Diabetic Peripheral Neuropathy
Evidence for Apoptosis and Associated Mitochondrial Dysfunction
Shanthi Srinivasan, Martin Stevens, and John W. Wiley

We hypothesized that diabetic sensory neuropathy is associated with activation of apoptosis and concomitant mitochondrial dysfunction. Studies were performed in excised intact and acutely dissociated dorsal root ganglion (DRG) neurons from control and streptozotocin-induced diabetic rats with decreased peripheral nerve conduction velocities (NCV). Apoptosis was increased in acutely dissociated DRG neurons from 3- to 6-week-old diabetic rats. Basal mitochondrial membrane potential (ΔΨm) was significantly more positive in DRG neurons from diabetic rats. Depolarization with glutamate resulted in significantly more positive ΔΨm and delayed recovery of ΔΨm in neurons from diabetic rats. Restoration of euglycemia for 2 weeks with insulin implants normalized NCV, ΔΨm, and apoptosis. Intact and acutely dissociated neurons from diabetic rats demonstrated decreased Bcl-2 levels and translocation of cytochrome C from the mitochondria to the cytoplasm. Neither levels of Bax nor levels of Bcl-XL were altered in diabetic rats. Basal mitochondrial membrane potential (ΔΨm) was significantly more positive in DRG neurons from diabetic rats. Apoptosis associated with mitochondrial dysfunction may contribute to the pathogenesis of diabetic sensory neuropathy. Diabetes 49:1932–1938, 2000

Diabetic neuropathy is the most common form of peripheral neuropathy in the Western world, with similar functional, morphological, and metabolic changes in peripheral nerves documented in both human and animal models of type 1 and type 2 diabetes (1,2). The pathophysiology of diabetic neuropathy remains controversial (3,4). Little is known about the etiology of the loss of peripheral sensory neurons that accompanies diabetic neuropathy. Programmed cell death, or apoptosis, has been implicated in diabetic retinopathy and neuropathy (5,6). Impaired mitochondrial function has been implicated in the promotion of apoptosis (7). Abnormalities in mitochondrial function have been reported in both type 1 and 2 diabetes (8,9). We hypothesized that apoptosis is involved in the pathogenesis of diabetic sensory neuropathy and that activation of the apoptosis cascade is associated with mitochondrial dysfunction. Specifically, we examined whether mitochondrial dysfunction in diabetic sensory neuropathy was associated with decreased levels of the antiapoptotic protein Bcl-2, translocation of cytochrome C from mitochondria to the cytoplasm, and induction of apoptosis (10,11). Few published studies examine a potential connection between activation of the apoptosis cascade in diabetic neuropathy and impaired mitochondrial function, an observation that would help explain the slow progressive loss of peripheral sensory neurons that accompanies this disorder.

Excitatory amino acids such as glutamate have been implicated in the induction of apoptosis in several neurodegenerative disorders (12–14). We examined the effect of glutamate on mitochondrial membrane potential (ΔΨm) and induction of apoptosis in primary sensory (dorsal root ganglion [DRG]) neurons from control and diabetic rats.

RESEARCH DESIGN AND METHODS
Animal model: streptozotocin-induced diabetic rat. Two-month-old male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed at the Unit for Laboratory Animal Medicine–approved Animal Research Facility at the VA Medical Center. Animals were randomized into three groups: diabetic, diabetic with insulin-mediated normalization of serum glucose levels, and healthy controls. Animals were fasted for 12 h before injection of streptozotocin. Intraperitoneal injection of streptozotocin at a dose of 45 mg/kg caused 80% of animals to become hyperglycemic within 1 week of injection, with blood glucose between 250 and 500 mg/dl. This model demonstrates highly reproducible functional (decreased nerve conduction velocities), morphological, and metabolic changes in peripheral nerves similar to those observed in humans with diabetic neuropathy (15). Experiments were performed 3–6 weeks after confirmation of hyperglycemia. Insulin was administered using LinBit, a sustained-release insulin implant (2 U · day–1 · implant–1) (Lin Shin, Canada). After the animals were anesthetized with ketamine and xylazine, the site for implantation was shaved and treated with Betadine solution. Typically, two implants were placed subcutaneously by making a small incision using a 12-gauge needle and positioning the implants with a trocar and stylet available from Lin Shin. Euglycemia was confirmed by glucose monitoring within 1–2 h after implantation, at ~24 h, and when the animals were killed. Insulin-treated animals were used for experiments after 2 weeks of insulin therapy. Control animals were sham-injected with vehicle. Some diabetic animals were implanted with sham implants.

Reagents. Penicillin, streptomycin, goat serum, and trypsin were obtained from Gibco Laboratories (Gaithersburg, MD). Collagenase, streptozotocin, trypsin, Trypan blue, neutral buffered formalin were obtained from Sigma (St. Louis, MO). TdT (terminal transferase) enzyme, Biotin-conjugated dUTP, and fluorescein isothiocyanate (FITC)-Avidin, were obtained from Boehringer Mannheim Biochemical (Indianapolis, IN). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) and JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) were obtained from Molecular Probes (Eugene, OR).

Tissue preparation. Isolated acutely dissociated thoracic and lumbar DRG neurons were aseptically prepared from rats using techniques described previously (15). Isolated DRG neurons were resuspended in minimal essential medium and plated onto matrigel-coated coverslips. The glucose concentration...
of the minimal essential medium used to resuspend DRG neurons harvested from diabetic rats was supplemented to 27.0 mmol/l. No sera or growth factors were added to the culture media. Cells were incubated at 37°C for 2-6 h. All studies were performed on neurons within 6 h after plating.

Mitochondrial membrane potential measurement. Isolated DRG neurons prepared as described above were loaded with JC-1 dye (2.5 µg/ml) for 20 min at 37°C (17,18). Using a confocal laser microscope to visualize intracellular organelles, 590 nm/530 nm ratios were obtained under the following conditions: 1) 30-min basal recording, 2) glutamate-stimulated (10 µmol/l glutamate for 5 min in Mg²⁺-free buffer supplemented with 10 µmol/l glycine), and 3) recovery (defined as return to 65% of basal ratio at 50 min after glutamate washout).

Cell viability. Neuronal viability was assessed in basal control and diabetic DRG neurons. Neurons were exposed to 0.2% Trypan blue for 10 min. The number of Trypan blue positive cells per 200 cells were counted in a blinded manner (19).

Determination and quantitation of apoptosis. Apoptosis was detected using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method (20). This method is based on the principle that terminal deoxynucleotidyl transferase catalyzes a template-independent addition of deoxyxynucleotides to the free 3’OH end present in DNA breaks formed during apoptosis. The morphological characteristics of apoptosis detected by this assay and the sensitivity and specificity of the TUNEL method were confirmed in our system using measurement of neuronal DNA content in conjunction with flow cytometry and electron microscopy. The reliability of the TUNEL method for detection of apoptosis was confirmed in our laboratory using cis-platinum as a positive control for the induction of apoptosis (21–23). In situ fixation of control and diabetic DRG neurons was performed. Rats were given anesthesia using ketamine and xylazine (20 mg/ml), and the left ventricle was cannulated with a 16-gauge needle tube. The left ventricle was perfused with porcine heparin (10 units USP/ml; Sigma), diluted in 0.15 mol/l phosphate-buffered saline (pH 7.3) (1/40 dilution), and perfused at a rate of 15 cc/min followed by ice-cold 4% paraformaldehyde in phosphate-buffered saline at 15 cc/min. Thirty minutes after perfusion with paraformaldehyde, the DRGs were removed and placed in 4% paraformaldehyde. The DRGs were processed and embedded in paraffin, using standard protocols at the clinical pathology department, and 5-µm paraffin sections were obtained. The sections were deparaffinized through standard protocols, and a 20-min serum block (20% goat serum) was performed. Studies were also performed using control and diabetic neurons that were fixed and labeled using the TUNEL method under basal conditions (1 h after removal from animal) and glutamate-stimulated conditions (6 h after exposure to 100 µm glutamate for 30 min at 37°C). Two hundred neurons were counted blinded (one-way), and the positive cells were expressed as a percentage of total neurons counted. The percentage of neurons undergoing apoptosis was standardized to the total number of neurons attached to the coverslip. The number of necrotic neurons was not subtracted from each group before calculation of the percent of apoptotic neurons. Negative controls received only the label solution without terminal transferase, and the positive controls were slides with confirmed apoptosis provided by the company (Oncogene).

Western blot analysis. For mitochondrial and cytosolic protein extraction, the DRGs were extracted using standard procedures (24,25). Protein extracts were probed by SDS-PAGE using 15% agarose. Polyclonal antibodies against rat Bcl-2 (1:750), Bax (1:500), and Bcl-xL (1:500) (Santa Cruz) and a monoclonal antibody against rat cytochrome C (1:250 dilution) (PharMingen) were used for Western blot analysis. Immunoblot analysis was performed with the appropriate horse-radish peroxidase–conjugated secondary antibody using enhanced chemiluminescence (ECL-Plus) Western blot detection reagents (Amersham). Quantitative Western blot analysis was done by scanning the immunoblot using ImageQuant software in conjunction with the STORM machine (Molecular Dynamics).

Measurement of Bcl-2 by immunohistochemistry. The slides were incubated with primary Bcl-2 antibody (1:20, Santa Cruz) for 2 h at room temperature, followed by secondary anti-rabbit-TRITC antibody for 2 h at room temperature. Images were obtained under confocal microscopy (Bio-Rad).

Electrophysiological measurements. Sciatic-tibial motor nerve conduction velocity (NCV) was measured after induction of anesthesia with intraperitoneal urethane (1–1.2 g/kg). Body temperature was monitored by a rectal probe and maintained at 37°C with a warming pad. Hind-limb skin temperature was also monitored by a thermistor and maintained between 36 and 38°C by radiant heat. The left sciatic-tibial motor conduction system was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimuli (8 V) at Hz. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. Motor NCV was calculated by subtracting the distal latency from the proximal latency, and the result was divided into the distance between the stimulating and recording electrode.

Statistical analysis. The data are summarized as the mean ± SE. Statistical analysis was performed using Student’s t test or the one-way analysis of variance with appropriate adjustment for nonparametric statistics. All statistics were done using the InSTAT software program. Statistical significance was accepted at the P < 0.05 level.

RESULTS

Baseline body weights were similar in all experimental groups. After 4 weeks of hyperglycemia, body weights were

FIG. 1. Effect of streptozotocin-induced diabetes and insulin treatment on NCV. Sciatic motor NCV was determined in nondiabetic (A), untreated streptozotocin-diabetic, and insulin-treated diabetic (B) rats as described in RESEARCH DESIGN AND METHODS. NCV was significantly slower in the diabetic rats. Insulin-mediated euglycemia for 2 weeks normalized the NCV in the treated diabetic rats. *P < 0.01 vs. nondiabetic rats; †P < 0.05 vs. diabetic rats.
significantly lower in diabetic rats (270 ± 15 g) than in controls (378 ± 12 g, n = 15, P < 0.01). Plasma glucose levels were markedly higher in diabetic rats (26.2 ± 1.4 mmol/l) than in controls (7.8 ± 0.3 mmol/l) (n = 15, P < 0.01).

Sciatic-tibial motor NCV temporarily decreased in diabetic rats. Sciatic-tibial motor NCV was reduced by 28% (P < 0.01) in untreated diabetic rats compared with nondiabetic control rats (Fig. 1A). The decreased NCV was normalized after 2 weeks of insulin-mediated euglycemia (diabetic: 45 ± 3 m/s; diabetic + insulin: 64 ± 4 m/s) (P < 0.05, n = 4) (Fig. 1B).

DRG neurons from diabetic rats demonstrated more positive mitochondrial membrane potentials compared with controls under basal and glutamate-stimulated conditions that were normalized after 2 weeks of euglycemia. The fluorescent dye JC-1 was used to measure Δψ. JC-1 is a cell-permeant dye that exists as an aggregate at the very negative membrane potentials (Δψ < −100 mV, red fluorescence, 590 nm) (Fig. 2A). At Δψ > −100 mV, JC-1 exists as a monomer (green fluorescence, 530 nm). Figure 2B shows control neurons at baseline (1 and 2) and during depolarization (3 and 4). The ratio of green/red (530 nm/590 nm) fluorescence can therefore be used as an indicator of mitochondrial membrane potential (Δψ). Δψ is expressed as a ratio of green (monomer) to red (aggregate) fluorescence. Larger numbers (ratio) correlate with more positive Δψ. As shown in Fig. 2C, the baseline Δψ was significantly higher in diabetic (2.34 ± 0.2, n = 63) than in control (1.17 ± 0.1, n = 45, four animals; P = 0.006) DRG neurons. During depolarization with glutamate (10 µmol/l for 5 min), peak ratios were significantly higher in diabetic neurons (6.1 ± 1.6, n = 54) than in controls (1.59 ± 0.5, n = 35; P = 0.02). After 2 weeks of insulin-mediated euglycemia, baseline and glutamate-induced changes in mitochondrial membrane potentials were not significantly different in DRGs from control and treated diabetic rats (baseline: control 1.17 ± 0.1, n = 45, four animals; insulin-treated
diabetic 1.20 ± 0.1, n = 40 neurons; four animals; glutamate [10 µmol/l for 5 min]; control 1.61 ± 0.2, n = 46 neurons; four animals; insulin-treated diabetic 1.75 ± 0.3, n = 98 neurons; four animals, P > 0.05). Seventy-five percent of the control neurons recovered compared with 43% of diabetic neurons (recovery defined as return to 65% of baseline ratio 1 h after washout of glutamate).

**Increased apoptosis in diabetic DRG neurons reversed after insulin-mediated euglycemia.** Apoptosis was measured in acutely dissociated DRGs using the TUNEL method. Two hundred neuronal cells were counted blinded (one-way), and the positive cells were expressed as a percentage of total neurons counted. Basal apoptosis was significantly elevated in DRGs from diabetic animals compared with controls (control 4.96 ± 0.75, n = 6; and diabetic 5.66 ± 1.05, n = 6; P < 0.05). After 2 weeks of euglycemia with insulin therapy, apoptosis levels in diabetic animals were similar to those in controls (insulin-treated diabetic 5.1 ± 1.3%, n = 5, P = 0.04, compared with untreated diabetic rats).

Neurons from diabetic rats with neuropathy demonstrated decreased Bcl-2 levels, but no change in Bcl-xL or Bax levels. We observed a decrease in Bcl-2 levels using quantitative immunohistochemistry and confocal laser microscopy (control 428 ± 167, n = 30, P = 0.03; diabetic 906 ± 1.05, n = 10, P = 0.04). After 2 weeks of euglycemia with insulin therapy, apoptosis levels in diabetic animals were similar to those in controls (insulin-treated diabetic 4.96 ± 0.75%, n = 5, P = 0.04, compared with untreated diabetic rats).

**DISCUSSION**

We have demonstrated that the pathophysiology of early sensory neuropathy in diabetes may involve activation of the apoptosis cascade associated with mitochondrial dysfunction. As early as 3 weeks after the induction of diabetes, we observed increased apoptosis associated with elevated (more positive) Δψ in diabetic DRG neurons. Previous reports indicate that the streptozotocin-induced diabetic rat...
model used in our studies demonstrated functional and morphological features of diabetic peripheral neuropathy (15). We observed that the diabetic rats demonstrated typical features of chronic hyperglycemia, including weight loss and slowing of NCVs. It is not likely that the observed changes in apoptosis, NCV, and \( \Delta \psi \) were related to nonspecific streptozotocin toxicity because normalization of serum blood glucose levels with insulin implants for 2 weeks reversed these abnormalities in diabetic rats to control levels. We focused on assessing the reversibility of diabetic neuropathy in early diabetes because previous studies suggest that insulin therapy can prevent the development of retinopathy and peripheral neuropathy (5,26). We believe that this is the first report demonstrating that neuronal apoptosis, mitochondrial dysfunction, and slowing of NCV observed in early diabetic neuropathy can be reversed with restoration of euglycemia for 2 weeks. The mechanism underlying increased apoptosis in diabetic neurons involved decreased levels of Bcl-2 and translocation of mitochondrial cytochrome C to the cytosol. The reduced Bcl-2 levels observed in diabetic neurons may reflect either reduced synthesis or increased proteolysis of this protein.

Previous studies have reported the presence of apoptosis in diabetic retinopathy and cultured neuroblastoma cells treated with sera from diabetic subjects (5,21,27). Although the percentage of apoptotic neurons in diabetic animals was modest compared to, for example, chemotherapy-induced apoptosis, significant neuronal loss could occur over the typical lengthy course of diabetic neuropathy, explaining the dropout of sensory neurons. Relevant to our observations, the symmetrical loss of sensory neurons over time in diabetic neuropathy is not associated with a significant inflammatory response, which is consistent with activation of apoptosis (2).

The threshold for activation of the apoptotic cascade appears to depend on the ratios and relative abundance of positive and negative regulators, such as expression of the Bcl family of proteins, including Bcl-2, Bax, and Bcl-xL (28). These proteins are expressed primarily in the inner mitochondrial membrane. Bcl-2 stabilizes the mitochondrial membrane by inhibiting the opening of a large conductance channel known as mitochondrial permeability transition (PT) pore (7) and thereby has an antiapoptotic effect. Opening of the PT pore results in loss of \( \Delta \psi \) and expansion of the intermembranous space. This results in outer membrane rupture and the release of caspase-activating enzymes, such as cytochrome C and/or APAF-1, from mitochondria into the cytosol (7). In addition to preventing cytochrome C translocation, evidence suggests that Bcl-2 may protect cells after cytochrome C has been released (29,30). Bcl-2 may also prevent apoptosis by enhancing proton extrusion from mitochondria that helps to maintain the mitochondrial buffering capacity (31).
We also examined the level of the putative antiapoptotic protein Bcl-xL. Bcl-xL prevents apoptosis by stabilizing the mitochondrial membrane, similar to the actions of Bcl-2. Like Bcl-2, Bcl-xL forms channels in the mitochondrial membrane and influences the translocation of cytochrome C to the cytosol through various mechanisms (29). Bcl-xL has been shown to block apoptosis where Bcl-2 is ineffectual (32). We observed no difference in Bcl-xL levels in neurons from control and diabetic rats.

Previous studies have implicated increased Bax levels in apoptosis. Bax dimerizes with itself or with Bcl-2 or Bcl-xL. Recent studies suggest that a shift in the balance toward Bax homodimers enhances apoptosis, whereas the formation of Bcl-2/Bax or Bcl-xL/Bax heterodimers inhibits programmed cell death (25). Hyperglycemia-induced apoptosis in mouse blastocysts involved increased Bax levels (33). We observed no change in the level of Bax in the diabetic DRG neurons. This is consistent with some studies demonstrating that overexpression of Bax did not increase the apoptosis of DRG neurons following nerve growth factor deprivation (34). Neurtrophin levels are known to be decreased in primary sensory neurons in diabetic neuropathy (35,36). Thus, Bcl-2 and Bax can act independently to inhibit or promote apoptosis, respectively (37). Our studies suggest that diabetic sensory neuropathy is associated with a selective decrease in the levels of Bcl-2. This is an interesting observation that supports selective activation of known participants of the apoptosis cascade in diabetic neuropathy. Additional studies will be required to delineate the specific components of the apoptotic cascade that are involved in diabetic neuropathy.

Excitatory neurotransmitters such as glutamate have been implicated in the pathophysiology of several models of neurodegenerative diseases (12,13). Of interest, apoptosis was increased by a similar amount in diabetic neurons both before and 6 h after depolarization with glutamate. Therefore, diabetic neurons demonstrated an increased magnitude of mitochondrial depolarization in response to glutamate and delayed recovery, but this was not associated with an increase in apoptosis measured 6 h after the depolarizing event. This result suggests that glutamate-induced depolarization does not contribute to the observed differences in apoptosis between control and diabetic neurons, at least under the experimental conditions used in these studies.

In summary, a selective decrease in Bcl-2 levels in diabetic neuropathy results in loss of Δψm translocation of cytochrome C into the cytosol, and activation of apoptosis. The observed abnormalities in apoptosis, Δψm, and NCV were normalized after 2 weeks of insulin-mediated euglycemia. Based on the observation that the apoptosis cascade is activated in diabetic DRG neurons, downstream targets such as caspases may represent potential targets for diagnosis and therapeutic intervention in diabetic neuropathy (38), in addition to insulin-mediated normalization of hyperglycemia.

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