising the possibility that nemaline rods can influence muscle strength. Restoration of myofibrillar structure (removal of rods) in the EDL with exercise recovery occurred without the activation of the normal regenerative process. The process of muscle regeneration following stretch-induced damage (soleus muscle) was also altered in nemaline mice. These two myofiber repair processes (repair without regeneration and regeneration with few centralized nuclei) are distinct from sarcolemmal repair in muscular dystrophy and repair in normal healthy muscle and may be specific to diseases of the sarcomeric thin filament.

RESULTS

Nemaline mice have greater loss of whole-body strength following immobilization and require exercise to regain strength

Many patients with nemaline myopathy have prolonged periods of immobility owing to muscle weakness (2,13). We modeled prolonged inactivity in our transgenic nemaline mouse with a hindlimb immobilization procedure and examined whether exercise could alleviate disuse-induced weakness with a standard whole-body strength and fatigability test (see Materials and Methods). This procedure tests the overall strength of the limb and abdominal muscles and mimics clinical tests of muscle weakness (14). The results of this test indicate that although the nemaline mice were not weak before immobilization, they lost a greater amount of whole-body strength following immobilization (Fig. 1). The nemaline mice were weak compared with WT mice after 10 days of cage-rest recovery and were still severely weak 3–4 weeks later. In contrast, 3–4 weeks of free-wheel or treadmill exercise led to almost complete recovery of whole-body strength in WT and nemaline mice \(P < 0.05\) versus cage-rest, LSD post hoc analysis). These results suggest that although the nemaline myopathy mutation \(α-Tm<sub>slow</sub>M9R\) promotes disuse-induced muscle weakness it does not alter exercise-induced recovery of muscle strength.

The atrophic effect of immobilization and fiber-size recovery was maintained in nemaline mice

As myofiber atrophy (decrease in myofiber size) is a major cause of muscle weakness, we examined whether the greater weakness in the nemaline mice after immobilization was due to myofiber atrophy and the regain of strength with exercise was the result of fiber-size recovery. Hindlimb immobilization of WT and nemaline mice lead to a reduction in myofiber size in both chronically stretched (soleus) and chronically shortened (EDL) muscles (Table 1). The decrease in fiber size was similar for the two sets of mice. However, the mechanisms for the decrease in fiber size were different for the two muscles. In the soleus muscle, a decrease in average fiber size was due mainly to the appearance of small regenerating fibers, induced by chronic muscle extension (see data to follow). While in the shortened EDL a process of true atrophy occurred, there was a decrease in the size of mature fibers without regeneration. This effect may be due to an increase in nemaline rods in response to muscle disuse (see data to follow).

Both cage-rest recovery (4 weeks) and free-wheel exercise returned fiber size (EDL and soleus muscles) to pre-immobilization levels (Table 1). This occurred to a similar extent for both WT and nemaline mice (Table 1). Fiber size also returned to the unimmobilized state with treadmill exercise in the nemaline mice (EDL and soleus muscles), but in the WT soleus fibers retained their immobilized size (Table 1). An atrophic effect of endurance exercise training on slow/oxidative fibers has been observed in unimmobilized mice (15). Thus, it would appear that the atrophic effect of endurance exercise itself in the present study prevented the restoration of fiber size in the soleus from WT but not from nemaline mice. Overall changes in fiber size (Table 1) did not correlate with muscle weakness (Fig. 1); e.g. mice were weak with 4 weeks cage-rest but fibers were back to pre-immobilized size. Thus, fiber size was not primarily responsible for the regain of strength with recovery. Moreover, the nemaline myopathy mutation \(α-Tm<sub>slow</sub>M9R\) had only a minor impact on the trophic response to immobilization and exercise.
Muscle fiber-type transformations are not grossly perturbed in the nemaline mice

In the nemaline mice, disruption of the early postnatal maturation of fiber types leads to an increase in the number of slow/oxidative fibers (8). The ability to modify fiber-type composition in response to altered loading is an important property of muscle and is a major contributor to the increased endurance and strength that occurs with exercise training. Therefore, we were interested to see whether mature nemaline mice (4 months old) would undergo the normal fiber-type conversion in response to immobilization and endurance exercise training. Chronic shortening of the EDL muscle with hindlimb immobilization had no significant effect on fiber-type proportions in WT or nemaline mice (data not shown).

In contrast, chronic overloading (stretch-immobilization) of the soleus muscle resulted in an increase in the number of slow/Iβ fibers in both WT and nemaline mice (Fig. 2A). Endurance exercise (both free-wheel and treadmill), which also results in a fast-to-slow fiber conversion, maintained the elevated number of slow/Iβ fibers in both WT and nemaline mice (Fig. 2A). These findings show that although muscles from nemaline mice have altered postnatal fiber maturation, the ability to undergo fiber-type transformations is retained in the adult mice.

Another indication of muscle transformation with immobilization was the appearance of hybrid fibers expressing both myosin heavy chain (MyHC) slow/Iβ and IIA (Fig. 2B and C). Hybrid fibers are thought to be intermediates in the process of transformation (16) and occur during development and myofiber regeneration (17). Quantification of the number of hybrid Iβ/IIA fibers revealed that the increase in hybrid fibers with immobilization was much greater in WT versus nemaline mice (Fig. 1D). That there was an increase in hybrid Iβ/IIA fibers in WT and nemaline mice and no change in the total number of fibers (hybrid plus pure) expressing the IIA isoform (Fig. 2A) indicates that immobilization led to a decrease in pure IIA fibers (expressing only MyHC IIa). From this it follows that the major fiber-type transformation induced by stretch-immobilization was conversion of pure IIA fibers to hybrid Iβ/IIA fibers. Likewise, the smaller increase in hybrid Iβ/IIA fibers in nemaline versus WT mice with immobilization (Fig. 2D) indicates that there was less extensive conversion of IIA to hybrid Iβ/IIA fibers in the nemaline mice. This may be due to the greater number of slow/Iβ fibers in the unimmobilized nemaline mice (Fig. 2A).

With cage-rest recovery there was a reduction in the number of hybrid Iβ/IIA fibers, but the levels were still elevated compared with unimmobilized mice (Fig. 2D). Endurance exercise (both free-wheel and treadmill) affected an increase in hybrid fibers compared with cage-rested mice (P < 0.05, post hoc Fisher’s LSD test). This is in keeping with a fast-to-slow fiber conversion that normally occurs during endurance exercise training (15,16). That the response to exercise post-immobilization was similar in WT and nemaline mice indicates that exercise-induced fiber-type transformations are not grossly perturbed in muscles from nemaline mice.

Stretch-immobilization of the soleus muscle led also to appearance of the embryonic MyHC (eMyHC) isoform (Fig. 3A). The appearance of regenerating fibers expressing eMyHC has been observed in muscle immobilized in the stretched position (18). This is presumably in response to areas of muscle damage and regeneration or fusion of nuclei to the ends of fibers during the process of sarcomere addition induced by chronic stretch (19). The amount of eMyHC expressed during immobilization was similar in WT and nemaline mice (Fig. 3B). eMyHC expression was not observed after 4 weeks of recovery in the soleus muscle and in the chronically shortened EDL muscle (data not shown). It is quite notable that the pattern of protein expression is more similar between WT and NM after immobilization (Fig. 3A).
Immobilization of the EDL, but not the soleus, results in an increase in the number of nemaline rods

In human nemaline patients and in the α-Tm<sub>slow</sub>(M9R) mouse model, the distribution of rods in various muscles is very diverse, but the number of rods (rod-containing fibers and number of rods per fiber) remains relatively stable throughout normal adult life (8,13). In the present study, we examined whether nemaline rods are a static feature of the disease or whether they can change in response to altered muscle loading.

This was performed using electron microscopy (Fig. 4) and immunofluorescent microscopy (Fig. 5). Nemaline rods were defined as electron dense elongated (long-axis parallel to the sarcomeric filaments) accumulations of filamentous material that at high magnification have a lattice appearance (Fig. 4F–H). To more clearly define the lattice of the rods, the muscle section was tilted sequentially until the best lattice view was obtained (Fig. 4I for representative example). Uniform regions of these rods images were used to calculate Fourier filtered images. Such analysis of the rods from the EDL muscle revealed a chevron shaped (V) lattice structure (Fig. 4J). This lattice arrangement is typical of vertebrate Z-bands (20).

Nemaline rods were observed in few fibers (<10%) of the unimmobilized soleus muscle. These rods were elongated and ‘spindle-shaped’ (Fig. 4A). With stretch-immobilization of the soleus muscle, the number of rod-affected fibers and the number of rods within each affected fiber did not change (data not shown). However, chronic stretch of this muscle did result in areas of Z-line streaming (Fig. 4B): filamentous structures extending from the Z-line with electron density less than the Z-line (inset in Fig. 4B) and without the lattice structure observed in nemaline rods. Z-line streaming is a relatively normal feature of muscle (21) and is thought to reflect remodeling of the sarcomere that occurs with muscles contracting under a load (eccentric contractions) (22,23). Unlike nemaline rods, Z-line streaming resolved with cage-rest and was not observed in the soleus muscle with free-wheel or treadmill exercise (data not shown).

In the chronically shortened EDL, there was no evidence of Z-line streaming, but there was a striking increase in the number of rods within a fiber (compare the representative images of affected fibers in Fig. 4C with Fig. 4D) and the number of rod-affected fibers (Fig. 5). Increase in rods/fiber was associated with a decrease in fiber size (Table 1),...
Figure 4. Immobilization leads to an increase in the number of nemaline rods. Shown are representative electron micrographs from 70 nm longitudinal sections of soleus (A and B) and EDL muscle (C–H). (A) Micrograph of unimmobilized soleus showing electron dense elongated spindle-shaped nemaline rods (marked with white arrow). Note that the nemaline rods often appear as expanded Z-lines and are slightly less electron dense than the Z-lines (marked with black arrow). Scale bar = 2 μm. (B) Micrograph of chronically stretched soleus muscle showing areas of Z-line streaming (arrow head). This was not observed in the shortened EDL muscle (not shown). Scale bar = 2 μm. The inset in (B) shows an enlarged image of Z-line streaming. Note that these structures are generally more diffuse and less electron dense than the Z-line (black arrow) and nemaline rods. (C–E) Electron micrographs of EDL muscle showing an increase in the number of nemaline rods (white arrows) with immobilization (compare rod abundance in C with D) and a decrease with treadmill exercise. A number of adjacent non-affected fibers (white stars in C–E) are also shown in the micrographs. Scale bars = 5 μm. Insets in (C) and (D) are enlarged portions of rod-affected fibers. Note that, in contrast to the unimmobilized muscle where many Z-lines are visible [arrowheads in main image and inset in (C)], in the immobilized rod-affected fiber (D) there is almost complete absence of Z-line structure [arrowhead in the inset of (D) points to an isolated Z-line]. (F–H) Nemaline rods from EDL muscle at high magnification. Micrographs from (F) unimmobilized, (G) immobilized and (H) immobilized + treadmill exercised nemaline mice are shown. The size, shape and lattice appearance of the rods appeared similar in unimmobilized and immobilized mice (with and without exercise). Black arrowhead in (F) points to a Z-line. The rods in the EDL, in general, were not as elongated as the soleus rods (compare rods in A with rods in F–H); however, the lattice structure of the rods from the two muscles appeared similar (data not shown). Scale bars = 0.5 μm. (I) Representative electron micrograph of a rod from unimmobilized EDL showing lattice structure. This image was obtained by tilting the muscle section to obtain an optimal view of the lattice. Scale bar = 200 nm. (J) Fourier filtered image of the boxed region depicted in (I) shows clearly chevron-like (V) links typical of vertebrate Z-bands.
indicating that immobilization led to a marked increase in rod density in the EDL. The size and shape of the rods appeared similar in unimmobilized and immobilized mice (Fig. 4F–H). This suggests that the rods formed with immobilization were similar to the constitutive rods that existed in the muscle prior to immobilization. The large accumulations of rods in the immobilized EDL led to an almost complete breakdown of sarcomere structure; Z-line structures were difficult to see and were very isolated (inset of Fig. 4D). Despite the extensive disruption to the sarcomere, the sarcolemma appeared to be largely intact in the affected fibers of immobilized mice (data not shown).

The nemaline rods formed during immobilization are resolved with endurance exercise without classical signs of muscle regeneration

Simple cage-rest had little effect on the number of rods/fiber (data not shown) or the number of rod-affected fibers in the EDL muscle (Fig. 5D). However, exercise (both free-wheel exercise and treadmill exercise) decreased the number of rod-containing fibers to pre-immobilized levels (data not shown).
Muscles of nemaline mice undergo altered myofiber repair during stretch-immobilization

Muscles under chronic maximal extension immobilization undergo a cycle of myofiber degeneration and regeneration (24). We were interested to determine whether the process of muscle repair during stretch-immobilization of the soleus muscle would be altered in the nemaline mice as this may explain the poor recovery after immobilization. On hemotoxylin and eosin (H&E) stained sections, few pathological features were observed in muscles from unimmobilized nemaline mice; the myofibers were polygonal in shape with nuclei located at the fiber periphery, and there was little evidence of myonecrosis or infiltrating mononuclear cells (Fig. 6A and B, respectively). This is consistent with the categorization of this disease as a myopathy without dystrophy. Immobilization of the EDL in the shortened position produced few histopathological effects; few signs of necrosis, myofiber regeneration or fibrosis and only baseline numbers of central-nucleated fibers (<1% of fibers; data not shown).

However, in the soleus, extensive myofiber degeneration and macrophage infiltration were observed after 10 days of stretch-immobilization in WT and nemaline mice (Fig. 6C and D, respectively). The level of necrosis appeared similar in the two mice, and at this stage there were few fibers with centralized nuclei indicating that active regeneration had not commenced. After a further 18 days of stretch-immobilization (4 weeks in total), infiltrating macrophages were still evident in the WT soleus, but myofiber necrosis had essentially disappeared, replaced by extensive areas of small regenerating fibers (small central-nucleated fibers) (Fig. 6E). In the nemaline muscle, at the same time point, there were fewer regenerating fibers; and in contrast to WT muscle, there were very few fibers with central nuclei (Fig. 6F). With 4 weeks of exercise recovery (both free-wheel and treadmill), in the WT soleus muscle, small foci of regenerating fibers (small central-nucleated fibers) were still present and there were many mature fibers with centralized nuclei (Fig. 6G); whereas in the nemaline mice, central-nucleated mature fibers were virtually absent (Fig. 6H).

To provide an objective assessment of regeneration in the WT and nemaline mice, the number of fibers with centralized nuclei was counted in whole sections of unstretched and stretched soleus muscles (500–700 fibers/muscle) (Fig. 7). This analysis confirmed the markedly reduced number of fibers with centralized nuclei in the soleus muscle of nemaline compared with WT mice after 4 weeks stretch-immobilized; in WT muscle ~50% of the fibers had central nuclei, whereas in the nemaline muscle the number of central-nucleated fibers was three times less (~15%) (Fig. 7). The number of central-nucleated fibers did not change appreciably with either freewheel or treadmill exercise, although there was a modest decrease ($P = 0.057$ and 0.063 for WT and nemaline mouse; post hoc Fisher’s LSD) in fibers with central nuclei in freewheel exercise compared with immobilized mice (Fig. 7). In the nemaline mice following both types of exercise, the central-nucleated fibers were almost entirely small regenerating fibers (Fig. 5H) (<1% of mature fibers had centralized nuclei; data not shown), whereas in the WT mice the central nuclei were in both immature and mature fibers (Fig. 6G). Together the data suggest that the expression of the mutant α-Tm_{slow} protein in muscle alters the normal myofiber regenerative process.

DISCUSSION

Muscle weakness is a significant cause of morbidity for patients suffering from nemaline myopathy and can lead to prolonged periods of immobility (2,25). Prolonged muscle inactivity may be particularly detrimental in these patients as an early radiological study indicated that nemaline patients experience greater muscle disuse-atrophy compared with healthy controls (12). The data from our nemaline mouse verifies that a nemaline mutation can indeed increase the sensitivity of muscle to periods of immobility and that endurance exercise is an effective means of stimulating recovery from such insults. The results of the present study also provide important data on potential mechanisms for poor recovery in nemaline myopathy. Immobilization of a muscle (EDL) in the shortened position in the nemaline mice led to a large increase in the number of nemaline rods, the defining pathological feature of the disease. Endurance exercise but not cage-rest restored whole-body strength and was associated with a reduction in the number of nemaline rods and sarcomere disruption. These observations raise the possibility that nemaline rod abundance can influence muscle strength and the increase in

and treadmill) in the post-immobilization period led to a decrease in the number of rods per fiber (Fig. 4E) and the number of rod-containing fibers to baseline-unimmobilized levels (Fig. 5D). That the increase in rod-affected fibers with immobilization was similar to the decrease in affected fibers with exercise (Fig. 5D) is compatible with the possibility that the rods formed during immobilization are cleared during endurance exercise. In addition, the data in Figure 5D display a close, but reciprocal, relationship to the clinical strength measurements in Figure 1.

The increase in the total number of rod-containing fibers with immobilization and decrease with exercise was due to an increase and decrease, respectively, in the number of rod-containing IIA and IIX fibers (Fig. 5D). This suggests that these fibers are more susceptible to rod formation than the fast glycolytic IIB fibers. This is consistent with a previous study on these mice where the majority of rods were found to be in the IIX and IIA fibers in the EDL and the fast fibers (IIA) of the soleus muscle (15). In this earlier study, unimmobilized mice underwent the same endurance exercise regimen as the present study, and this had no effect on the total number of rod-affected fibers in two fast muscles (EDL and extensor carpi ulnaris). In the present study, exercise decreased the number of rod-containing fibers, but not below baseline unimmobilized levels (Fig. 5D). Together the findings of these two studies suggest that the constitutive rods present in the unstressed muscle are more stable than the rods formed during immobilization. The decrease in nemaline rod abundance (rods within a fiber and rod-containing fibers) with exercise in the EDL occurred without histological evidence of myofiber regeneration, indicating that the repair of damaged myofibrils (sarcomere arrays) occurred by processes intrinsic to the muscle fiber.
Figure 6. The process of muscle regeneration in the chronically stretched soleus muscle is altered in nemaline mice. Representative H&E stained transverse sections through soleus muscle from wild-type (A, C, E and G) and nemaline (B, D, F and H) muscle are shown. (A and B) Unimmobilized muscle showing fibers with peripherally located nuclei (indicated by black arrows in the inset) and absence of muscle pathology. (C and D) Sections from muscle after 10 days of stretch-immobilization showing large areas of necrosis (white arrows) and extensive mononuclear cell infiltration. At this stage, there is little evidence of muscle regeneration (few fibers with centralized nuclei). (E) Muscle from WT mice following 4-week of immobilization showing few necrotic fibers and the presence of many small fibers with centralized nuclei (regenerating fibers, black arrow in inset). (F) Muscle from nemaline mice following 4-week of immobilization showing that in comparison with WT mice at the same time point in nemaline mice there are far fewer fibers with centralized nuclei [compare (E) with (F), respectively]. Most fibers are of mature size and there are only a few foci of regeneration (small fibers with centralized nuclei, black arrow in inset). (G) In muscle from immobilized + treadmill exercised WT mice regenerating fibers are absent and have been largely replaced by regenerated central-nucleated fibers of mature size (black arrow in inset). (H) In the nemaline mice after immobilization and treadmill exercise a few foci of regeneration are present (circled), but most of the fibers are of mature size and virtually all mature fibers have peripheral nuclei (arrow in inset). Scale bars = 50 µm.
Nemaline rods with immobilization may have contributed to greater muscle weakness in the nemaline mice. Another major finding that may explain poor recovery was the altered regenerative process in the nemaline mice during immobilization. Immobilization of the soleus muscle in the over-extended position resulted in significant myofiber degeneration in nemaline and wild-type mice; but in the diseased mice, repair occurred without the classical features of muscle regeneration: regeneration with few central-nucleated fibers.

**Nemaline mice are weaker following immobilization**

A major feature of the α-Tm<sub>slow</sub>(Met9Arg) mouse model of nemaline myopathy is late-onset muscle weakness; weakness is not observed until 6–8 months of age (8). In this regard, the mice are similar to humans with this mutation who develop signs of clinical weakness in early childhood (9). In the present investigation, mice were studied prior to the onset of muscle weakness (4 months of age) and yet they experience greater loss of whole-body strength with hindlimb immobilization. Thus, immobilization revealed a functional defect that was not apparent in unstressed mice. The greater weakness in the nemaline mice was prolonged (present 5 weeks post-immobilization with just cage-rest) and required active exercise to regain whole-body strength. This models the clinical situation of muscle weakness leading to periods of immobility in nemaline patients (25), and supports regular endurance exercise as a means of improving muscle strength in patients who are not severely weak. From the data in this study, it is unclear whether exercise would be as effective in patients with more debilitating nemaline mutations or in older patients who have reduced regenerative capacity (17).

**Nemaline rod formation is associated with greater weakness**

The development of greater weakness with disuse and the recovery from weakness with exercise in nemaline mice was associated with the respective formation and resolution of rods in the EDL muscle. The level of increase in rods with immobilization was substantial (rod-affected fibers almost doubled) and led to extensive disruption of the ordered sarcomeric structure of muscle. Thus, the formation of rods and the resulting increase in sarcomere disruption may have contributed to the greater loss of weakness in the nemaline mice with immobilization. There is much debate about the precise effect of nemaline rods and other nemaline pathologies on muscle function. For example, in a number of studies, the number of rod-containing fibers has been shown to correlate poorly with the age of presentation and severity of the disease (13,26,27). Furthermore, in early life (<5–6 months of age) the nemaline mice are not grossly weak and yet there are many rod-affected fibers in the diaphragm and many forearm and hindlimb muscles (8). However, in humans, the more severe forms of NM tend to be associated with more extensive sarcomeric disruption (26), and the greater number of rods in the diaphragm is associated with ventilatory insufficiency (10,26). In the nemaline mice in this study, the number of rods in the fiber increased as the fiber size decreased leading to an increase in rod density. It seems reasonable that a threshold level of rods/fiber (i.e. rod density) has to be exceeded before sarcomere disruption becomes extensive enough to result in clinical signs of muscle weakness. It is possible that this limit was exceeded during immobilization of the nemaline mice.

**Rods formed with immobilization are dynamic structures**

Previous studies in our mouse model (8) and in human patients (13) with nemaline myopathy have shown that rod abundance is relatively stable during adult life. This has led to the suggestion that rods are a pathological end-point and that once formed cannot be removed by the normal processes of degradation. The results of the present study indicate that this is not the case under all conditions. Immobilization of the EDL led to an increase in rods that were reduced to basal unimmobilized levels with exercise. This strongly suggests that many of the rods formed during immobilization were resolved with exercise. Nemaline rods can form during myofiber regeneration (28), but there was no histological evidence of muscle regeneration in the chronically shortened EDL in the present study (discussed later). Nemaline rods have also been observed in muscles from patients with HIV myopathy (29) and mitochondrial myopathy (30), in normal extra-ocular muscle (muscles), a muscle under constant contractile stress (31), and during prolonged (7 months) immobilization (32). It is possible that the formation of rods in the immobilized nemaline muscle is a normal response to stress that is somehow exaggerated by the presence of the mutant α-Tm<sub>slow</sub> protein.

**Clearance of rods with endurance exercise occurred without activation of the normal regenerative process**

In contrast to the stretched soleus muscle, the chronically shortened EDL muscle showed no histological evidence of myonecrosis or myofiber regeneration. It would appear that chronic underloading although producing extensive sarcomere disruption did not induce significant sarcomermal damage and therefore activation of myofiber degradation. This is distinct to dystrophies where the plasma membrane is destabilized and
this leads to infiltration of phagocytic cells and myonecrosis (33). The lack of myofiber regeneration in the EDL indicates that the decrease in rod abundance and repair of damaged sarcomeres occurred by processes intrinsic to the muscle fiber. This repair also appears to be distinct from the process of limited necrosis and autodigestion that occurs after minor focal injury (28) as necrotic debris was absent in the EDL from nemaline mice. Collectively, these observations suggest that the type of repair that occurs in the nemaline mice is novel and may be more similar to the normal processes of removal of malformed or damaged sarcomeric proteins that occur in healthy undamaged muscle.

However, this raises the question of why the rods induced by immobilization were resolved, while rods in human patients and in unstressed nemaline mice remain throughout life (8,25). As the number of rod-containing fibers increased with immobilization, it would appear that the rods formed in the fibers that were not affected prior to immobilization. Perhaps there is something different about these fibers or the structure of the rods formed in these fibers that allowed them to be cleared with exercise recovery. It is also intriguing that cage-rest had little effect on rod numbers, whereas both endurance exercise regimens were able to restore rod numbers to basal levels. Under normal conditions, the turnover of sarcomeric proteins occurs at a relatively slow rate (half-life, 6–9 days depending on the protein) (34). Thus, one could argue that the breakdown of rods formed during immobilization with simple cage-rest may take significantly longer than the 4-week-period allowed in the present study. In contrast, exercise leads to an increase in protein turnover and remodeling of the sarcomere (35), and this may have stimulated the removal of the rods formed during immobilization. Regardless of the mechanism, this study clearly suggests that exercise in adult nemaline mice is effective in resolving the sarcomere disruption produced by immobilization.

Muscles from nemaline mice do not exhibit the classic features of muscle repair during immobilization of the soleus muscle

Central to the normal process of muscle regeneration after injury is the activation and proliferation of myogenic satellite cells. Activated satellite cells migrate to the site of injury, fuse with damaged myofibers or to themselves, withdraw from the cell cycle and form new differentiated myotubes with centrally located nuclei (17). Normally, as the nascent myofiber matures it increases in size and the nuclei migrate to the periphery of the fiber. However, in rodents, nuclei can remain in the center of a regenerated fiber indefinitely and is often used as an index of fibers that have undergone necrosis (28). The results of this study indicate that in nemaline myopathy the process of regeneration is altered. Regeneration of the soleus muscle following stretch-induced muscle damage in nemaline mice was associated with far fewer fibers with central nuclei compared with WT mice. This was apparent in the early phase of regeneration (at the end of 4 weeks immobilization) and also after 1 month of exercise recovery (both low- and high-intensity). Despite the relative lack of centralized nuclei, regeneration did occur in the nemaline muscle; necrotic fibers were replaced by fibers that appeared morphologically normal. There are two possible explanations for fewer centralized nuclei in nemaline mice: (i) satellite cells or other myogenic cells fuse to damaged fibers, and nuclei stay at the periphery or migrate more quickly to the periphery or (ii) myogenic cells are activated but do not fuse as readily with the fibers of nemaline mice. The second mechanism is less likely because without fusion of activated satellite cells regeneration would be attenuated and there is no sign of this in the nemaline mice. With the first mechanism, one could hypothesize that the nemaline myopathy mutation alters the structural or regulatory networks responsible for nuclear migration during myofiber regeneration. These networks are still to be described but are likely to involve similar cytoskeletal systems that mediate organelle transport in non-muscle cells (microtubules, intermediate filaments and actin microfilaments). Whether the altered regenerative process in nemaline mice impacts on the restoration of myofiber function needs to be established.

Muscle from nemaline patients and the unimmobilized nemaline mouse model do not display the characteristic pathological features of muscle regeneration: muscle degeneration, macrophage infiltration and central-nucleated fibers. However, affymetrix array analysis of human nemaline patients (7) and our nemaline mouse (unimmobilized; unpublished data) showed increased expression of genes characteristic of activated satellite cells and immature fibers (e.g. PAX7, MYF6, NCAM1, ANKR2). These observations together with the presented data on the nemaline mice clearly suggest that nemaline muscle undergoes repair without displaying the traditional features of regeneration.

In summary, we have shown that expression of a nemaline myopathy mutation exacerbates the effects of chronic inactivity. The data suggests that this may be due to the formation of nemaline rods and/or altered myofiber repair. In the nemaline mice, endurance exercise alleviated muscle weakness and reduced the number of nemaline rods, supporting its use in patients with the disease. Two distinct myofiber repair processes have been identified in the nemaline mice: (i) regeneration without centralized nuclei and (ii) sarcomere repair (clearance of nemaline rods) without classic signs of regeneration. These repair processes maybe specific to diseases of the sarcomeric thin filament and are distinct from sarcomemal repair in muscular dystrophy and repair in normal healthy muscle.

MATERIALS AND METHODS

This study was approved by the Children’s Medical Research Institute/New Children’s Hospital Animal Care and Ethics Committee, Westmead.

Transgenic mice

Generation and characterization of the human skeletal actin (HSA)–α-Tmslow(Met9Arg) transgenic mice has been described previously (8). The HSA–α-Tmslow(Met9Arg) construct uses the HSA promoter to drive expression of the α-Tmslow(Met9Arg) mutant cDNA (TPM3 gene) with a SV40 3'-UTR sequence specifically in all fibers to varying degrees. This approach was taken to achieve a mouse model
in which a significant number of fibers would be affected by the presence of the mutant protein as the majority of mouse muscles have a predominance of fast fibers. Non-transgenic mice arising from the matings of transgenic mice were used as control WT animals.

Hindlimb immobilization protocol

The hindlimbs of 8-week-old WT and transgenic mice were immobilized for 28 days as originally described by Booth (36) with minor modifications. While anaesthetized with ketamine/xylazine (100 and 10 mg/kg BW, respectively) both feet were fixed in maximal dorsal flexion using a standard porous adhesive tape. The knees were then bent so that the distal lower leg was apposed to the proximal upper leg and the feet were bound in a dorsal-flexed position resulting in the chronic shortening of the tibialis anterior and EDL muscles and lengthening of the soleus, gastrocnemius and plantaris muscles. The bandages were inspected daily for fraying, soiling or loosening and replaced as required. Following immobilization, one group of mice were sacrificed (Immobilized group) and the remaining mice were randomly assigned to one of three recovery regimens: (i) cage-rest (minimal exercise), (ii) free-wheel exercise (low-intensity voluntary exercise) or (iii) motorized treadmill exercise (high-intensity exercise). Unimmobilized age-matched (12 months old) WT and α-Tmslow(Met9Arg) mice were also studied.

Free-wheel exercise

Immediately following immobilization, mice were placed in separate cages containing custom-made (Siwino Pty Ltd, Sydney) exercise free-wheels (30 cm circumference) equipped with counters to register the number of wheel revolutions. The number of revolutions was recorded every 10 s, 24 h/day, by computer. Following 24 days of free-wheel exercise, strength and fatigability of each mouse were measured according to the test procedures described later.

Endurance exercise training

The exercise-training regimen is similar to that reported previously (15). Following immobilization, the transgenic and WT littermates were allowed to recover from the immobilization for 1 week before exercise training commenced. During this period, the mice were housed in a room with reversed light and dark cycle (light off: 7:00; light on: 19:00) so that they could be exercised during the period of maximum activity (9:00–14:00). The mice were primed to exercise on a motorized treadmill for 1 week (Columbus Exer-4/8, Columbus, OH): a daily 30 min bout of exercise of increasing intensity (6–15 m min−1 on days 1–2 to 15–25 m min−1 on day 7) at a 5% incline.

Exercise training was performed over 17 days at a speed of 25 m min−1. The treadmill incline and length of exercise was increased progressively over the training period: 5% and 45 min on day 1 to 10% and 90 min on day 17. At the end of the exercise regime, strength and fatigability of each muscle and lengthening of the soleus, gastrocnemius and plantaris muscles. The bandages were inspected daily for fraying, soiling or loosening and replaced as required. Following immobilization, one group of mice were sacrificed (Immobilized group) and the remaining mice were randomly assigned to one of three recovery regimens: (i) cage-rest (minimal exercise), (ii) free-wheel exercise (low-intensity voluntary exercise) or (iii) motorized treadmill exercise (high-intensity exercise). Unimmobilized age-matched (12 months old) WT and α-Tmslow(Met9Arg) mice were also studied.

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Strength and fatigability test

Whole-animal strength and fatigability were measured according to the test procedure of Hubner et al. (14). In brief, this test required the mice to pull themselves on top of a suspended rod (3 mm in diameter). The measurement of muscle weakness was based on the mean percentage of passes over 15 trials of the test in a 3 min period. Fatigability was assessed as the average pass rate over time for each group of mice.

Muscle collection

Following immobilization and at the end of the recovery periods (cage-rest, free-wheel exercise and treadmill exercise) mice were sacrificed by cervical dislocation and the EDL and soleus muscles were collected. Muscles were coated in tissue freezing medium (ProSciTech, Brisbane, Australia), frozen in melting isopentane pre-chilled in liquid nitrogen and stored in liquid nitrogen for immunohistochemical and histopathological analysis.

Fiber morphometry

Quantification of fiber-type and morphometry were performed as described by Nair-Shalliker et al. (15). Fiber-typing was performed on transverse sections (8 μm thickness) of muscle with antibodies to specific MyHC isoforms: slow/type I(β) (undiluted BA-F8) (37), type IIB (undiluted BF-F3) (38), type IIA (undiluted SC 71) (38) and IIX (undiluted; gift from Dr Joseph Hoh, Sydney) (39). The secondary antibody was peroxidase-conjugated goat/anti-mouse immunoglobulins (Dako; 1:100 dilution) and detection was carried out using the DAB peroxidase substrate kit (Vector Laboratories). Detection of IIX antibody was enhanced using Vectastain® ABC kit (PK-400) prior to DAB staining. Images from the muscle sections were captured using a digital camera. Fiber cross-sectional area (CSA) were obtained from the stained sections using the Image-Pro software (Media Cybernetics) as described previously (15).

Immunohistochemical analysis of nemaline rods

Nemaline rods contain Z-line proteins, principally α-actinin. Antibodies to α-actinin were used to detect rod-containing fibers in longitudinal sections (6 μm thickness) from EDL and soleus muscles. The primary antibody mixture comprised the monoclonals that recognize the MyHCs of interest (details of antibodies have been mentioned earlier) and the rabbit-polyclonal to α-actinin-2 and -3 (1:200; gift from Alan Beggs, Boston) (40). The sections were washed thoroughly and incubated in (1:1000 dilution) secondary antibody (Alexa Fluor 488 goat anti-mouse immunoglobulins and Alexa Fluor 594 goat anti-rabbit immunoglobulins; Molecular Probes) for 1 h at RT. The number of rod-containing fibers of specific fiber-types was determined and expressed as a percentage of the total number of fibers (all fiber types).
present in the muscle section. At least 300 fibers from each muscle were counted.

Electron microscopic analysis of nemaline rods
EDL and soleus muscles were removed from anaesthetized animals (100 mg/kg BW ketamine/10 mg/kg BW xylazine) and immediately cut into very thin slices while immersed in modified Karnovsky’s fixative (2.5% glutaraldehyde/4% paraformaldehyde in 1 M cacodylate buffer, pH 7.4). Samples were further fixed overnight in the same fixative and post-fixed with 2% osmium tetroxide, dehydrated through an ascending series of ethanol, and embedded in Spurr’s epoxy resin. Ultrathin sections (70 nm) were cut with a Reichert-Jung ULTRACUT ultramicrotome, double contrasted with uranyl acetate and lead citrate, viewed and photographed with a Philips CM120 BioTwin transmission electron microscope.

For more detailed analysis of rod ultrastructure sections were viewed in a Jeol 1200 electron microscope fitted with a Teitz Fastscan CCD camera. The rod images were captured directly onto the 1K x 1K CCD camera. To optimize the lattice view of the nemaline rods, a rotation tilt holder was used to align the tilt axis along the long axis of a selected rod and then the section was tilted in sequential steps of 5° from −60° to +60°. The image showing the best lattice view was selected. Uniform regions of rod images were boxed and in-house software (Imperial College London) was used to calculate Fourier filtered views.

Histopathology
Transverse sections (8 μm thickness) were cut from the midsection of the muscles. Sections were placed on poly-L-lysine pre-coated glass microscope slides, air-dried, fixed and stained with H&E. The number of fibers with centralized nuclei in the whole muscle section was counted.

Myosin heavy chain SDS–PAGE
MyHC extracts were prepared and SDS–PAGE preformed as described by Nair-Shalliker et al. (15). Briefly the MyHC extract was suspended in Laemmli sample buffer (Biorad), loaded onto an 8% vertical acrylamide gel (3% stacking) and electrophoresed at 40 mA for 3 h. The gel was stained with Coomassie Blue and examined with a densitometer. Evidence for a dominant-negative effect in ACTA1 nemaline myopathy caused by abnormal folding, aggregation and altered polymerization of mutant actin isoforms. Hum. Mol. Genet., 13, 1727–1743.

REFERENCES