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Up-regulation of MHC class I in transgenic mice results in reduced force-generating capacity in slow-twitch muscle

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Abstract

Expression of major histocompatibility complex (MHC) class I in skeletal muscle fibers is an early and consistent finding in inflammatory myopathies. To test if MHC class I has a primary role in muscle impairment; we used transgenic mice with inducible over-expression of MHC class I in their skeletal muscle cells. Contractile function was studied in isolated extensor digitorum longus (EDL, fast-twitch) and soleus (slow-twitch) muscles. We found that EDL was smaller, whereas soleus muscle was slightly larger. Both muscles generated less absolute force in myopathic compared to control mice, however when force was expressed per cross-sectional area, only soleus muscle generated less force. Inflammation was markedly increased, but no changes were found in the activities of key mitochondrial and glycogenolytic enzymes in myopathic mice. The induction of MHC class I results in muscle atrophy and an intrinsic decrease in force-generation capacity. These observations may have important implications for our understanding of the pathophysiological processes of muscle weakness seen in inflammatory myopathies.

Introduction

Widespread expression of major histocompatibility complex (MHC) class I in muscle fibers is a frequent finding in muscle tissue of adult patients with idiopathic inflammatory myopathies. This is present even in early and late phases of disease without detectable inflammatory cell infiltrates 29,26,10,9. MHC class I over-expression has also been found to be an early event in juvenile dermatomyositis patients 19. Moreover, MHC class I over-expression can also be seen in other myopathies, dystrophies, and neuropathies 6,14,20.

The initiating trigger that induces MHC class I expression in adult muscle fibers is not known. *In vitro* models show that pro-inflammatory cytokines, including tumor necrosis factor (TNF), interferon (IFN)- γ , IFN- α and interleukin (IL)-1, could all induce MHC class I

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expression in cultured muscle fibers 23. MHC class I expression has also been found in muscle fibers of myositis patients who lack inflammatory cell infiltrates but have impaired muscle performance. This indicates that other factors than those produced by inflammatory cells may induce MHC class I expression in muscle fibers 9,10,26. Furthermore, these observations indicate that MHC class I expression in muscle fibers may affect muscle contractility.

To test if MHC class I has a primary role in muscle impairment, we used transgenic mice with inducible over-expression of MHC class I in their skeletal muscle cells. These mice develop a self-sustaining inflammatory myopathy 24. They show decreased movement activity in open field behavioral activity measures. However, it is not known whether they have any specific impairment in muscle function or if the decreased movement activity could be explained by other factors.

In this report, we have characterized muscle function in conditional transgenic mice that specifically over-express mouse syngenic MHC class I in skeletal muscle cells. We found a reduction in force production in these myopathic mice. This reduction was associated with atrophy in fast-twitch extensor digitorum longus (EDL) but not in slow-twitch soleus muscle.

Material and Methods

Animals

Female myopathic mice (n=8) that specifically over-express MHC class I in their skeletal muscle using a doxycycline-regulated system 24 were investigated. As controls (n=8), single transgenic age and sex matched littermates were used. Four animals per cage were housed at room temperature with a 12-12 hour light-dark cycle. Standard rodent chow and water were provided *ad libitum*. At 10-14 weeks of age, the MHC class I expression was induced by withdrawing doxycycline from the drinking water. Mice were killed by rapid neck disarticulation after 16 weeks. At this time they had clear signs of disease, such as lack of weight-gain and weakness of the lower back and hind limbs. Soleus, EDL, and tibialis anterior muscles were isolated. All procedures were approved by the Stockholm North local ethical committee.

Force measurements

The contractile function was studied in the EDL and soleus muscles of all animals. Isolated intact fast-twitch EDL and slow-twitch soleus muscles were mounted between a force transducer and an adjustable holder (World Precision Instruments, Florida, USA). The stimulation chamber temperature was set at 25°C with a water-jacketed circulation bath. The muscles were bathed in a Tyrode solution with the following composition (in mM): 121 NaCl, 5 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 0.4 NaH₂PO₄, 0.1 NaEDTA, 24 NaHCO₃, 5.5 glucose and 0.2% fetal calf serum (bubbled with 95% O₂-5% CO₂, pH 7.4). Muscle length was adjusted to give a maximum tetanic force response. The force-frequency relationship was obtained by sequential stimulation of the muscle at 1 minute intervals, giving a single twitch or a 300ms tetanus at 20, 30, 40, 50, 70, 100, or 120Hz for EDL muscles and 1000ms tetanus at 10, 15, 20, 30, 50, 70 or 100Hz for soleus. After the force-frequency relationship was established, fatigue (one tetanus every two seconds) was induced by 50 repeated 70Hz tetani each with a duration of 300ms (EDL) and 100 repeated 50Hz tetani each with a duration of 600ms (soleus). Recovery of force was followed by a single 70Hz (EDL) or 50Hz (soleus) tetanic contraction at 1, 2, 5, 10, 20 and 30 minutes after the end of fatiguing stimulation. Thereafter, muscle lengths and weights were measured for each muscle, and then they were snap frozen in pre-cooled isopentane in liquid nitrogen and stored at -70°C.

Fiber typing by immunohistochemical stainings

7- μ m thick cryostat sections from EDL and soleus muscles were mounted on gelatin-coated glass slides, air-dried for 30 minutes at room temperature, and kept at -70°C until use.

Phosphate-buffered saline (PBS) was used as a buffer, and sections were rinsed in PBS/0.5% bovine serum albumin (BSA) between incubations. Prior to staining, the sections were fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 10 minutes and then incubated in 10 mM NH₄Cl for 30 minutes. Endogenous peroxidase was blocked with 1% hydrogen peroxide for 30 minutes, after which the sections were blocked with 1% BSA for 30 minutes. Primary antibodies for slow type I fibers (A4.951) and fast type II fibers (N3.36) (both purchased from Developmental Studies Hybridoma Bank) were diluted 1/20 and incubated overnight at room temperature. Biotinylated rabbit anti-mouse (E0354, DakoCytomation A/S, Glostrup, Denmark) was diluted 1/500 and incubated for 1 hour prior to addition of ABC standard kit (Vector Laboratories, Burlingame, USA) and incubation for 45 minutes. For development, 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, USA) was added and incubated for eight minutes. Sections were counterstained with hematoxylin and mounted before analyses. Mouse IgG₁ (X0931, DakoCytomation A/S, Glostrup, Denmark) and Mouse IgM (Zymed Laboratories Inc., San Francisco, USA) were used as negative controls. The sections were also stained with Mayer's hematoxylin and eosin to confirm the absence or presence of inflammatory infiltrates.

For EDL, measurements of fiber type and cross-sectional area were performed on 30 fibers from 5 muscles obtained from 5 mice. Soleus measurements were performed on 60 fibers from 6 muscles of 6 mice. The analyses were done using a Polyvar II microscope (Reichert-Jung, Vienna, Austria) connected to a 3CCD color camera (DXC-750P; Sony, Tokyo, Japan). The identity of the specimens was unknown when they were analyzed. The determination of cross-sectional area was performed using Image J (NIH, USA; <http://rsb.info.nih.gov/j>). The mean cross-sectional area and fiber type composition of each muscle were used in subsequent statistical analyses.

Western blotting and enzyme activities

Citrate synthase (CS), a mitochondrial enzyme, and glycogen phosphorylase (cytosolic rate limiting enzyme for glycogenolysis) activities were assayed as follows. Muscles were homogenized with ground glass homogenizers in ice-cold buffer (20 μ l/mg wet wt.) consisting of (in mM): 50 KH₂PO₄, 1 EDTA, Triton X-100, 0.05% (v/v), pH 7.5. The homogenate was centrifuged for 30 seconds at 1400 $\times$ g (4°C), and aliquots of the supernatant were frozen for subsequent analyses. CS was analysed with a standard spectrophotometric technique 5, and phosphorylase was assayed with a filter technique 30. The supernatant protein content was determined using the Bradford assay (BioRad, United Kingdom). All enzyme activities were assayed at room temperature (~22°C) under conditions that yielded linearity with respect to extract volume and time (data not shown).

Local inflammation in skeletal muscle was assessed by western blotting for the pro-inflammatory cytokine high mobility group box chromosomal protein-1 (HMGB-1) on 8 tibialis anterior muscles from myopathic and control mice, respectively. The supernatant protein (see above) was separated by SDS-PAGE (4-12% Bis-Tris Gels-Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked overnight at 4°C with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% Tween 20, followed by incubation with primary antibody rabbit-anti-HMGB1 (1:1000 dilution, 556528, PharMingen, San Diego, USA) at room temperature for 2 hours. Membranes were then washed and incubated for 1 hour at room temperature with the secondary antibody HRP-conjugated donkey-anti-rabbit IgG (1:10000 dilution,

NA934V, GE Healthcare UK Limited, Little Chalfont, UK). Immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal, production number 34075, Pierce, Rockford, USA) in a Bio-Rad Gel Doc system.

As controls, recombinant HMGB-1 18 and two cell lines were used: one that expressed HMGB-1 and a second that was a knock out for HMGB-1 (both kind gifts from Marco E. Bianchi, San Raffaele Research Institute, Milan, Italy). All controls were tested at 5, 10, and 25µg protein.

To control for equal loading of protein applied onto the gel, membranes were stripped (Restore western blot stripping buffer, Pierce, Rockford, USA) and re-blotted against the constitutively expressed L-type Ca^{2+} channel dihydropyridine receptor (mouse anti-DHPR, 1:500 dilution, AB2864, Abcam, Cambridge, UK), followed by incubation with HRP-conjugated goat anti-mouse IgG (1:1000 dilution, production number 1858413, Pierce, Rockford, USA). Band densities were analyzed with Image J (NIH, USA; <http://rsb.info.nih.gov/j/>).

Statistics

Values are presented as means $\pm$ standard error of the mean (SEM). Difference between single measurements in two groups was determined with Student's unpaired *t* test. For repeated measurements in the same preparation we used two-way repeated measures ANOVA. When the ANOVA analysis showed a significant difference between the two groups, the Holm-Sidak post-hoc test was performed. The significance level was set at $P < 0.05$.

Results

Body weight and inflammation in myopathic and control mice

MHC class I expression was induced by doxycycline removal from drinking water when mice were 10-14 weeks of age (18-21g). There was no statistically significant difference in body weight between myopathic (19.1 ± 0.9) and control (21.1 ± 0.9) mice at that time ($P > 0.05$). Myopathic mice subsequently showed no weight gain after withdrawal of doxycycline, whereas control mice increased their weight by ~30% after 16 more weeks.

Staining with Mayer's hematoxylin and eosin confirmed the presence of mononuclear cells in both EDL and soleus muscle tissue of myopathic mice compared to control mice, where no infiltration of mononuclear cells was visible (Fig. 1).

HMGB-1 is a non-histone nuclear protein that displays potent pro-inflammatory activity when released from cells. Upon release from cells HMGB-1 can stimulate monocytes to produce pro-inflammatory molecules in a downstream cascade fashion 3. Myopathic mice had a significantly higher expression of HMGB-1 in tibialis anterior muscle compared to control mice ($n=8$ per group) by western blot ($P < 0.05$) (Fig. 2A and 2B).

Contractile properties of unfatigued EDL muscles

In unfatigued EDL muscles, the absolute force was significantly less in myopathic than in control muscles at all stimulation frequencies (Fig. 3A; $n=16$ muscles, 8 animals in both groups). The muscle length was not different between myopathic and control EDL muscles (9.0 ± 0.1 vs. 9.1 ± 0.1 mm), whereas the muscle weight was lower in myopathic muscles (8.4 ± 0.2 vs. 10.2 ± 0.4 g). Hence the cross-sectional area of myopathic EDL muscles was decreased by ~20% (0.90 ± 0.02 vs. 1.10 ± 0.04 mm² in control; $P < 0.001$). After accounting

for the difference in cross-sectional area, force was not significantly lower ($P>0.05$) in myopathic compared to control EDL muscles (Fig. 3B).

The kinetics of twitches at 100 Hz tetanus was similar in myopathic and control EDL muscles. The twitch contraction time (24.3 ± 1.0 vs. 26.2 ± 0.8 ms) and the twitch half-relaxation time (23.1 ± 0.9 vs. 22.5 ± 1.2 ms) were not significantly different, whereas the tetanic half-relaxation time was significantly longer in myopathic muscles (49.0 ± 2.1 vs. 44.0 ± 1.5 ms; $P<0.05$). Furthermore, when force was expressed as a percentage of the force at 100 Hz tetanus, the force frequency curves were superimposable, and the frequency giving 50% force was not significantly different between myopathic and control muscles. Thus, the major change in myopathic EDL muscles was a decrease in cross-sectional area, which was accompanied by a corresponding decrease in force production.

Contractile properties of unfatigued soleus muscles

At all stimulation frequencies, the absolute force was significantly lower ($P<0.001$; 1 Hz, $P<0.05$) in soleus muscles of myopathic compared to control mice (Fig. 3C; $n=16$ muscles, 8 animals in both groups). The muscle length was not different between myopathic and control soleus muscles (8.7 ± 0.2 vs. 8.8 ± 0.1 mm), but the muscle weight was higher in myopathic soleus muscles (9.7 ± 0.2 vs. 9.1 ± 0.2 g). The cross-sectional area of myopathic soleus muscles was significantly larger than that of control muscles (1.10 ± 0.03 vs. 0.98 ± 0.02 , $P<0.05$). Thus, when force was adjusted for cross-sectional area, the difference in force production between myopathic and control soleus muscles becomes even larger (Fig. 3D), which is in sharp contrast to the situation in EDL muscles.

The twitch contraction time was markedly shorter in myopathic than in control soleus muscles (41.0 ± 0.8 vs. 51.0 ± 1.2 ms; $P<0.001$), but the twitch half-relaxation time (55.2 ± 1.9 vs. 61.0 ± 4.3 ms), and half-relaxation times in 70 Hz tetanus (102.7 ± 2.2 vs. 99.2 ± 2.3 ms) were not different between the two groups. When force was expressed as a percentage of the force at 70 Hz tetanus, the force-frequency relationship was shifted to the right in myopathic muscles, and the relative force was significantly lower in myopathic compared to control muscles at frequencies < 30 Hz. In other words, force was relatively more reduced at low than at high stimulation frequencies in myopathic muscles. Thus, soleus muscles of myopathic mice show a markedly impaired contractile function that cannot be explained by muscle atrophy.

Fatigue and recovery properties

The mean decline of force during a series of repeated tetani is shown in Fig. 4A (EDL) and 4B (soleus). The difference in absolute force production between myopathic and control muscles became smaller as fatiguing stimulation progressed, and there was no statistically significant difference between the two groups at the end of the stimulation period. When force was expressed as a percentage of the force in the first tetanus, the relative force at the end of fatiguing stimulation was significantly higher in both myopathic EDL (42.4 ± 2.0 vs. $32.9 \pm 1.9\%$; $P<0.001$) and soleus (46.0 ± 3.5 vs. $33.0 \pm 3.5\%$; $P<0.001$) compared to controls. After 10 min of recovery, force recovered to a similar extent in myopathic and control EDL (80.0 ± 3.7 vs. $76.0 \pm 3.1\%$ of the initial; $P>0.05$) and soleus (97.8 ± 6.5 vs. $96.4 \pm 16.1\%$ of the initial; $P>0.05$) muscles.

Fiber-type composition in myopathic and control mice

Immunohistochemical fiber-typing of EDL revealed that these muscles consisted entirely of fast-twitch type II fibers both in myopathic and control mice. The fiber cross-sectional area was $\sim 30\%$ smaller in myopathic compared to control EDL muscles (549 ± 63 vs. 824 ± 49 μm^2 ; $P<0.01$) (Fig. 1A and 1B), which is in accordance with the smaller size of the isolated

myopathic EDL muscles (~20%; see above). Soleus muscles contained about 45% fast-twitch type II fibers and 55% slow-twitch type I fibers, and there was no difference between myopathic and control mice in this aspect. Moreover, there was no significant difference ($P>0.05$) in fiber cross-sectional area between myopathic and control soleus muscles (Fig. 1C and 1D), neither regarding type II fibers nor type I fibers.

Metabolic profile

Neither the mitochondrial enzyme citrate synthase nor the glycogenolytic enzyme phosphorylase showed any difference in activity between myopathic and control muscles (Fig. 5A and 5B).

Discussion

In this study, we used transgenic mice that conditionally over-express MHC class I in their skeletal muscle cells to study primary effects on MHC class I on muscle function. We observed a marked decrease in force production in muscles isolated from these myopathic mice. The major novel finding of our experiments is that this force decrease was associated with a proportional decrease in cross-sectional area in fast-twitch EDL muscles, whereas it was due to a decrease in the intrinsic force-generating capacity in slow-twitch soleus muscles.

While differentiated skeletal muscle fibers do not constitutively express or display MHC class I molecules under physiological conditions, it is a characteristic early event in patients with inflammatory myopathies as well as other myopathies with impaired muscle performance 8,17,4. Several findings indicate that MHC class I itself affects muscle function in both clinical and experimental settings. As an example, an endoplasmic reticulum (ER) stress response is induced in skeletal muscle cells of the MHC class I transgene mouse model used in the present study 25. Similar changes compatible with ER stress were recorded in muscle biopsies from patients with dermatomyositis with MHC class I expression in muscle fibers 25.

It is well documented that fast-twitch fibers are more susceptible to inflammation-induced atrophy than slow-twitch fibers. For instance, protein synthesis is significantly more decreased in fast-twitch EDL muscles compared to slow-twitch soleus muscles in sepsis 16,31,35, after burn injury 12,13, and during turpentine-induced inflammation 22. This is in agreement with our finding that myopathic mice, which have muscle inflammation as judged from the presence of mononuclear cells (Fig. 1) and increased HMGB-1 expression (Fig. 2), show fiber atrophy in fast-twitch EDL but not in slow-twitch soleus muscle.

Our results show a marked force decrease in soleus muscles of myopathic mice that was not due to reduced muscle size. In principle this force decrease can arise from three factors, viz. (i) a decreased Ca^{2+} release from the sarcoplasmic reticulum, (ii) a decreased myofibrillar Ca^{2+} sensitivity, and (iii) a decreased force-generating capacity of the contractile proteins. One potential mechanism underlying the muscle weakness is increased expression of pro-inflammatory molecules in, or adjacent to, inflamed muscles that adversely affects muscle fiber function. Interestingly, TNF was shown to reduce muscle force production by decreasing Ca^{2+} sensitivity 27,15. Furthermore, HMGB-1 was found to have negative effects on cardiac myocytes 32 and we found increased expression of HMGB-1 in muscles of myopathic mice compared to control mice. However, exactly how these cytokines cause muscle weakness is still unclear, but the results of numerous studies suggest the involvement of increased formation of reactive oxygen and nitrogen species 21,28.

Muscle fatigue, which is a common feature in myopathies, could be due to a primary defect in muscle fibers resulting in decreased muscle endurance. Alternatively, the muscles might be weak already at the start, and therefore fatigue occurs more rapidly due to muscles being used at a higher proportion of maximal capacity 2. Our results from the MHC class I transgene model show that the rate of force decrease during fatiguing stimulation was in fact slower in myopathic than control muscles (Fig. 4). Moreover, we did not detect any differences between the two groups regarding the activity of the mitochondrial enzyme citrate synthase or phosphorylase, which is responsible for glycogen breakdown (Fig. 5). Thus, our results indicate that the decreased fatigue in this model is due to muscle weakness. This could imply that an improvement of the physical performance can be achieved through strength training rather than through endurance training. In line with this, strength training programs for patients with idiopathic inflammatory myopathies result in improved muscle function 1,36,37, reduction in disability scores 11 and improved scoring in the Health Assessment Questionnaire 34.

We used the MHC class I transgene mouse model to study the physiological effects of MHC class I expression in muscle fibers. The clinical implication of this model is the frequently observed expression of MHC class I in skeletal muscle fibers in patients with myopathies 19,29,26,9,10,33. The observed weakness in the MHC class I transgene model with a differential effect on type I, slow twitch fibers and type II, fast twitch fibers respectively has two implications for our understanding of the pathophysiological processes in human myopathies. First, slow-twitch fibers may have marked defects in their force-generating capacity despite relatively normal appearance in histochemical analyses of muscle biopsies. Second, muscle weakness can be severe despite relatively small changes in muscle cross-sectional area and fiber type composition 7.

In conclusion, we show decreased force in both fast-twitch and slow-twitch muscle of a transgenic mouse model with skeletal muscle MHC class I expression. The force decrease could be explained by a reduced cross-sectional area in fast-twitch muscle, whereas it was due to an intrinsic decrease in force-generating capacity in slow-twitch muscle. These observations have important implications for our understanding of the pathophysiological processes in myopathies, as we demonstrate that the induction of MHC class I expression in muscle fibers results in muscle weakness. Moreover, our results suggest that strength training might be better than endurance training in order to improve the physical performance of patients with inflammatory myopathies.

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Abbreviations

BSA	bovine serum albumin
CS	citrate synthase

DAB	3,3'-diaminobenzidine
DHPR	dihydropyridine receptor
EDL	extensor digitorum longus
EDTA	ethylene dinitrilotetraacetic acid
ER	endoplasmic reticulum
HMGB-1	high mobility group box chromosomal protein-1
IFN	interferon
Jo-1	histidyl-transfer ribonucleic acid (tRNA) synthetase
MHC class I	major histocompatibility complex class I
NaEDTA	sodium ethylene dinitrilotetraacetic acid
PBS	phosphate-buffered saline
PVDF	polyvinylidene fluoride
TNF	tumor necrosis factor

Figure 1. Mononuclear cells are present in both EDL and soleus muscles of myopathic mice (A) Control EDL muscle section stained with Mayer's hematoxylin and eosin (H&E). No mononuclear inflammatory cells are visible. (B) Myopathic EDL muscle section stained with H&E. Several mononuclear inflammatory cells are visible within this section, and also many fibers are atrophic. Bold arrows indicate mononuclear cell infiltrates, and thin arrows indicate atrophic fibers. (C) Control soleus muscle section stained with H&E. No mononuclear inflammatory cells are visible. (D) Myopathic soleus muscle section stained with H&E. Increases of mononuclear inflammatory cells are visible within this section. Bar = 100 μ m. Original magnification $\times 50$.

Figure 2. Western blotting of high mobility group box chromosomal protein 1 (HMGB-1) in myopathic and control mouse muscle

(A) The upper gel shows HMGB-1 expression in tibialis anterior from myopathic (M) and control (C) mice. The lower gel shows the DHPR expression in the same muscles. Lane one (Pos) of both gels shows the positive control cell line and lane two (Neg) shows the HMGB-1 knock out cell line. (B) Myopathic mice had a significantly higher expression of HMGB-1 compared to control mice. Data are expressed relative to the DHPR expression. Black bar, myopathic mice; white bar, controls. Values are mean $\pm$ SEM, (n=8 muscles from 8 mice, respectively). *P < 0.05.

Figure 3. Tetanic force production was less in unfatigued extensor digitorum longus (EDL) and soleus muscle from myopathic mice

(A) Absolute force was significantly smaller in myopathic compared to control EDL muscles at all stimulations frequencies. (B) After correcting for the differences in cross-sectional area, force was similar in myopathic and control EDL muscles. Absolute force (C) and force per cross-sectional area (D) were significantly smaller in myopathic compared to control soleus muscles at all stimulation frequencies. Black circles, myopathic mice; white circles, controls. Values are mean $\pm$ SEM, (n=16 muscles from 8 mice, respectively). *P < 0.05 and ***P < 0.001.

Figure 4. The difference in force production between myopathic and control muscles was decreased during fatigue induced by a series of repeated tetanic contractions
 Force during repeated tetanus in EDL (A) and soleus (B) muscles (n=16 muscles from 8 mice, respectively). Black circles, myopathic mice; white circles, controls. Values are mean $\pm$ SEM, (n=16 muscles from 8 mice, respectively). *P < 0.05 and ***P < 0.001.

Figure 5. Metabolic profile of myopathic and control muscle

Neither the mitochondrial enzyme citrate synthase (A) nor the glycogenolytic enzyme phosphorylase (B) showed any difference in activity between myopathic and control muscles. The black bars indicate myopathic mice, and white bars indicate controls. Values are given in $\mu\text{mol}/\text{min}/\text{mg}$ wet weight and are expressed as mean $\pm$ SEM, ($n=4$ muscles from 4 mice, respectively).