Myosin heavy chain isoform immunolabelling in diabetic rats with peripheral neuropathy

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Received 24 January 2005; received in revised form 25 May 2005; accepted 6 June 2005

Summary
This study evaluated mature and immature myosin heavy chain (MHC) isoform immunolocalisation in soleus muscle of diabetic rats with documented motor neuropathy. Sprague Dawley rats were assigned to one of three groups: control (C), diabetic with insulin (DI), or diabetic without insulin (DNI). Twelve weeks after diabetes induction, soleus muscles were excised and quick-frozen. Cross-sections were labelled immunohistochemically for slow, fast, developmental and neonatal MHC isoforms to determine fiber-type composition. Fiber cross-sectional areas were determined morphometrically. Results revealed that DNI and DI muscles contained greater percentages of myofibers positive for fast MHC compared with controls. DNI animals also showed a lower percentage of myofibers positive for slow MHC compared to the DI group. The number of fibers immunolabelled for developmental MHC isoforms was greater in DNI animals than in the other groups. The differences in slow and fast MHC-labelling appear to indicate a condition of altered neuromuscular activity affecting the diabetic muscles. The increase in developmental MHC-labelling in the DNI muscles could indicate myofiber regeneration or reinnervation that would be more pronounced in the DNI animals in context of their more severe neuropathy. Insulin appeared to influence muscle fiber cross-sectional area and possibly fiber-type grouping frequency; the potential mechanism for these effects was not elucidated.

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Introduction

Diabetes mellitus (DM) is a condition of disordered glucose metabolism that is characterized by hyperglycemia. Diabetes can be associated with complications including cardiovascular disease, retinopathy nephropathy and neuropathy (Boel et al., 1995; Sheetz and King, 2002). Skeletal muscle is a vital organ system in diabetes because it is responsible for a major portion of glucose uptake from the blood (Mercier et al., 1999). Commonly, the emphasis on skeletal muscle in experimental diabetes has been on its function in glucose metabolism. Less information is available regarding possible changes in fiber types in skeletal muscle in diabetes (Punkt et al., 1999), particularly in experimental models with documented peripheral neuropathy.

Standard techniques for histological evaluation of skeletal muscle include stains for structural characteristics (hematoxylin and eosin—H&E) and for fiber types (myofibrillar ATPase). Immunohistochemical labelling techniques provide additional information about myosin distribution in single myofibers within skeletal muscles by use of antibodies to the myosin heavy chains (MHC). It allows identification of co-localisation patterns of individual MHC isoforms within single fibers in serial sections (Sawchak et al., 1989; Staron, 1997). Immunohistochemistry also allows visualization of fiber-type grouping that may not be shown by the myofibrillar ATPase technique (Sawchak et al., 1989).

It was therefore the purpose of this study to evaluate MHC composition, the co-localisation patterns and selected other histological characteristics of skeletal muscle in diabetic rats with documented peripheral neuropathy. It was hypothesized that the MHC composition would be distinct in diabetic neuropathic muscle compared with controls. It was also hypothesized that, due to the neuropathy, the diabetic muscle would exhibit structural and/or anatomical differences in comparison with the controls.

Materials and methods

Animal protocol

Twenty-three male Sprague Dawley rats (Charles River, Wilmington, MA) of weights 200–250 g were randomly assigned to control or diabetes groups. Diabetes was induced by intravenous administration of the pancreatic beta cell toxin streptozoto-
cin (STZ), 50–60 mg/kg. (Sigma, St. Louis, MO) in saline solution. Control rats received intravenous saline injections. To be included in the study, plasma glucose of 250 mg/dl or greater was required. After verification of consistent hyperglycemia on blood drawn for 3 days after STZ injection, plasma glucose determinations in the diabetics were obtained three times weekly by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Palo Alto, CA). Blood for these determinations was collected 12–16 h after the insulin injection. This timing was selected to coincide with the estimated peak insulin effect. No insulin was administered to one group of diabetics (diabetic without insulin—DNI, n = 7). Neutral Protamine Hagedorn insulin (Novolin, recombinant DNA origin, Novo Nordisk Pharmaceuticals, Inc., Princeton, NJ) was administered to the other diabetic group (diabetic with insulin—DI, n = 8) based on a sliding scale (Mayer and Tomlinson, 1983; Fink et al., 1987). The dose of insulin administered was sufficient to prevent life-threatening metabolic complications but was not intended to provide tight glucose control (Weis et al., 1995). Insulin was administered daily for serum glucose > 200 mg/dl and given at the beginning of the animals’ active phase in the evening. Serum glucose monitoring and insulin administration were continued for 12 weeks. Serum glucose was determined in control rats (C, n = 8) at the beginning and end of the experimental period. All animals were fed standard rat chow and water ad libitum. They were all housed in the same room. Inverted 12 h dark–light cycles occurred according to standard animal care protocol at the University of Minnesota. The rats were weighed at the beginning and end of the experimental period. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

At the end of the 12-week experimental period, each animal was deeply anesthetized with intraperitoneal sodium pentobarbital (Nembutal sodium, Fisher Scientific, Fair Lawn, NJ) 50–60 mg/kg. Electrophysiologic testing was performed on the left leg to determine nerve conduction velocities in the tibial and sciatic nerves. The procedure used was that of Brown et al. (1980) with minor modifications (Snow et al., 2001). Rats were positioned prone on a heating pad; left hindlimbs were positioned in hip abduction and knee extension, with ankle plantar flexed. A proximal stimulating electrode was placed over the left sciatic nerve, which was visualized through a small incision in the gluteal muscles. A recording electrode was placed intramuscularly in the medial plantar...
surface of the foot using consistent anatomic landmarks. Reference electrodes were placed subcutaneously near the lumbosacral spine, and in the left third toe tip. A ground electrode was placed under the abdomen. Supramaximal square wave stimuli of 0.05 ms duration were applied. Five determinations of latency were made and mean values calculated. Care was taken to avoid trauma to the soleus muscle during these procedures. After the completion of the electrophysiologic testing, and with the animal still deeply anesthetized, the left soleus muscles were excised, weighed, placed on corks in OCT, and snap frozen in isopentane over liquid nitrogen. Specimens were stored at −80 °C until cross-sections were cut.

**Tissue staining and analysis**

Muscle cross-sections were taken from mid-belly, sectioned at 10 μm using a cryostat (Leica Microsystems, Nussloch, Germany) at −20 °C, and placed on gelatinized slides. The tissue was stained routinely using H&E for morphological assessment. Immunohistochemical labelling for MHC isoforms was performed using monoclonal antibodies to slow, fast, developmental and neonatal MHCs (Novocastra, Newcastle upon Tyne, UK). Antibody dilutions used were as follows: slow 1:20, fast 1:10 developmental 1:10 and neonatal 1:10. Controls were included in which the primary antibody incubation step was omitted. Fibers were typed according to the MHC isoform combinations they displayed (Fig. 1).

Digital images were obtained of cross-sections that were matched for cells that could be identified across the four MHC labels and the H&E stain. The images were obtained using a microscope at 225 × magnification. These images were used to determine fiber types and cross-sectional areas. An average of 300–400 cells were counted per muscle. Fiber types were identified initially by each of the four fiber types (slow, fast, developmental, neonatal). Identification was further broken down into combinations of MHC co-labelling (slow+fast, fast+developmental, etc.), and fiber-type percentages were calculated from the numbers of myofibers counted.

Due to small proportion of cells that labelled for developmental MHC, further quantification of these fibers was performed by taking digital images using a microscope at 180 × magnification. The number of images taken was the largest number that could fit onto a whole cross-section of each muscle labelled for developmental MHC. The total number of developmental MHC-labelling cells was tabulated for each muscle cross-section and expressed as number/mm².

Digital images of the sections stained for H&E were analyzed for muscle fiber shape, nucleus position, connective tissue amount and distribution, presence of inflammatory infiltrates, and indications of fiber necrosis. The sections labelled for MHC were additionally evaluated for presence or absence of fiber-type grouping, an indicator of reinnervation (Karpati and Engel, 1968; Kugelberg et al., 1970; Jaweed et al., 1975). For the purposes of this report, fiber-type grouping was defined as larger aggregations of fibers with similar MHC isoform labelling than present in controls (Karpati and Engel, 1968; Kugelberg et al., 1970).

**Statistical analysis**

Descriptive statistical analysis included means and standard errors of the means. Differences between groups were determined using one-way ANOVA. If a significant overall F-test was obtained, post hoc comparisons were performed with the Fisher’s LSD test. If the data did not meet the assumptions of ANOVA, Kruskal–Wallis one-way ANOVA on ranks was used. Statistical analyses were performed using SPSS (Chicago, IL) statistical program. Significance level was set at p ≤ 0.05.
Results

Animal characteristics

Body weights, soleus weights, soleus/body weight ratios, serum glucose values, and motor nerve conduction velocities are detailed in Table 1. Body weights and soleus weights were significantly lower in the diabetics without insulin than in the other groups. However, when soleus weight was normalized to body weight, there was no difference between groups. Serum glucose values were significantly greater in the diabetics than in controls, but there was no difference between diabetic groups. Mean insulin dose for the DI group was 2.3 units/day. Motor nerve conduction velocities were 21% slower in the DNI group than in controls. Motor nerve conduction velocities in the DI group were 15% slower than in controls.

Fiber types

When all slow MHC-labelling fibers were considered (labelling for slow MHC alone or co-labelling), the DNI animals showed a smaller percentage of slow fibers than did the C or DI groups (Fig. 2). There was no difference in the percentage of total slow MHC-labelling between the DI and C groups. When all fast MHC-labelling fibers were evaluated, the diabetics in both groups showed higher percentages than did the control group (Figs. 2 and 3). There were no significant differences between groups in the percentages of developmental or neonatal MHC isoform labelling. It is noted, however, that, when expressed as number of developmental MHC fibers/mm^2, there were significantly greater numbers of developmental MHC-labelling fibers in the DNI animals than in the other two groups (Figs. 4 and 5).

The patterns of single mature MHC isoform labelling, but not co-labelling, were different in the diabetic muscles compared with controls (Fig. 6). Both diabetic groups showed a significantly lower proportion of fibers labelling for only the slow MHC isoform compared to controls. Fibers co-labelling for both slow and fast MHC isoforms were present in all three groups and did not differ in proportion between groups. Fibers labelling for only the fast MHC isoform were found in greater proportions in the DNI group than in controls.

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean serum glucose (mg/dl)</th>
<th>Body weight (g)</th>
<th>Soleus weight (mg)</th>
<th>Soleus weight/body weight (g/g × 10^3)</th>
<th>Motor nerve conduction velocity (m/s) (sciatic–tibial nerve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 8)</td>
<td>127 ± 6</td>
<td>553 ± 26</td>
<td>230 ± 14</td>
<td>0.419 ± 0.020</td>
<td>59.8 ± 2.5</td>
</tr>
<tr>
<td>DI (n = 8)</td>
<td>309 ± 21^a</td>
<td>492 ± 35</td>
<td>216 ± 10</td>
<td>0.446 ± 0.019</td>
<td>51.1 ± 3.1^b</td>
</tr>
<tr>
<td>DNI (n = 7)</td>
<td>339 ± 17^a</td>
<td>373 ± 17</td>
<td>165 ± 14^a</td>
<td>0.440 ± 0.020</td>
<td>47.1 ± 2.7^a</td>
</tr>
</tbody>
</table>

Values presented are mean ± s.e.m. Mean serum glucose values were obtained from all glucose determinations over the 12-week experimental period. Body weights and muscle weights were obtained at the time of sacrifice.

^aDenotes significantly different from controls p < 0.01.

^bDenotes significantly different from controls p < 0.05.
All the myofibers positive for the immature MHC isoforms were co-labelled with mature MHC isoforms; no fibers were found that labelled for the developmental or neonatal isoforms alone. The most common pattern of immature MHC labelling was slow+fast+developmental positive (0.4% of the overall total in C, 1.0% in DI, 1.4% in DNI). The next most common patterns of labelling were for both slow+developmental positive (0.3% in C, 0.2% in DI, 0.8% in DNI) and fast+developmental positive (0.1% in C, 0.2% in DI, 0.8% in DNI).

**Fiber cross-sectional areas**

Myofibers labelling for mature MHC isoforms were smaller in the DNI group than in DI rats (Fig. 7). DNI myofiber cross-sectional areas were also smaller than controls except for the slow-only myofibers. The myofibers labelling for only the mature MHC isoforms were larger than the fibers labelling for immature MHC isoforms (range of means, mature fibers = 2289–4095 \( \mu \text{m}^2 \); range of means, immature fibers = 1454–3533 \( \mu \text{m}^2 \)). Variability co-efficients for overall fiber cross-sectional areas were as follows: C = 14.7%, D = 9.3%, DNI = 20.6%. Histograms of these data did not reveal much difference in the variability of cross-sectional areas between groups (Fig. 8).

**Qualitative histological characteristics**

H&E staining revealed evidence of greater myofiber pathology in the diabetic animals compared to controls. These findings included small, angulated fibers, pale fibers and inflammatory cell infiltrates (Fig. 9). Signs of myofiber regeneration such as central nuclei were seen occasionally in the soleus from the diabetics, but did not regularly...
coincide with myofibers labelling for immature MHC isoforms. The muscles from six DI animals showed small groups of either slow/fast, or slow+fast+developmental fibers (Fig. 10). Only one DNI animal exhibited a fiber-type group, but it was large and consisted of myofibers co-labelling for the slow, fast and developmental MHC isoforms (Fig. 11). It was found, particularly in the fiber-type groups in the DI animals, that fast or developmental MHC labelling appeared to change within the same fiber along the length of the muscle.

Discussion

In this study of soleus muscle in rats with diabetic neuropathy, it was shown that fast MHC immunolabelling was increased in both groups of diabetic animals, those given insulin and those not given insulin. In contrast, the number of myofibers labelling for developmental MHC increased only in the rats not given insulin. The overall proportion of slow MHC isoform labelling decreased only in the DNI animals as well. Myofibers labelling for fast MHC alone or co-labelled with other MHC isoforms had smaller cross-sectional areas in the DNI animals than those in the other two groups. Myofibers labelling for immature MHC showed no difference in mean cross-sectional areas between groups, although their areas were generally smaller than those of fibers labelling for mature MHC isoforms. The muscles of the rats given insulin exhibited more frequent fiber-type grouping than did the other animals, and the fiber-type groups appeared relatively small. Other qualitative findings in the diabetic muscles included the presence of small, angulated fibers and non-uniform MHC labelling.
along the length of some fibers. Body weight and soleus muscle weight were smaller in the DNI rats compared with the others, but this difference disappeared when presented as the ratio of muscle weight to body weight.

Animal characteristics

The findings on body weight are consistent with those of many prior studies that show decreased body weight in diabetic rats not given insulin (Armstrong et al., 1975; Hegarty and Rosholt, 1981; Cotter et al., 1989; Medina-Sanchez et al., 1991). These data indicate the known effect of insulin as an inhibitor of general protein degradation (Charlton et al., 1997; Zhang et al., 1999).

Mature fiber types, general patterns

There are few studies of myofiber type in muscle of the STZ rat model of diabetes (Armstrong et al., 1975; Chao et al., 1976; Cotter et al., 1989; Medina-Sanchez et al., 1991; Morris et al., 1996; Punkt et al., 1999). These studies differ in the muscles and techniques used; several were done prior to the advent of immunohistochemical techniques for analysis of MHC protein labelling. Therefore, comparison of the current results to prior studies is difficult. One study, however, employed acrylamide gel electrophoresis (SDS-PAGE) to determine MHC composition in soleus of STZ-diabetic rats not given insulin (Punkt et al., 1999). The results showed that diabetes was associated with a decreased percentage of fibers containing slow MHC isoforms, and an increased percentage of fibers containing fast MHC isoforms. The authors concluded that such results could be consistent with decreased activity or with neuropathy. These findings are similar to the fiber-type characteristics of several STZ diabetic muscles concluded that alterations in fiber types were similar to those seen in denervated muscle (Chao et al., 1976).

Denervated soleus exhibits an increased percentage of fast fiber types (Jaweed et al., 1975; Jakubiec-Puka et al., 1990; Talmadge et al., 1995; Bodine and Pierotti, 1996) and a decreased percentage of slow fiber types (Jakubiec-Puka et al., 1990; Talmadge et al., 1995; Bodine and Pierotti, 1996). The increased proportion of fast MHC isoform in both DNI and DI groups and the decreased proportion of slow MHC isoform expression in the DNI animals therefore appear similar to fiber-type composition described in denervated soleus. However, further study will be needed to more specifically determine the degree to which denervation influences the fiber-type composition in diabetic, neuropathic muscle.

Other factors affecting neuromuscular activity could contribute to the changes in MHC isoform-labelling patterns described here. One such factor is decreased muscle loading due to inactivity (Punkt et al., 1999; DeSchene et al., 2001). It has recently been demonstrated that chronic endurance exercise does not alter the increased proportion of fibers labelling for fast MHC in diabetic, neuropathic rats given insulin (Snow et al., 2004). Therefore, the increased fast MHC labelling in the diabetic animals may be more a manifestation of neuropathy than of inactivity. However, endurance training may be performed in varying degrees of intensity, and it may be that moderate intensity training may not be sufficient to influence the fiber-type shifts seen in this model of diabetes. Additional investigation will be needed to further delineate specific mechanisms by which these factors influence fiber type composition.

Mature MHC Co-labelling

All animal groups had a small percentage of myofibers that co-labelled for slow with fast MHC.
isoforms. Because proportions of these fibers did not differ between groups, it seems likely that this is a normal finding. Such co-localization has been previously described in normal rodent muscle (Schiaffino and Reggiani, 1996; Baldwin and Haddad, 2001).

Immature MHC localization

The low levels of developmental MHC positive fibers in control soleus are in agreement with previous findings (Wanek and Snow, 1995; Wanek and Snow, 2000). It is postulated that this result is due to the postural function of the soleus, in which a small amount of muscle injury and regeneration occurs in an ongoing fashion.

The presence of developmental MHC has also been described in mature adult muscle under conditions of denervation and regeneration (Jakubiec-Puka et al., 1990; Schiaffino and Reggiani, 1994, 1996). The DNI animals in the current study exhibited an increased number of myofibers labelling for the developmental MHC isoform compared to both C and DI animals. This finding is consistent with increased muscle regeneration in the context of diabetic neuropathy. Since the DNI rats exhibited a more severe neuropathy than did the DI group, it is possible that myofiber degeneration and regeneration in these muscles was more pronounced as well. The specific etiology of these findings is not yet clear. Muscle injury with subsequent repair may be a possibility, as could denervation with reinnervation; additional studies are needed for better understanding of these findings.

Cross-sectional areas

Muscle fiber cross-sectional areas of the DNI rats were smaller in general than those in C and DI animals. It appears that this change may be related to the presence or absence of insulin, but not to the level of hyperglycemia. The anti-catabolic effect of insulin may contribute to these results.

Histologic characteristics

The presence of small angulated fibers in the diabetic soleus muscles is consistent with myofiber changes described in human neuropathic conditions (Dubowitz, 1985). As was seen in diabetic rectus femoris muscle (Medina-Sanchez et al., 1991), the pattern of fast MHC-labelling fibers in the soleus was generally diffuse in the muscles of both diabetic groups. This pattern is consistent with a diffuse and distal neuropathic process.

The fiber-type clustering in the diabetic groups indicates fiber reinnervation (Karpati and Engel, 1968; Jaweed et al., 1975; Sawchak et al., 1989). The difference in the frequency of this grouping between the DI and DNI animals may be associated with the presence or absence of insulin since the level of hyperglycemia was comparable between these groups. Further investigation is needed to better define the effects of varying concentrations of insulin on skeletal muscle MHC expression in the context of diabetic neuropathy.

Variation in MHC expression along the length of a muscle has been described earlier as a feature of denervated muscle (Schiaffino et al., 1988). The occurrence of similar variations in MHC labelling in the DI muscles, along with the occurrence of fiber-type grouping indicates shared characteristics between denervated–reinnervating muscle and these diabetic muscles. However, it is not known if the reasons for these manifestations are identical between denervated muscle and diabetic muscle.

Conclusion

The soleus muscle in animals with diabetic peripheral neuropathy exhibits myofiber types consistent with altered neuromuscular activity, which may be related to a neuropathic process. The presence of increased developmental MHC isoforms in the DNI animals may represent increased fiber regeneration, which may be related to greater fiber degeneration in the more severe neuropathy in these animals compared with the DI animals. The fiber-type grouping pattern differs between DI and DNI animals; this difference may possibly be related more to the presence of insulin than to the degree of hyperglycemia. The mechanisms for the contribution of insulin to these changes are unclear at present.

Acknowledgements

The authors thank Janice Shoeman, Toan-Thien Vo, and Cindy Franzen for their technical assistance. This project was partially funded by a Muscle Center Research Award from the Center for Muscle and Muscle Disorders, University of Minnesota, and NIA training grant in Aging/NIA5T32AG00198.

References

Myosin heavy chains in diabetic neuropathic rats


