Early morphological remodeling of neuromuscular junction in a murine model of diabetes

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Streptozotocin (STZ) is a diabetogenic agent that had been used in an animal model to study insulin-dependent diabetes mellitus and had demonstrated wide variety of physiological effects on peripheral nerves. STZ treatment, in rodents, also resulted in complex structural histochemical and functional alterations in skeletal muscles (21).

The skeletal muscle contractile properties of STZ-treated rats manifested nonuniform modification, depending on the muscle type studied (2–5). For instance, changes included an increase in the contraction and relaxation times in soleus and a decrease in tetanic tension in extensor digitorum longus muscles with no effect on muscle strength and performance (28). Diaphragm contractility was shown to be impaired in STZ-treated rats (14). The reduced activity of skeletal muscle had been correlated with the press function of oxidative fast-twitch fibers (5). Therefore, utilization of sensitive fast-twitch fibers such as dorsiflexor digitorum superficialis muscle may help in detecting electrophysiological modifications and changes in contractile parameters as well as morphological observations at different stages.

Morphological alterations in myofibers of mice with STZ-induced diabetes showed a significant decrease in size that became more pronounced with time. The shift in fiber size toward smaller diameters also developed in a progressive manner over time. It appears that muscle contractile impairments, noticed after STZ, occur in a time-dependent fashion (28). Despite mice with STZ-induced diabetes exhibiting various neuromuscular abnormalities, most of the resultant complications appeared at 8 wk posttreatment. By virtue of the complications, the STZ model in this form was very much time consuming. Further reduction in muscle weight, seen at 8 wk, has added to the problem of detecting muscle strength alterations (11). It appears from the observation that the progress in muscle function occurs in a time-dependent manner.

STZ-induced effects were previously studied in rat using a dose of 200 mg/kg (28). In a formal report, using the same dose of 200 mg/kg and TO (HsdOla;

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Harlan, Bicester, Oxon, UK) mice strain, few skeletal muscle function abnormalities seemed to occur at early stages; however, peak effect appeared at ~8 wk after the initiation of STZ treatment (8). In the present work, our aim was to study the effect of 2 wk of 60 mg/kg STZ treatment in the c57BL mice strain, which could be a more sensitive animal model. Application of larger doses is expected to cause severe hyperglycemia and reduce survival rate. The hypothesis is that, if a different dose of STZ is utilized and another animal species is used, we might be able to detect earlier signs of muscle weakness before muscle weight is lost. Because these signs are usually seen within 8 wk, our question here is whether we can predict them earlier.

In the present work, we have studied both electrophysiological and ultrastructural characteristics of the fast-twitch dorsiflexor muscle fibers after 2 wk of STZ-induced diabetes in adult c57BL mice compared with age-matched, nondiabetic controls. We aimed to identify early signs of morphological changes at the neuromuscular systems that may happen after STZ treatment. By conducting intracellular recording to study the effect STZ on resting membrane potential (RMP) and on the amplitude and frequencies of miniature end-plate potentials (MEPPs) and by examining the ultrastructure of the intramuscular nerves, neuromuscular junction, and muscle fibers, we attempted to correlate morphological signs to functional modifications.

MATERIALS AND METHODS

Animals. Adult male c57BL strain mice (25 ± 0.6 g body wt) were used throughout these experiments. Animals were housed in groups of five in plastic cages with a controlled light and dark cycle of 12 h each at 25°C. Food and water were available ad libitum. Diabetes was induced with a single intraperitoneal injection of STZ (60 mg/kg) into 10 animals. Plasma glucose was determined to confirm the diabetic status (29). A second group of 10 mice served as control. All animals were examined 2 wk later. Animals were handled according to established welfare procedures and were continuously observed for safety by the institution. The Faculty of Medicine and Health Sciences institution research committee granted the study.

Electrophysiological recording. At the time of experiments, mice were anesthetized with urethane (2 mg/g ip), and the dorsiflexor digitorum superficialis muscle was exposed, dissected out, and pinned in a Lucite chamber containing Krebs solution (pH 7.2) kept at 23–25°C. Because transmitter release is affected by muscle stretch, the excised muscle was pinned at 1.1 times the resting length for all experiments. The solution was oxygenated (95% O2-5% CO2) by a gas-lifting device that circulates freshly oxygenated solution, at a rate of 10–15 ml/min without agitating the recording chamber. Glass capillary microelectrodes filled with 3 M KCl were inserted into muscle fibers at the end-plate region to record MEPPs. A combination of oblique and transillumination was used in conjunction with a Leitz-Wild microscope to locate end-plate regions. In addition, a MEPP rise time of <1 ms was used as a criterion for locating end-plate regions. Subsequently, the RMP and MEPPs were recorded in normal Krebs solution, which had the following composition (in mM): 135 NaCl, 5 KCl, 0.5 Ca-glucuronate, 1 MgSO4, 1 NaH2PO4, 15 NaHCO3, and 11 glucose. From each muscle fiber, 200 MEPPs were digitized on-line via an analog-to-digital conversion board (10-kHz sampling rate) in a Northstar Horizon microcomputer, which provided MEPP frequency and amplitude values. In each group, RMPs and MEPPs were recorded from 10 animals, with 20 fibers per animal used.

Electron microscopy. Electron microscopy study was carried out after the physiological experiments were performed. The fixation method was conducted by bathing muscles in situ with 4% glutaraldehyde, in a 0.1 M phosphate buffer of pH 7.2, for 10 min. Later on, the muscles were removed and placed in buffered glutaraldehyde at 4°C for 2 h. The samples were then washed, in 0.1 M phosphate buffer, for 1 h and stained for 3 min for cholinesterase activity according to Tennyson and co-workers (30). The specimens that contained cholinesterase-positive end plates were later dissected free, postfixed for 1 h in 2% osmium tetroxide in a phosphate buffer of pH 7.2, dehydrated, and embedded in Spur. Thin sections (80 nm) of muscles containing end plates were cut, contrasted with 2% alcoholic uranyl acetate for 10 min and with alkaline lead citrate for an additional 10 min. The specimens were examined with a Philips (Eindhoven, The Netherlands) CM-10 electron microscope. Various sections were taken at random from 100 blocks of embedded tissues. Only one thin section containing one end plate was taken for further analysis.

Statistical analysis. Data analysis in the present study pertained to the distribution in the general population. Accordingly, the sample size was the number of different individuals (mice) and not the total number of observations. Observations from a single mouse (experimental unit) were treated as replicates or repeated measures rather than as independent samples. The mean was obtained for each mouse, and then the group mean and SD were calculated using means from different mice in that group. The results from various groups were compared using both t-test and ANOVA. For experiments, the sample size was 10, and the significance level of 0.05 (95% confidence) was considered as a cutoff.

RESULTS

Glucose levels. Two weeks after STZ treatment, diabetic animals exhibited severe hyperglycemia compared with control group (plasma glucose level was 100 ± 10 mg/dl for control mice and 290 ± 12 mg/dl for mice having STZ-induced diabetes for 2 wk; P < 0.01).

Animal weights. After 2 wk of STZ-induced diabetes, mice did not change significantly in their weight from the control groups (25.4 ± 1.1 vs. 24.8 ± 0.9 g). The ratio of the dorsiflexor digitorum superficialis muscle to body weight remained unchanged.

RMP. The RMP was measured in rats with STZ-induced diabetes and compared with that of control mice. RMP of muscle fibers was measured to determine whether the response of membrane ion channels such as Ca2+ and K+ is altered by STZ-induced diabetes. RMP of STZ-induced diabetics was significantly less negative than that of control mice. The values of RMP in both mice with STZ-induced diabetes and control mice are presented in Table 1.

MEPPs. Although there are different modes of MEPP amplitude, the rise time was the same (<1 ms) for all (Fig. 1). MEPP frequencies of flexor digitorum superficialis muscles were found to be lower in mice.
with STZ-induced diabetes compared with control animals (Table 1). MEPP amplitudes also decreased in mice with STZ-induced diabetes compared with control, nondiabetic animals. STZ treatment reduced the unimodal and bimodal significantly, but the small and large modes of MEPP were unaffected. The extent of this reduction on different types of MEPP frequencies is shown in Table 1.

**Ultrastructural studies.** In control mice, nerve terminals containing numerous synaptic vesicles were covered by a Schwann cell and were found to lie within a depression of the muscle fiber (Fig. 2). The muscle sarcolemma extended inward to form junctional folds underneath the nerve terminals (Fig. 2). Two weeks of diabetes affected the neuromuscular junction. Nerve terminals of diabetic mice showed a decreased number of synaptic vesicles and degeneration of mitochondria (Fig. 3). Some nerve terminals were completely devoid of synaptic vesicles with electron-dense bodies and myelin-like figures. The Schwann cell nucleus appeared to intrude into the region that is normally occupied by the synaptic terminal. At the muscle level, mitochondria were swollen, with disorganization of

<table>
<thead>
<tr>
<th>Physiological Parameters</th>
<th>Control Mice</th>
<th>Diabetic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>79.4 ± 3.1</td>
<td>67.1 ± 2.9*</td>
</tr>
<tr>
<td>MEPP amplitude, mV</td>
<td>0.9 ± 0.1</td>
<td>0.4 ± 0.2*</td>
</tr>
<tr>
<td>MEPP frequency, Hz</td>
<td>2.9 ± 0.2</td>
<td>1.0 ± 0.1*</td>
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<tr>
<td>Unimodal MEPPs</td>
<td>1.4 ± 0.1</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>Bimodal MEPPs</td>
<td>1.1 ± 0.2</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>Small-mode MEPPs</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Large-mode MEPPs</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD for 10 control mice and 10 diabetic mice (20 muscle fibers/animal). MEPP, miniature end-plate potential. *Significantly different from control, P < 0.001 (ANOVA).
their cristae and disruption of the T tubules, and myo-fibers had deposition of increasing numbers of glycogen granules (Fig. 3). These STZ-induced ultrastructural changes were also seen in the peripheral intramuscular myelinated axons. These changes included disruption of mitochondria, and the axonal cytoplasm contained an increased number of membranous materials (Fig. 4).

Morphometric analysis. Morphometric analysis at the nerve terminal showed an overall decrease in terminals of diabetic mice. Fiber diameter, nerve terminal area, synaptic vesicle density, and synaptic cleft width were all reduced in STZ-treated mice compared with controls. A quantitative summary of ultrastructural and morphometric results are presented in Tables 2 and 3.

DISCUSSION

STZ treatment in animals appears to serve as a proper model for studying nerves and muscles in diabetes. Two weeks after introduction of STZ in c57BL mice, plasma glucose levels increased to >290 mg/dl without significant changes in body weights. Although reduction in body weight was previously reported, it seems that a period of >2 wk is required to notice significant changes (14). The present project revealed early signs of nerve and muscle modifications appearing even after 2 wk of STZ-induced diabetes. Decreased RMP and reduced MEPP frequencies and amplitudes that were accompanied with significant alteration of neuromuscular junction morphology were evidenced 2 wk after STZ treatment.

The present study confirms that diabetes reduces RMP and conductance of skeletal muscle membrane (16). MEPP frequencies of flexor digitorum superficialis muscles were found to be lower in mice with STZ-induced diabetes compared with control animals. Modifications of MEPP frequencies of nerve terminals are thought to be a result of modifications in Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels in the terminal membrane (25). It may be argued that Ca\(^{2+}\) buffering by intraterminal organelles is altered in diabetes mellitus. Ca\(^{2+}\) appears to be involved in altered muscle contraction of various species, including denervated skeletal muscle of adult rat (18). Similar observations can also be expected in terminals from aging animals compared with terminals from young mice.

It could be argued that STZ affects the nerve presynaptically and the muscle postsynaptically and that the modifications of neuromuscular structure and function become more perceptible with time. For instance, diabetic TO mice were found to have reduced dorsi flexor digitorum superficial twitch tension after 8 wk compared with age-matched controls (11). TO mice are probably inbred strains that have inert resistance to STZ-induced diabetic complications. Similar time periods were also demonstrated by data obtained from rats with STZ-induced diabetes (5, 28).

In the present study, utilization of a different dose of STZ (60 mg/kg) compared with 200 mg/kg and probably a more sensitive species (i.e., c57BL) resulted in earlier signs of muscle weakness before the weight loss that may complicate the STZ animal model. This dose was selected because introduction of 200 mg/kg resulted in severe hyperglycemia and a very low survival rate. Previously, 8 wk posttreatment, STZ induced significant changes in body weight, which in turn may impact muscle structure and function. Thus the present observation serves as a unique model for studying diabetes-
induced changes sooner without significant loss of body weight. The changes observed are similar to neurodegenerative disorders and diabetes-induced complications, but the pattern and time course of appearance seem to be different. Formerly, modification of Ca$^{2+}$ mobilization was reported to result in the loss of tension in muscle fibers of diabetic rats (2). Such a mechanism may also account for detected alterations in muscle function in the present study. Further measurement of intracellular Ca$^{2+}$ is required for confirming such a hypothesis. Morphologically, Ca$^{2+}$ overload has also been shown to induce rapid swelling and disruption of mitochondria (7).

The ultrastructural features of the mature neuromuscular junctions of mice have been described earlier (8). The present study focused on ultrastructural changes in relation to alterations in RMP and MEPPs after 2 wk of STZ treatment in c57BL diabetic mice. The ultrastructural changes observed here are similar to those of previous experiments and support the notion that neuromuscular impairment could be partially achieved as early as 2 wk, possibly via modification of transmitter release mechanisms. The demyelination and the reduction of fiber dimension were comparable to those formerly reported in peripheral nerves and could represent causal factors in slowing the conduction velocity of action potentials (6). Also, the interposition of Schwann cell between nerve terminal and muscle was in agreement with previously seen changes in extraocular muscles in diabetic mutant mice (23). Furthermore, the significant reduction of synaptic vesicles at nerve terminal levels viewed here resembles those in diabetic rats (21).

Various MEPP amplitude distributions have been recognized at the neuromuscular junction. In the present study, after STZ treatment, unimodal and bimodal MEPPs were significantly modified, but the small and large modes of MEPPs were unaffected. The mechanism of different sensitivities of multimodal MEPPs to STZ treatment is not adequately understood. The differential effect is possibly ascribed to variations in transmitter release from partially filled synaptic vesicles and/or partial release of synaptic content of the neurotransmitter. From the data presented, it may be suggested that diabetes produces the neuromuscular impairment, possibly at both pre- and postsynaptic sites, because flexor digitorum superficialis muscles showed decreased RMP and MEPPs. Reduction of MEPP amplitude could be a result of the depolarization of RMP. Changes in synaptic vesicle number that occurred in mice with STZ-induced diabetes are also consistent with the decrease in MEPP frequencies. Additionally, the evidence illustrated here of diabetes resulting in swollen mitochondria with disorganized cristae implies that there is a postsynaptic element for diabetic-induced myopathy. Furthermore, presynaptic contribution, including remodeling of some of the nerve terminal population and reduction in capacity of sustained neuromuscular transmission, cannot be overlooked either. Alterations shown by the present study in the structures of nerves and muscles are related to the functional modifications observed in muscle contractile properties and may contribute to the pathogenesis of diabetic neuromyopathy.

The muscle weakness previously reported in the pathogenesis of diabetes can be attributed to changes in the intramuscular nerves and in the neuromuscular junction (23). Morphologically, mitochondrial degeneration, electron-dense bodies, myelin figures, irregular synaptic vesicles, and breakdown of the presynaptic nerve membrane were all observed in diabetic Wistar rats (23). These changes were similar to alterations reported as signs of nerve degeneration (8, 19) that ultimately affected muscle function (17). Physiologically, impaired calcium handling of muscle membrane (5), changes in threshold potential of the muscle, decreased intracellular ATP concentration (4), and direct effect of the diabetic condition on the contractile regulatory system (28) are suggested as possible mechanisms for muscle STZ-induced weakness. Although physiological and morphological modifications seen in the present study appear to be consistent with altered Ca$^{2+}$ mobilization across muscle membrane, nevertheless other mechanisms such as free radical-mediated actions may also be implicated in STZ-induced changes in skeletal muscle.

The findings of this study are consistent with the earlier reports and also with previous work by our laboratory. The difference is in the manner of occurrence and fact that they can be detected earlier. A chronological description is extremely useful and will allow for further developments with time. However, if any degenerative changes are seen as early as 2 wk, with a further postexposure period a significant impairment in body weight may be observed, a factor that may impact modifications expected at nerve terminal or neuromuscular junction or muscle itself. The inconsistency with the previous work is in terms of the manner of appearance and the occurrence of earlier signs, which can be explained by the utilization of a different animal species and/or the use of a different dose of STZ.

This study described electrophysiological recording (RMP, MEPPs) in STZ-induced diabetes. In the previous work (11), isometric twitch tension was measured, but no electrophysiological data were recorded. It may be found that utilization of different doses of STZ (60 mg/kg compared with 200 mg/kg) in a more sensitive species enables the observation of the signs of muscle weakness before the weight loss that may further complicate alterations seen in an animal model of STZ-induced diabetes. Eight weeks of STZ treatment induces significant changes in body weight, which in turn may impact muscle structure and function. The present observation serves as a unique model for studying diabetes-induced changes sooner without significant loss of body weight. The changes observed are similar to diabetes-induced complications in other models, but the pattern and time course for appearance are different.

The electrophysiological and ultrastructural changes observed here are also similar to various conditions,
such as those of diabetic neuropathy in humans (27), motoneuron disease (15), spontaneously diabetic rats (22), rats with STZ-induced diabetes (2), myotonic rats (24), muscle disuse (8), and steroid-induced stress and aging (9, 12). Both pre- and postsynaptic effects of STZ-induced diabetes, resulting in neuromuscular impairment and muscle weakness, could form the bases for explaining these changes. Exercise programs may improve and strengthen muscles, particularly if introduced at the early stages (10).

REFERENCES