Prosaposin Gene Expression and the Efficacy of a Prosaposin-derived Peptide in Preventing Structural and Functional Disorders of Peripheral Nerve in Diabetic Rats

NIGEL A. CALCUTT, PHD, W. MARIE CAMPANA, PHD, NAHIDA L. ESKELAND, PHD, LIZA MOHIUDDIN, BM, BS, KEVIN C. DINES, PHD, ANDREW P. MEIZIS, PHD, AND JOHN S. O’BRIEN, MD

Abstract. We have recently demonstrated that prosaposin is a neurotrophic and myelinosinotropic factor with the active trophic sequence located at the N-terminal region of the saposin C domain. There are also reports that prosaposin mRNA is increased distal to a physical nerve injury and that exogenous prosaposin treatment induces subsequent neuronal sprouting, suggesting involvement in repair processes. In the present study, we show that prosaposin mRNA is significantly (p < 0.05) elevated in the peripheral nerve of streptozotocin-diabetic rats, a model of insulin-deficient diabetes in which nerve injury arises from the metabolic trauma of hyperglycemia and its consequences. A 14 amino acid peptide derived from the neurotrophic region of prosaposin prevented the development of deficits in both large and small fiber function caused by diabetes in rats. The dose-dependent prevention of nerve conduction slowing by TX14(A) was accompanied by preservation of axonal caliber and sodium-potassium ATPase activity, while prevention of thermal hypoalgesia was associated with attenuation of the decline in nerve substance P levels. It is concluded that nerve subject to the metabolic injury of uncontrolled diabetes responds by increasing prosaposin gene expression, and that prosaposin-derived neurotrophic peptides may provide a novel therapeutic approach to treatment of diabetic and other peripheral neuropathies.

Key Words: Diabetes; Neuropathy; Pain; Prosaposin; Substance P.

INTRODUCTION

Peripheral neuropathy is a common complication of diabetes and represents the most frequently encountered peripheral neuropathy in the developed world. Physiologic disturbances, including slowed nerve conduction velocities (NCV), resistance to ischemic conduction block and elevated thermal perception and thermal pain thresholds (1–3) may occur early in the course of diabetes, while pathologic changes in both axons and Schwann cells are noted as the disease progresses (4).

Slowed nerve conduction (5), resistance to ischemic conduction block (6), and thermal hypoalgesia (7) are also found in diabetic rats within weeks of the onset of hyperglycemia. This resemblance to early human diabetic neuropathy provides experimental end points for both investigation of proposed etiologic mechanisms and evaluation of potential therapeutic strategies. Studies in diabetic rodents have suggested that exaggerated flux through the polyol pathway is the primary etiologic mechanism of many nerve disorders (8), but it is unclear how this leads to the subsequent neurochemical, physiologic, and structural abnormalities. As aldose reductase inhibitors have displayed limited clinical efficacy in treating diabetic neuropathy (9), establishing intermediate steps in the pathogenesis of nerve dysfunction may lead to alternative therapeutic strategies.

It is becoming apparent that adult peripheral nerves receive ongoing neurotrophic support and that diabetes disrupts this process. In diabetic rats, mRNA levels for members of the neurotrophin gene family are altered in the target organs for peripheral nerves (10–12). This is accompanied by decreases in protein levels and bioactivity of a range of neurotrophic factors in both target organs and peripheral nerve (12–14). Impaired supply of neurotrophic factors, coupled with disrupted production of appropriate receptor proteins by peripheral neurons (15–17) and diminished retrograde axonal transport (12, 17, 18) are all likely to contribute to a deficiency in neurotrophic support to peripheral nerves in diabetic rats and have prompted investigation of the therapeutic properties of neurotrophic factors. Treatment of hyperglycemic rats with exogenous nerve growth factor (NGF) improves neupeptide levels in small fibers (12, 19) while brain-derived neurotrophic factor (BDNF) prevents conduction slowing and structural disorders of large motor neurons (20). However, NGF is unlikely to protect against disorders of large sensory or motor neurons such as conduction slowing, as they do not possess the trkA receptor, while BDNF is without effect on neurochemical and functional disorders of sensory nerves in hyperglycemic rats (19, 20). The apparent selectivity of the neurotrophins, coupled with reduced levels of neurotrophin receptors (15–17) may restrict their therapeutic potential for treating the wide range of fiber disorders induced by diabetes. Neurotrophic factors with a broader spectrum of
action may therefore be more useful as agents to treat diabetic neuropathy.

Prosapasin is the precursor of the saposins A, B, C, and D, which are found within lysosomes and mediate the hydrolysis of sphingolipids by activating lysosomal hydrolases (21). Uncleaved prosapasin is also found in the brain and is secreted into cerebrospinal fluid (22). The potential importance of prosapasin to the nervous system is suggested by the severe neurological degeneration noted in humans with a point mutation in the start codon of the prosapasin gene (23, 24) and by the hypomyelination that occurs in prosapasin-deficient mice (25). Prosapasin and saposin C bind to neuroblastoma cells with high affinity, initiate intracellular signaling pathways that involve MAPK activation, and stimulate both neurite outgrowth and choline acetyltransferase activity (26, 27). Prosapasin mRNA is increased in peripheral nerve following injury (28) and treatment with exogenous prosapasin increased the number of regenerating axonal sprouts after sciatic nerve transection (29). The neurotrophic activity of prosapasin has been localized to a 12-amino acid sequence in the amino terminal portion of saposin C and a variety of peptide fragments encompassing this region, termed prosaptides, have been produced (30). These prosaptides are similar to whole prosapasin in their binding kinetics, activation of intracellular signaling pathways, and evoked responses when applied to neuronal cells in culture (27). Prosa
tides may, therefore, also serve as small peptide molecules to mimic the neurotrophic properties of prosapasin in vivo.

The present study investigated whether peripheral nerve responds to the metabolic injury of insulin-deficient diabetes by altering prosapasin mRNA levels, as is the case in normal nerve following physical injury. We subsequently examined the efficacy of a prosapatin in preventing biochemical, functional, and structural disorders of peripheral nerve in diabetic rats.

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures were approved by the local Animal Subjects Committee and used adult (cohort mean body weight = 266 ± 2 g, N = 60 and 267 ± 1 g, N = 50 for 2 major experiments) female Sprague-Dawley rats (Harlan Industries, San Diego, CA). Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg in 0.9% sterile saline) after an overnight fast and hyperglycemia was confirmed 2 days later by measurement of tail vein blood glucose concentration (Ames Glucostix, Myles Inc., Elkhart, IN). Rats were allowed free access to food and water and maintained under a 12-hour (h) light/dark cycle for the duration of the studies. Groups of control and diabetic rats were treated with the prosapatin TX14(A), a 14 amino acid peptide fragment from the neuroactive region of saposin C (TXLIDNNATBEILY; where X = D-alanine, Myelos Neurosciences, San Diego, CA). The peptide was synthesized to 98% purity (AnaSpec, San Jose, CA) and has previously been reported to stimulate both neurite outgrowth and MAPK-associated intracellular signaling pathways (27, 30). TX14(A) was administered thrice weekly (20–1000 μg/kg body weight) by subcutaneous injection in 250 μl of phosphate buffered saline, beginning at confirmation of hyperglycemia. Animals that did not receive TX14(A) were treated with an equivalent volume of vehicle. All animals were treated for an 8-wk period of diabetes, after which a range of biochemical, physiologic, and structural analyses were performed.

Thermal Nociceptive Testing

To determine responses to an acute thermal nociceptive stimulus, rats were placed in an open-top Plexiglas cylinder on top of a Thermal Stimulation System (UARD, San Diego, CA) with a surface temperature of 30°C. A mobile radiant heat source was maneuvered below the plantar surface of the right hindpaw and heat applied until the limb was moved. The latency to response was recorded automatically by movement sensors. Responses were measured 4 times, each measurement being 5 min apart. The first series of measurements represents a period of behavioral adjustment to the new surroundings and the movement of the mobile heat source and tends to be notably different from subsequent responses. The median response time of measurements 2–4 was therefore used for analysis. Treated rats were tested 24–48 h after the last injection with TX14(A) or vehicle for studies of chronic preventative treatment.

Nerve Conduction Studies

Nerve conduction studies were performed on rats anesthetized with halothane (4% in O2 for induction and 2% to maintain) before the injection of streptozotocin and also at the end of the study, 24–48 h after the final injection of TX14(A) or vehicle. A thermistor probe was placed adjacent to the left sciatic nerve via a skin incision and blunt separation of the connective tissue fascia between the biceps femoris and glutus maximus muscles. The incision was closed and local temperature held at 37°C using a heat lamp and temperature controller during stimulation of the sciatic nerve (5V, 0.05 ms single square wave pulses) at the sciatic notch or ankle. Evoked responses were recorded with needle electrodes placed in the interosseous muscles of the ipsilateral foot, amplified (×100) with a P15 AC Amplifier (Grass Instruments, Quincy, MA) and displayed on a 5110 Storage Oscilloscope and 5D10 Waveform Digitizer (Tektronix Inc., Beaverton, OR). The difference in response latency of
the M wave following stimulation at the 2 sites was recorded as the time required for motor nerve conduction to proceed from notch to ankle, and the difference in H wave latency as the time required for sensory conduction to travel from ankle to notch. This procedure was repeated 3 times for each rat and the median latency differences used to calculate conduction velocity. Motor or sensory nerve conduction velocities (MNCV and SNCV) were calculated by dividing distance between stimulation sites by latency difference.

Nerve Laser Doppler Flux

Rats were anesthetized with an intraperitoneal injection (2 ml/kg) of a solution consisting of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in 0.9% sterile saline 24–48 h after the final injection of TX14(A) or vehicle. Core temperature was maintained at 37°C using a heating pad, rectal probe, and temperature controller. Mean arterial pressures were measured with a MacLab/8s system and transducer amplifier after cannulation of the right femoral artery. The left sciatic nerve was exposed and a pool of mineral oil applied over the exposed tissues. Temperature readings were made with a thermistor probe placed in the mineral pool close to the sciatic nerve. When temperature had stabilized at 32°C, a laser Doppler probe (0.85-mm diameter attached to TSI Blood Perfusion Monitor Model 403 A, St Paul, MN) was positioned with a micromanipulator above the surface of the mid-thigh region of the sciatic nerve. Ambient lighting was kept constant and 5 measurements were made at 1-mm increments along the nerve. Laser Doppler flowmetry was chosen because the technique readily yields measurements that are proportional to nerve blood flow without the prolonged anesthesia or surgery required by other methods that measure nerve blood flow and because there is no direct trauma to the nerve. The probe was placed to avoid large and/or obvious epineurial vessels and had a tissue penetration depth of 1 mm, thereby measuring flow within the whole fascicle (31). As nerve blood flow values vary according to arterial pressure, laser Doppler flow was normalized to a particular arterial pressure at each time point as laser Doppler vascular conductance (LDVC) in arbitrary Doppler flow units/mm Hg. The mean of the 5 measurements was taken to represent nerve LDVC.

Sugar Assays

Blood samples were collected from the femoral artery of anesthetized rats for determination of plasma glucose concentration by spectrophotometric assay (glucose kit, Sigma, St Louis, MO). Segments of the left sciatic nerve were removed and stored at −70°C before assay. Nerve sugars and polysols were measured as their trimethylsilyl derivatives with α-methyl mannose as an internal standard, exactly as described elsewhere (31), and values referenced to nerve dry weight.

Northern Analysis

RNA was isolated from the sciatic nerve of individual control or diabetic rats using theTotally RNA kit (Ambion, Austin, TX). 10 μg samples of total RNA were denatured in 50% formamide and 2.2 M formaldehyde solution before electrophoresis in a 1.1% agarose gel containing 2.2% formaldehyde, 20 mM morpholinopropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA at pH 7.0. RNA samples were transferred to nylon membranes (Stratagene, San Diego, CA) and UV cross-linked before prehybridization with Denharts solution containing 0.5% SDS, 500 μg denatured salmon sperm DNA and 50% formamide. This was followed by hybridization with [32P]-labeled prosaposin cDNA probe (a 650-bp fragment of the rat SGP-1 cDNA that was kindly donated by Dr. Carlos Morales, McGill University, Montreal, Canada). Membranes were then washed in hybridization buffer before exposure onto Biomax MS film (Eastman Kodak, Rochester, NY) via an intensifying screen. Blots were reprobed for mRNA of 18S ribosomal RNA using a [32P]-labeled cDNA probe (# 1227, ATCC, Bethesda MD). Densitometric quantification was performed to provide the ratio of mRNA for prosaposin to that of 18S ribosomal RNA; levels of which do not significantly change in diabetic nerve (5.7 ± 0.5 vs control = 5.8 ± 0.4 densitometric units per 10 μg total RNA loaded per lane, N = 3/group).

Enzyme and Immuno Assays

For assay of substance P content, nerve segments were transferred to boiling 1.0 M acetic acid for 5 min, homogenized, and boiled for a further 5 min. Homogenates were centrifuged (3,000 × g, 10 min) and the supernatant freeze-dried. Samples were reconstituted in assay buffer and substance P content measured in triplicate by ELISA using a commercial kit (Cayman Chemical Company, Ann Arbor, MI). Sodium-potassium (Na/K)-ATPase activity was measured in homogenates of previously stored (−70°C) sciatic nerve as the ouabain-inhibitable fraction of total nerve homogenate ATPase activity, as determined by coupling ATP hydrolysis to NADH oxidation (32). Enzymatic activity was calculated as the rate of ADP production per unit time. Both nerve substance P content and Na/K ATPase activity were expressed relative to nerve length.

Morphometry

Anesthetized rats were perfused through the heart with 200 ml of phosphate-buffered saline (37°C) followed by 250 ml of 2.5% phosphate-buffered glutaraldehyde (37°C). The right sciatic nerve was removed and fixed.
overnight in 2.5% phosphate-buffered glutaraldehyde (4°C). Tissue was postfixed in 1% aqueous osmium tetroxide for 3–4 h before dehydration using a series of graded alcohols and propylene oxide. After infiltration with a 1:1 mixture of propylene oxide and araldite for 4 h, nerves were placed in 100% araldite overnight before embedding in fresh araldite resin. Thick sections (1 μm) were stained with p-phenylenediamine prior to light microscopic examination. Computer-assisted analyses of myelinated fiber size-frequence distributions were performed on a single section, sampled from the tibial fascicle midway between the sciatic notch and popliteal fossa. Video images from a light microscope were captured via a television camera and analyzed using NIH Image 1.55 software. Nonoverlapping fields (at ×40 objective) were sampled by a systematic serpentine progression across the whole fascicle. All axons of myelinated fibers greater than 1 μm diameter were identified and sorted into size-bins by an automated process (20). Approximately 3,000 myelinated fibers were examined in each tibial fascicle, representing about 60% of the total number of fibers present.

Statistical Analysis

All measurements were performed on animals that were coded to avoid the possibility of bias. Statistical analyses were performed by unpaired t test or one factor analysis of variance as appropriate. When the F ratio indicated p < 0.05, post hoc analysis was performed using the Student-Newman-Keuls test or Dunnett test to compare multiple drug doses against untreated diabetics. Data are presented as mean ± SEM.

RESULTS

Prosaposin Gene Expression in Rat Sciatic Nerve

Northern analysis detected mRNA for prosaposin in the rat sciatic nerve (Fig. 1) and densitometric quantification indicated that levels were significantly (p < 0.05) higher after 8 wk of diabetes than in age-matched controls (Fig. 2). This finding prompted studies involving treatment of control and diabetic rats with prosaptide TX14(A).

Effects of TX14(A) on Control and Diabetic Rats

After 8 wk of untreated diabetes, rats had reduced body weight compared with untreated controls, were hyperglycemic, and accumulated glucose, sorbitol, and fructose in the sciatic nerve with concomitant myo-inositol depletion (Table 1). Blood pressure and heart rate were unaffected by diabetes whereas sciatic nerve LDVC was significantly reduced compared with controls. Treating control or diabetic rats with 1 mg/kg TX14(A) thrice weekly for 8 wk had no significant effect on any of the above parameters, although TX14(A) treatment slightly attenuated weight loss and hyperglycemia in diabetic rats.

Hindpaw thermal response latencies, measured after 8 weeks of diabetes, were significantly (p < 0.05) increased in untreated diabetic rats compared with untreated controls (Fig. 3). TX14(A) had no effect on thermal response latencies in control rats but prevented the increased response latency in the hindpaw of diabetic rats so that values were not different from controls and significantly (p < 0.05) lower than untreated diabetics. At the end of the 8-wk study, NCV had not changed markedly in the untreated control animals compared with values at the
TABLE 1
Effects of Diabetes and TX14(A) Treatment on Body Weight, Plasma Glucose, Nerve Sugars, Polyols, and Vascular Function

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>Body weight (g)</th>
<th>Plasma glucose (mmol/l)</th>
<th>Nerve glucose (mmol/mg dry weight)</th>
<th>Nerve sorbitol (mmol/mg dry weight)</th>
<th>Nerve fructose (mmol/mg dry weight)</th>
<th>Nerve myoinositol (mmol/mg dry weight)</th>
<th>Blood pressure (mmHg)</th>
<th>Heart rate (bpm)</th>
<th>Sciatic LDVC (U/mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (14)</td>
<td>280 ± 5a</td>
<td>6.4 ± 0.4a</td>
<td>7.1 ± 0.4a</td>
<td>3.5 ± 0.2a</td>
<td>9.3 ± 0.4a</td>
<td>102 ± 4</td>
<td>315 ± 8</td>
<td>0.45 ± 0.05a</td>
<td></td>
</tr>
<tr>
<td>Control (15)</td>
<td>280 ± 5a</td>
<td>6.2 ± 0.3a</td>
<td>6.3 ± 0.3a</td>
<td>3.5 ± 0.2a</td>
<td>9.2 ± 0.5a</td>
<td>102 ± 3</td>
<td>313 ± 7</td>
<td>0.43 ± 0.03a</td>
<td></td>
</tr>
<tr>
<td>+TX14 (A)</td>
<td>214 ± 9b</td>
<td>39.6 ± 2.8b</td>
<td>55.7 ± 4.3b</td>
<td>8.8 ± 0.7b</td>
<td>27.1 ± 1.4b</td>
<td>7.0 ± 0.3b</td>
<td>94 ± 2</td>
<td>319 ± 6</td>
<td>0.24 ± 0.03b</td>
</tr>
<tr>
<td>Diabetic (13)</td>
<td>195 ± 8b</td>
<td>33.9 ± 2.9b</td>
<td>46.6 ± 3.6b</td>
<td>7.9 ± 0.8b</td>
<td>27.4 ± 2.1b</td>
<td>6.5 ± 0.2b</td>
<td>96 ± 3</td>
<td>313 ± 5</td>
<td>0.20 ± 0.02b</td>
</tr>
<tr>
<td>+TX14 (A)</td>
<td>214 ± 9b</td>
<td>33.9 ± 2.9b</td>
<td>46.6 ± 3.6b</td>
<td>7.9 ± 0.8b</td>
<td>27.4 ± 2.1b</td>
<td>6.5 ± 0.2b</td>
<td>96 ± 3</td>
<td>313 ± 5</td>
<td>0.20 ± 0.02b</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Statistical significance by ANOVA with Student Newman Keuls post hoc test. nd = not detected.

This study was performed to establish the lowest effective dose of TX14(A) on nerve dysfunction in diabetic rats. Dose-effect of TX14(A) in Diabetic Rats, showing that the study was performed in adult animals with mature peripheral nerves. Both MNCV and SNCV were significantly lower in diabetic rats compared with untreated controls. In diabetic rats, TX14(A) treatment had no effect on MNCV or SNCV in control rats but attenuated the decrease in diabetic rats so that values were significantly lower (p < 0.05) than controls.

Both MNCV and SNCV were significantly lower in untreated diabetic rats compared with untreated controls. In diabetic rats, TX14(A) treatment had no effect on MNCV or SNCV in control rats but attenuated the decrease in diabetic rats so that values were significantly lower (p < 0.05) than controls.

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rats. After 8 wk of diabetes, rats were hyperglycemic, and both body weight and weight of the extensor digitorum longus (EDL) muscle were lower than age-matched controls, indicating that muscle wasting contributed to the general weight loss (Table 2). TX14(A) treatment was again without significant effect on these parameters, although a trend towards attenuated hyperglycemia, body weight loss, and atrophy of the EDL muscle was noted in diabetic rats treated with the highest dose of TX14(A). The significantly (p < 0.01 vs control) increased hindpaw thermal response latency of untreated diabetic rats (Fig. 5) was dose-dependently reduced by TX14(A) and completely prevented at doses of either 200 μg/kg and 1000 μg/kg (both p < 0.01 vs untreated diabetics). Thermal hypoalgesia in diabetic rats was accompanied by a significant (p < 0.01) decrease in nerve substance P levels which was dose-dependently attenuated by TX14(A) (Table 2). In a separate study, no effects on sciatic nerve substance P levels were seen in control rats treated with 1,000 μg/kg TX14(A) for 8 wk (controls = 322 ± 42 vs control + TX14(A) = 364 ± 55 pg/cm: N = 7/group).

TX14(A) treatment dose-dependently attenuated the reduction in MNCV and SNCV of diabetic rats from pre-diabetic values (Fig. 6). Reduced NCVs in diabetic rats were accompanied by significantly (p < 0.01) decreased sciatic nerve homogenate Na/K-ATPase activity compared with controls that was attenuated by TX14(A), although not in a dose-dependent manner (Table 2). Morphometric analysis of axonal caliber in the mid-thigh sciatic nerve indicated a trend towards reduced mean axonal diameter in diabetic rats that was absent in TX14(A)-treated rats (Table 3). Construction of axonal size-frequency profiles revealed that diabetic rats had a significant (p < 0.05) reduction in the frequency of large myelinated axons and increased frequency of small and medium-sized (p < 0.05) axons compared with controls (Table 3). Treatment of diabetic rats with 1,000 μg/kg TX14(A) prevented the decline in frequency of large myelinated axons compared with untreated diabetics (p < 0.05).

DISCUSSION

Whole prosaposin is found in milk, seminal plasma, and cerebrospinal fluid (22), suggesting a secretory pathway distinct from its primary role as the precursor for lysosomal saposins (21). Prosaposin is immunolocalized to smooth muscle cells surrounding blood vessels within peripheral nerve (33) and, following injury to peripheral nerves, there is an increase in both nerve prosaposin mRNA levels (28) and prosaposin protein in extracellular fluid (33). Because prosaposin possesses neurotrophic activity (30) and local delivery of prosaposin to injured nerve enhances sprouting (29), it appears that prosaposin is a peripheral nerve-derived neurotrophic factor.

The twofold induction of prosaposin mRNA in the sciatic nerve of diabetic rats is similar to that noted following physical nerve injury to normal rats (28). This increase could arise from either increased transcription or stability of mRNA, and may remain exclusive to the vascular smooth muscle cells that contain prosaposin immunoreactivity in normal nerve (33), or incorporate other cells whose nuclei are found in peripheral nerve such as Schwann cells. Until reliable methods are developed, we cannot confirm any subsequent increase in translation leading to additional prosaposin protein. However, should increased translation occur, the increased production of prosaposin after nerve crush and during diabetes may suggest a reparative response to injury or loss of normal trophic support, as has been suggested for the elevated mRNA levels of BDNF in the soleus muscle of diabetic rats (11). Alternately, increased production of prosaposin protein could provide a precursor for lysosomal saposins,

**TABLE 2**

Effects of TX14 (A) on Body weight, Plasma Glucose, EDL Muscle Weight, and Sciatic Nerve Substance P and Na/K ATPase Activity in Diabetic Rats

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Body weight (g)</th>
<th>Plasma glucose (mmol/l)</th>
<th>EDL weight (mg)</th>
<th>Substance P (pg/cm)</th>
<th>Na/K ATPase activity (μmol/min/cm)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>292 ± 5*</td>
<td>9.6 ± 0.7*</td>
<td>119 ± 4*</td>
<td>182 ± 29*</td>
<td>2.65 ± 0.40*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>10</td>
<td>201 ± 6*</td>
<td>42.4 ± 1.2*</td>
<td>71 ± 2*</td>
<td>53 ± 7*</td>
<td>1.25 ± 0.18*</td>
</tr>
<tr>
<td>Diabetic + 20 μg/kg TX14(A)</td>
<td>9</td>
<td>218 ± 6</td>
<td>36.0 ± 1.9*</td>
<td>78 ± 4</td>
<td>44 ± 8</td>
<td>1.66 ± 0.24</td>
</tr>
<tr>
<td>Diabetic + 200 μg/kg TX14(A)</td>
<td>7</td>
<td>218 ± 15</td>
<td>40.4 ± 1.7</td>
<td>78 ± 8</td>
<td>88 ± 15</td>
<td>2.33 ± 0.15</td>
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<tr>
<td>Diabetic + 1000 μg/kg TX14(A)</td>
<td>9</td>
<td>230 ± 13</td>
<td>36.6 ± 2.0*</td>
<td>86 ± 8</td>
<td>124 ± 19*</td>
<td>1.44 ± 0.18</td>
</tr>
<tr>
<td>Statistical significance</td>
<td></td>
<td></td>
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<tr>
<td><strong>p &lt; 0.001</strong></td>
<td></td>
<td><strong>p &lt; 0.001</strong></td>
<td><strong>p &lt; 0.001</strong></td>
<td><strong>p &lt; 0.001</strong></td>
<td><strong>p &lt; 0.001</strong></td>
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</table>

Data are mean ± SEM. Statistical analysis by ANOVA with Dunnetts post hoc test.
TABLE 3

Sciatic Nerve Mean Axonal Diameter and Size-Frequency Distribution

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean axonal diameter (µm)</th>
<th>Relative proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤3 µm</td>
<td>3–7 µm</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>4.80 ± 0.08</td>
<td>21.5 ± 1.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>9</td>
<td>4.48 ± 0.12</td>
<td>24.1 ± 1.7</td>
</tr>
<tr>
<td>Diabetic + TX14 (A)</td>
<td>8</td>
<td>4.76 ± 0.09</td>
<td>20.3 ± 1.1</td>
</tr>
</tbody>
</table>

* vs Statistical significance, p < 0.05
* * vs Statistical significance, p < 0.01

Data are mean ± SEM. Statistical significance by ANOVA with Student Newman Keuls post hoc test.

![Graph showing glucose levels](image)

**Fig. 5.** Thermal response latencies of control and 8-wk diabetic rats ± treatment with TX14(A). Data are mean ± SEM of N = 7–10/group. * = p < 0.01 vs untreated diabetics.

perhaps reflecting an increased sphingolipid turnover in diabetic nerve. Supporting this possibility are reports suggesting that turnover of myelin constituents is increased in the nerves of diabetic rats (34, 35), although morphologic and morphometric studies have failed to demonstrate obvious disruption of the myelin sheath in the sciatic nerve of such animals (36).

To address the possibility that additional neurotrophic support from prosaposin may be of benefit to peripheral nerve during hyperglycemia, we performed a series of studies where diabetic rats were treated with TX14(A), a peptide fragment of the neurotrophic region of the saposin C domain of prosaposin. TX14(A) was chosen as it replicates the biological properties of prosaposin when applied to neuronal cells in vitro (37), but does not incorporate the region of saposin C that activates lysosomal sphingolipid hydrolases (38). TX14(A) treatment was without dramatic effect on the general physiologic and metabolic characteristics of uncontrolled diabetes in mature adult rats. It also did not act as an inhibitor of exaggerated flux through the polyol pathway or prevent nerve ischemia. These findings distinguish TX14(A) from other agents that protect nerve function and structure in diabetic rats by aldose reductase inhibition and improved neurovascular perfusion (8, 39). There was a tendency for TX14(A)-treated diabetic rats to have slightly lower plasma glucose levels and higher body weights than untreated diabetic rats. While it is not clear whether this represents limited actions of TX14(A) on systemic metabolic responses to insulin-deficient diabetes, TX14(A)-treated diabetic rats remained markedly hyperglycemic with blood sugar levels in excess of those commonly reported to induce nerve disorders.

TX14(A) dose-dependently attenuated the reduced NCV of diabetic rats compared with age matched controls, which represented a decrease from onset NCV values indicative of conduction slowing rather than impaired maturation. NCV slowing is a disorder of large fiber function common both to short-term adult diabetic animals that do not display fiber loss (5, 36), and to newly

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diagnosed diabetic patients (1). Potential etiologic mechanisms to explain NCV slowing during hyperglycemia include decreased nerve homogenate Na/K-ATPase activity and atrophy of the largest myelinated fibers (40, 41). TX14(A) treatment attenuated both of these disorders, although whether by direct effects on nerve or indirectly via induction of other neurosupportive factors is not yet known. Prosaposin and prosaptides modulate ganglioside levels in neuronal membranes (42), and exogenous ganglioside treatment enhances nerve homogenate Na/K-ATPase activity and protects axonal transport of cytoskeletal proteins in diabetic rats (32, 43). However, unlike its effect on NCV, TX14(A) did not show a clear dose-response effect on nerve homogenate Na/K-ATPase activity, indicating the absence of a simple association between the 2 parameters. Alternately, as prosaposin and TX14(A) activate G-protein and MAPK-mediated signaling cascades (27, 37), they have the potential to regulate synthesis and/or phosphorylation states of structural proteins disrupted by diabetes (44, 45). Further studies are required to determine how TX14(A) protects large fiber function and structure during hyperglycemia.

Thermal hyperalgesia occurs in diabetic patients (3) and rats (7) and, in the latter, is accompanied by depletion of the neuropeptide sensory neurotransmitter substance P in the peripheral nerves (46). Substance P is localized to small myelinated and unmyelinated sensory neurons in the peripheral nerve that are associated with nociception (47). In the present study, TX14(A) dose-dependently prevented thermal hyperalgesia in diabetic rats and attenuated nerve substance P depletion in a similar dose-dependent manner. Unlike NGF, which regulates substance P synthesis in sensory neurons (48), and can exaggerate substance P in nerves from either normal or diabetic animals (12, 19, 49), TX14(A) was without effect on nerve substance P levels in control nerves, suggesting that it acts selectively in preventing only the diabetes-induced disorder. TX14(A) did not alter thermal responses in control rats following extended treatment and there was no effect on the thermal response of either control or diabetic rats when TX14(A) was given as a single bolus dose at time points between 30 min and 3 h prior to testing. (Cutc, unpublished data). This further distinguishes the actions of TX14(A) from those of NGF, as the latter induces acute hyperalgesia (50). Thus, TX14(A) prevents abnormal nociceptive processing and biochemical disorders of small nociceptive fibers in diabetic rats without acute or chronic effects on normal nociception.

Our studies demonstrate altered prosaposin mRNA expression in diabetic nerve and that treatment with the neurotrophic portion of prosaposin prevents disorders of large and small fibers in diabetic rats. This indicates that the cellular signal transduction pathways activated by the neurotrophic region of prosaposin are intact in the nerves of diabetic rats. The precise site of action of TX14(A) is unknown and our recent studies indicate that it can induce phosphorylation of MAPK and synthesis of myelin lipids in Schwann cells (51, 52), as well as having actions on neurons (26, 27, 30). Given mounting evidence that axonal structure and function is regulated by Schwann cells (53), the efficacy of TX14(A) in diabetic rats could be mediated via actions on either neurons and/or Schwann cells. Whatever the site of action, our findings highlight prosaptides as potential therapeutic agents for the broad range of fiber disorders that occur during hyperglycemia.

ACKNOWLEDGMENTS

Our thanks to Maryanne Bache, Rose Cesca, Bob Garrett, and David Garrett for excellent technical support.

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Received December 3, 1998
Revision received February 24, 1999
Accepted February 26, 1999