Apoptotic Stress Is Counterbalanced by Survival Elements Preventing Programmed Cell Death of Dorsal Root Ganglions in Subacute Type 1 Diabetic BB/Wor Rats

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Several groups have reported apoptosis of dorsal root ganglion (DRG) cells as a prominent feature of diabetic polyneuropathy (DPN), although this has been controversial. Here, we examined subacute (4-month) type 1 diabetic BB/Wor rats with respect to sensory nerve functions, DRG and sural nerve morphometry, pro- and antiapoptotic proteins, and the expression of neurotrophic factors and their receptors. Sensory nerve conduction velocity was reduced by 13% and was accompanied by significant hyperalgesia. The numbers of DRG neurons including substance P- and calcitonin gene–related peptide–positive neurons were not altered, although they showed significant atrophy. Sural nerve morphometry showed decreased numbers of myelinated and unmyelinated fibers. Active caspase-3 and Bax expressions were increased, whereas antiapoptotic Bcl-xl and heat shock protein (HSP) 27 expressions in DRGs were decreased. Immunohistochemistry showed increased numbers of active caspase-3-, HSP70-, and HSP27-positive neurons. Examinations of DRGs revealed no structural evidence of apoptosis but rather progressive hydropic degenerative changes. We conclude that apoptotic stress is induced in DRGs but is counterbalanced by survival elements in subacute type 1 diabetic BB/Wor rats and that distal nerve fiber loss reflects a dying-back phenomenon caused by impaired neurotrophic support. Diabetes 54: 3288–3295, 2005

Diabetic polyneuropathy (DPN) is the most common late complication of diabetes and shows an increasing prevalence. Like other diabetes complications, DPN has been ascribed to hyperglycemia and subsequent metabolic abnormalities, particularly oxidative stress. Although other factors contribute to its development, such as insulin and C-peptide deficiencies and consequent deprivation of neurotrophic support (1), these have received less attention. Several investigators have reported neuronal apoptosis in relatively acute (2- to 3-month) streptozotocin-induced diabetes (STZ-D) in rats, sometimes exceeding 30% of dorsal root ganglion (DRG) cells as a contributing factor to DPN (2–4), while others have reported no significant neuronal loss in DRGs even after 12 months of STZ-D (5,6). The validity of major programmed cell death can be disputed, since the magnitude of reported apoptotic neuronal death does not correlate with a corresponding loss of axonal extensions in the peripheral nerve of the STZ-D rat. In contrast to the STZ-D rat, the spontaneously type 1 diabetic BB/Wor rat develops progressive sensory nerve fiber loss (7), as in humans, which therefore could reflect loss of parent DRG cells.

Apoptotic phenomena occur in pancreatic β-cells in type 1 diabetes (8), in diabetic retinopathy (9), and in hippocampal neurons in primary diabetic encephalopathy (10,11). Programmed cell death is believed to result from hyperglycemia-induced mitochondrial dysfunction (4,12) and depletion of antiapoptotic trophic factors like IGFs, insulin, and C-peptide and downregulation of their receptors (13,14). Apoptogenic stresses are mediated via increased expression of proapoptotic proteins such as Bax, oxidative DNA damage, activation of death receptors, and various upstream caspases inducing activation of caspase-3 as well as noncaspase-dependent apoptotic stressors (11). Proapoptotic activities are counteracted by stress-induced intrinsic antiapoptotic proteins, such as mitochondrial Bcl-xl, the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) (5), and heat shock proteins (HSPs) (15,16).

Here, we examined subacute (4-month) spontaneously diabetic BB/Wor rats, a close model of human type 1 diabetes (17), with respect to sensory nerve functions, DRG and sural nerve morphometry and morphology, expression of active caspase-3, pro- and antiapoptotic proteins, HSPs, and trophic factors and their receptors in DRGs. Particular attention was paid to nociceptive DRG ganglion cells and their content of substance P (SP) and calcitonin gene–related peptide (CGRP), since these appear to be particularly vulnerable under diabetic conditions (18,19).

RESEARCH DESIGN AND METHODS

Pre-diabetic BB/Wor rats and age-matched nondiabetes-prone BB rats were obtained from Biomedical Research Models, Worcester, Massachusetts. All rats had free access to water and rat diet. Body weight, urine volume, and

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glucosuria (Keto-Diastix; Bayer, Elkhart, IN) were monitored daily to ascertain onset of diabetes and for titration of daily insulin doses. After onset of diabetes, diabetic rats received titrated doses (0.5–3.0 units/day) of protamine zinc insulin (Novo Nordisk, Princeton, NJ) to maintain blood glucose levels at ~25 mmol/l glucose and to prevent ketoacidosis. Blood glucose levels were measured biweekly. Animals were cared for in accordance with guidelines of the Animal Investigation Committee, Wayne State University, and those of the National Institutes of Health (publ. no. 85-23, 1995).

Measurement of plasma insulin level. Insulin levels were measured in plasma obtained at the time the rats were killed, 20 h after the last insulin injection, using an enzyme-linked immunosorbent assay kit (Linco Research, St. Charles, MO).

Electrophysiological recordings. Rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body wt). Rectal temperature was maintained at 36–37°C by a heating pad and monitored by a rectal probe. Sensory nerve conduction velocity was recorded in the left hind limb as the mean of these measurements was calculated and used as the measure of the withdrawal latency.

Tissue collection. Four months after onset of diabetes, animals were anesthetized with urethane. Three control and BB/Wor rats were perfused with ~500 ml of 4% paraformaldehyde fixative. The left L5 DRGs were postfixed in situ for 10 min with 1% cacyclate-buffered (pH 7.4) 2.5% glutaraldehyde then excised and immersed in the same fixative for 2 h at 4°C. They were postfixed in 1% cacyclate-buffered (pH 7.4) osmium tetroxide, dehydrated, and embedded in Epon for morphologic and morphometric analyses. DRGs of the right side were fixed by immersion in 4% paraformaldehyde fixative. The left L5 DRGs were postfixed in situ for 10 min with 1% cacyclate-buffered (pH 7.4) 2.5% glutaraldehyde, dehydrated, and embedded in Epon for morphologic and morphometric analyses. DRGs of the right side were fixed by immersion in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) overnight at 4°C, rinsed in PBS, dehydrated, immersed in xylene, and embedded in paraffin.

The right sural nerve was dissected from five diabetic and five control rats and fixed in 1% cacyclate-buffered (pH 7.4) 2.5% glutaraldehyde, dehydrated, and embedded in Epon for morphometric analysis as previously described (20,21). DBGs and sciatic nerves were collected from five diabetic and five control rats for protein extraction. Tissues were snap frozen in liquid nitrogen and kept in –80°C until use.

Morphometry. Morphometric analyses of DRG neurons were performed on toluidine-blue–stained sections (0.5 μm thick; 40 μm apart) using a video image analysis system (Image-Pro Plus 3.0; Media Cybernetics, Silver Spring, MD). L5 ganglia were sectioned, 35–39 sections per ganglia. Neurons with distinguishable nuclei were counted and their area measured. Diameters of neuronal nuclei were measured and averaged. Number of neurons per DBG was calculated as the sum of numbers of neurons per section × 40/mean nuclear diameter.

Semithin (0.5-μm) cross-sections of Epon-embedded sural nerves were stained with toluidine-blue for light microscopic morphometric analysis using a computerized image analysis system (Image-I; Universal Imaging, West Chester, PA). This system is programmed to assess the total complement of sural nerve myelinated fibers and provides the following parameters: fasicular area (μm²), number of fibers (#/mm²), mean fiber area (μm²), mean axonal area (μm²), mean myelin area (μm²), axon-to-myelin ratio, index of circularity, and fiber occupancy rate (in percent) as previously described (20,21).

For morphometric analyses of unmyelinated fibers, five animals per group were examined. Ultrathin cross-sections of sural nerves were obtained with the aid of an LKB ultramicrotome (Marviac Limited, Halifax, Canada) and stained with uranyl acetate and lead citrate. They were examined in a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). Systematically selected frames representing 40–50% of the sural nerve cross-sectional area were obtained. Photographs were enlarged 10,000 times, scanned, and downloaded to the computerized image analysis system. The following morphometric parameters of unmyelinated fibers were obtained: unmyelinated fiber number, fiber density (#/mm²), mean fiber size (μm²), axon numbers per Schwann cell unit and the frequencies of collagen pockets, denervated Schwann cell profiles, type 2 axon-Schwann cell relationship, and regenerating C-fibers (19,21).

Morphological changes. Semithin (0.5-μm) toluidine-blue–stained sections of DRGs were examined for margination of nuclear chromatin, apoptotic bodies, other degenerative changes, and nodules of Nageotte as an indication of neuronal loss.

Immunohistochemistry for SP, CGRP, HSP27, HSP70, and active caspase-3 in lumbar DRG. Semithin (0.5-μm) cross-sections of Epon-embedded sural nerves were immunostained using an avidin biotin complex kit (Vector Laboratories, Burlingame, CA). Antibodies used are listed in Table 1. Quantifications of positive neurons were performed using the same image analysis system as above. Images of three serially sectioned DRG (60 μm apart) were captured and assessed using a binary scale. In each ganglion, 200–300 ganglion cells with visible nuclei were captured. The number and areas of SP- and CGRP-positive neurons were determined in each section. SP- and CGRP-positive neurons per ganglion were calculated as neurons per ganglion times positive neurons per section divided by total neurons per section. The frequencies of HSP27- and HSP70-positive neurons and caspase-3–positive neurons and neuronal nuclei were calculated from the same serial sections.

Measurement of SP and CGRP contents of DRGs. Enzyme immunometric assays were used to assess SP (Cayman Chemical, Ann Arbor, MI) and CGRP (SPIbio, Massy Cedex, France) in DRGs. Results were expressed as picograms per ganglion (18).

Western blotting. DBGs were lysed in detergent lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The lysates were centrifuged at 14,000 rpm for 20 min at 4°C, and protein concentrations were measured using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) with BSA as standard. Ten to 40 μg was separated by 7.5–15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with Tween-20-Tris–buffered saline (10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk (Bio-Rad) before incubation with primary antibodies.
antibodies (Table 1). Antigen detection was performed using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) with horseradish peroxidase–conjugated secondary antibodies. Membranes were scanned and densities determined by the Bio-Rad Fluoro-S multiimager (Bio-Rad). Expression of proteins was corrected for by actin density, and expression in control animals was arbitrarily set to 1.0.

**Nerve growth factor in sciatic nerve.** Nerve growth factor (NGF) content was determined with a quantitative two-site enzyme immunoassay (Enax Immunooassay System; Promega, Madison, WI). Assays were performed according to manufacturers’ protocol. Preweighed (~50 mg) sciatic nerve segments were homogenized in lysis buffer (137 mmol/l NaCl, 20 mmol/l Tris HCl, pH 8.0, 1% NP40, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mmol/l sodium vanadate) and centrifuged (14,000 rpm for 20 min). The supernatants were loaded on MaxiSorp 96-well plates (Nalge Nunc International, Rochester, NY), and measurements were performed as previously described (18,19). Results were expressed as picograms per milligram protein.

**Statistical analysis.** All values are expressed as means ± SD. Significance of differences was analyzed by ANOVA. Group differences were assessed by Scheffes test. Significance was defined as a P value <0.05. All analyses were performed by personnel unaware of the animal identities.

**RESULTS**

**Clinical findings.** At 4 months, BB/Wor rats showed severe hyperglycemia (control rats [n = 8]: 5.0 ± 0.4 mmol/l, diabetic rats [n = 8]: 24.4 ± 1.9 mmol/l; P < 0.001) and significant reductions in body weight (control rats [n = 8]: 458.1 ± 16.2 g, diabetic rats [n = 8]: 373.8 ± 16.4 g; P < 0.001). Serum insulin level was decreased in diabetic rats (control rats [n = 8]: 2.39 ± 0.28 ng/ml, diabetic rats [n = 8]: 0.67 ± 0.26 ng/ml; P < 0.001).

**Functional data.** Sensory nerve conduction velocity decreased progressively from 91% (control rats [n = 8]: 39.8 ± 0.9 m/s, diabetic rats [n = 8]: 36.2 ± 1.2 m/s; P < 0.001) of normal at 2 months to 87% at 4 months (control rats [n = 8]: 40.9 ± 1.5 m/s, diabetic rats [n = 8]: 35.6 ± 1.6 m/s; P < 0.001). Hyperalgesia, reflecting unmyelinated fiber function, was measured as latencies of hind-paw withdrawal to thermal stimuli. Diabetic rats showed progressive and significant decreases in the latencies to thermal stimuli that reached 11.9 ± 2.7 s (n = 8) at 4 months compared with 22.7 ± 2.2 s in control rats (n = 8; P < 0.001).

**Morphometry of DRG.** Neuronal size distribution of DRGs showed a shift toward smaller sizes in diabetic rats (Fig. 1A), consistent with a reduced mean neuronal size in diabetic rats (P < 0.05; Table 2). Although there was 14.7% fewer DRG neurons in diabetic rats, this did not reach statistical significance (Table 2). SP- and CGRP-positive neurons showed small shifts toward smaller sizes (Fig. 1B and C); however, the number of SP and CGRP neurons were not significantly altered (Table 2) in diabetic rats. Mean SP neuronal size was not significantly changed, whereas mean CGRP neuronal size was decreased by 15.6% (P < 0.05) in diabetic rats (Table 2).

**Morphological changes.** Approximately 1,000 DRG neurons per ganglion were examined. The vast majority of ganglion cells showed normal morphology (Fig. 2A). No chromatin margination or apoptotic bodies were observed in diabetic or control rats. Instead, subplasmalemmal small vacuoles were observed in 10.5 ± 2.1 and 10.9 ± 1.7% of neurons (Fig. 2B) in control and diabetic rats, respectively. These coalesced to form larger cytoplasmic vacuoles in 0.31 ± 0.13% of neurons in diabetic rats (Fig. 2C) compared with 0.03 ± 0.06% (P < 0.05) in control rats.

**TABLE 2**

Morphometry of DRG and SP and CGRP contents in DRGs

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<thead>
<tr>
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<th>Control</th>
<th>BB/Wor</th>
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<tr>
<td>Mean neuron area (μm²) (n = 3)</td>
<td>892.7 ± 60.3</td>
<td>738.0 ± 74.0*</td>
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<tr>
<td>Number of neurons per ganglion (n = 3)</td>
<td>12,667 ± 1086</td>
<td>10,806 ± 1,054</td>
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<tr>
<td>SP content (pg/ganglion) (n = 5)</td>
<td>85.5 ± 13.8</td>
<td>74.3 ± 51.0</td>
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<td>SP-positive neurons (%) (n = 5)</td>
<td>24.0 ± 9.5</td>
<td>22.1 ± 1.7</td>
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<tr>
<td>Mean area of SP-positive neurons (μm²) (n = 3)</td>
<td>448.8 ± 11.8</td>
<td>380.6 ± 56.3</td>
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<tr>
<td>SP-positive neurons per ganglion (n = 3)</td>
<td>3,042 ± 327</td>
<td>2,407 ± 399</td>
</tr>
<tr>
<td>CGRP content (pg/ganglion) (n = 5)</td>
<td>717.6 ± 160.1</td>
<td>628.1 ± 85.1</td>
</tr>
<tr>
<td>CGRP-positive neurons (%) (n = 3)</td>
<td>29.1 ± 0.7</td>
<td>27.5 ± 2.6</td>
</tr>
<tr>
<td>Mean area of CGRP-positive neurons (μm²) (n = 3)</td>
<td>519.2 ± 18.9</td>
<td>439.2 ± 28.2*</td>
</tr>
<tr>
<td>CGRP-positive neurons per ganglion (n = 3)</td>
<td>3,653 ± 188</td>
<td>2,991 ± 517</td>
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Data are means ± SD. *P < 0.05 vs. control rats.
Nodules of Nageotte (Fig. 2D) were equally common in control and diabetic rats (0.17 ± 0.06 vs. 0.19 ± 0.13%). The structural abnormalities are consistent with hydropic degeneration.

**Sural nerve morphometry.** The results of myelinated and unmyelinated fiber morphometry of sural nerves are given in Table 3. Myelinated fibers in diabetic rats showed a 15.6% fiber loss ($P < 0.01$) and decreased mean axonal ($P < 0.05$) and fiber ($P < 0.005$) size, whereas mean myelinated area was unchanged. The axonal atrophy was reflected by decreased index of circularity ($P < 0.05$).

Unmyelinated fiber morphometry of diabetic rats revealed a 28.5% fiber loss ($P < 0.001$), decreased fiber density ($P < 0.05$), axonal atrophy ($P < 0.05$), and decreased number of axons per Schwann cell unit ($P < 0.05$). These changes were accompanied by increased frequencies of denervated Schwann cell profiles ($P < 0.005$), collagen pockets ($P < 0.05$), type 2 Schwann cell/axon relationships ($P < 0.001$), and regenerating fibers ($P < 0.001$) in diabetic rats.

**Expression of active caspase-3 and caspase-3-positive neurons.** Western blots for active caspase-3 showed increased (P < 0.05) levels in diabetic DRGs (Fig. 3A). Immunohistochemically, the frequencies of caspase-3-positive DRG neurons showing cytoplasmatic staining (Fig. 4A) and nuclear staining (Fig. 4B) were increased (P < 0.001 and P < 0.05, respectively) in diabetic rats.

**Expression of pro- and antiapoptotic elements.** Proapoptotic Bax was increased 1.9-fold ($P < 0.005$) in diabetic rats (Fig. 3B). This was associated with a 1.6-fold ($P < 0.05$) increase in antiapoptotic Bcl-xl in diabetic ganglion cells (Fig. 3C). The expression of the low-affinity NGF receptor (NGF p75R), Fas, PARP, cleaved PARP, or caspase-12, reflective of endoplasmic reticulum dysfunction, were not altered (Fig. 3D–G).

**Neurotrophic factors.** The NGF content in the sciatic nerve was significantly decreased ($P < 0.05$) in diabetic rats (Fig. 5A). The high-affinity NGF receptor (TrkA) expression in diabetic DRGs was significantly decreased ($P < 0.05$; Fig. 5E), whereas the IGF-1 receptor and insulin receptor expressions in DRGs were not altered (Fig. 5F and G).

**SP and CGRP contents in DRGs.** SP and CGRP contents in DRGs were not significantly changed in diabetic rats (Table 2).

**HSPs in DRGs.** HSP27 protein expression was significantly ($P < 0.05$) increased in diabetic DRGs (Fig. 5A). This change correlated with an increased frequency ($P < 0.05$) of immunohistochemically positive neurons (Fig. 6A). HSP27-positive staining was observed in the cytoplasm of neurons and axons of all sizes. The expression of inducible HSP70 was not altered in diabetic DRGs (Fig. 5B). Immunostaining of HSP70 in DRGs showed localization mainly to Schwann cells, satellite cells, and small

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### Table 3

<table>
<thead>
<tr>
<th>Morphometric parameters</th>
<th>Control</th>
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<th>Myelinated fibers</th>
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<tr>
<td>Fasicular area (m$^2$)</td>
<td>44,003 ± 3,072</td>
<td>34,934 ± 2,780*</td>
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<tr>
<td>Number of myelinated fibers (#)</td>
<td>668 ± 35</td>
<td>564 ± 57†</td>
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<tr>
<td>Myelinated fiber density (#/m$^2$)</td>
<td>15,209 ± 449</td>
<td>16,148 ± 927</td>
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<tr>
<td>Mean myelinated fiber area (m$^2$)</td>
<td>35.2 ± 1.0</td>
<td>32.1 ± 1.3*</td>
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<tr>
<td>Mean axonal area (m$^2$)</td>
<td>14.1 ± 1.5</td>
<td>12.2 ± 0.9‡</td>
</tr>
<tr>
<td>Mean myelin area (m$^2$)</td>
<td>21.1 ± 1.3</td>
<td>19.9 ± 0.6</td>
</tr>
<tr>
<td>Axon-to-myelin ratio (m$^2$/m$^2$)</td>
<td>0.67 ± 0.11</td>
<td>0.61 ± 0.04</td>
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<td>Index of circularity</td>
<td>0.94 ± 0.01</td>
<td>0.91 ± 0.02‡</td>
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<tr>
<td>Odds ratio (myelinated fiber occupancy) (%)</td>
<td>53.6 ± 2.0</td>
<td>51.8 ± 2.4</td>
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<tr>
<th>Unmyelinated fibers</th>
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<tr>
<td>Number of unmyelinated fibers (#)</td>
<td>5,065 ± 374</td>
<td>3,622 ± 218§</td>
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<tr>
<td>Unmyelinated fiber density (#/m$^2$)</td>
<td>115,375 ± 0.43</td>
<td>103,859 ± 3,998‡</td>
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<tr>
<td>Mean unmyelinated fiber area (m$^2$)</td>
<td>0.74 ± 0.09</td>
<td>0.61 ± 0.04‡</td>
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<td>Axon numbers/Schwann cell unit (#/unit)</td>
<td>5.26 ± 0.64</td>
<td>4.22 ± 0.29‡</td>
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<td>Denervated Schwann cell profiles (% of total unmyelinated fibers)</td>
<td>0.24 ± 0.18</td>
<td>0.97 ± 0.26*</td>
</tr>
<tr>
<td>Collagen pockets (% of total unmyelinated fibers)</td>
<td>0.62 ± 0.42</td>
<td>1.59 ± 0.57‡</td>
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<tr>
<td>Type 2 Schwann cell/axon relationship (% of total unmyelinated fibers)</td>
<td>1.18 ± 0.13</td>
<td>6.45 ± 2.0§</td>
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<tr>
<td>Regenerating fibers (% of total unmyelinated fibers)</td>
<td>0.56 ± 0.43</td>
<td>2.07 ± 0.36§</td>
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Data are means ± SD. *P < 0.005; †P < 0.01; ‡P < 0.05; §P < 0.001 vs. control rats.
neurons (Fig. 6B). Although the percentage of HSP70-positive neurons was low, it was significantly increased (P < 0.05) in diabetic DRGs. Constitutively expressed HSC70 or HSP40 were not altered (Fig. 5C and D).

**DISCUSSION**

Human DPN shows progressive loss of myelinated and unmyelinated sensory fibers in peripheral nerves, accompanied by degeneration and loss of parent DRG neurons, and is classified as a peripheral axonopathy of dying-back type (1,7,22–24). Hyperglycemia with consequent perturbations of the polyol pathway, nerve hypoxemia, and oxidative stress have been suggested as the main culprits (1). In type 1 DPN, impaired insulin and C-peptide signaling plays contributing roles, causing perturbed gene regulation of neurotrophic factors and their receptors and aberrations of the expression and posttranslational modifications of neuroskeletal and adhesive proteins (1,18,19,24,25). It has been claimed that apoptosis of sensory ganglion cells is a prominent component in the pathogenesis of DPN in STZ-D rats (2–4,12).

Using the spontaneously type 1 diabetic BB/Wor rat we demonstrate here 1) increased stainability and upregulation of active caspase-3 and proapoptotic Bax in diabetic DRGs, 2) that these apoptotic stresses are accompanied by the induction of antiapoptotic Bcl-xl and survival promoting HSP27 and HSP70, 3) that NGF content in sciatic nerve and high-affinity NGF receptor TrkA expression in diabetic DRGs are decreased, 4) that morphometric analyses of sural nerve reveal myelinated and unmyelinated fiber loss and axonal atrophy in diabetic rats, and 5) that morphometric and morphologic examinations of DRGs show no neuronal loss or qualitative changes indicative of apoptosis. Instead, DRG neurons show progressive hydropic damage similar to that described in human DPN (22).

Caspase-3 is the common final executing caspase and is activated by several upstream apoptotic pathways. Here, we demonstrate increased expression of active caspase-3 as well as increased stainability in neuronal cytoplasm and nuclei in diabetic rats. These findings are in agreement with previous data (2,5,6) and were accompanied by increased expression of Bax, indicative of mitochondrial dysfunction. On the other hand, these apoptotic stressors were associated with upregulation of antiapoptotic Bcl-xl and HSP27 as well as an increased number of neurons staining positive for HSP70.

HSPs are proteins induced by thermal stimuli and act as chaperones to support folding of proteins. HSP27 and HSP70 have been widely investigated, and their protective effect against neuronal death has been repeatedly reported (15,16,26–30). HSP27 is constitutively expressed and can be induced not only by thermal stimuli but also by trophic factor withdrawal or by apoptotic stress (26). HSP27 interacts with cytochrome c, blocking its interaction with Apaf-1, procaspase 9, and downstream activation of caspase-3 (26), and it inhibits Fas-induced caspase-independent apoptosis by interacting with Daxx (30). However, Fas was not increased in DRGs in this study, suggesting that caspase-independent mechanisms are not involved. Inducible HSP70 is a survival element (28,29) induced by several stresses (31). In this study, we found localization of HSP70 in Schwann cells and small neurons. Although its expression in DRGs was not changed, immunohistochemistry revealed increased HSP70 positivity in small DRG neurons. HSP70 may therefore be increased in

**FIG. 3. Expressions of pro- and antiapoptotic proteins in DRGs.**

**A:** Active caspase-3. **B:** Bax. **C:** Bcl-xl. **D:** p75. **E:** FAS. **F:** PARP. **G:** Caspase-12. **H:** Actin. n = 4. Results are means ± SD. *P < 0.05; **P < 0.005 vs. control rats.

**FIG. 4. Quantitative analyses of neurons showing active caspase-3 stainability in the cytoplasm (A) and nuclei (B) of DRG neurons (n = 3).** Thin arrows show positive staining in the cytoplasm and bold arrows show nuclear staining. Results are means ± SD. *P < 0.05; **P < 0.001 vs. control rats. Bar = 30 μm.
the more susceptible small nociceptive neurons under diabetic conditions. In keeping with the present findings, Guzhova et al. (32) reported that HSP70 may be exported from glia cells and that extracellular HSP70 has protective effects on neurons.

Taken together with the morphometric and morphologic data, which failed to demonstrate significant neuronal loss and qualitative changes suggestive of ongoing apoptosis, it appears that apoptotic stresses are induced in DRGs but that the final execution of DRG neuronal apoptosis is prevented by the induction of counterregulatory elements such as Bcl-xl and HSPs. This is supported by the findings that the common death substrate protein PARP and its cleavage product, indicative of DNA damage, were unaltered in diabetic DRGs.

These findings differ from those previously reported in the hippocampus of the same rat model (11), in which activation of caspase-3 and increased expression of Bax were not counteracted by induction of Bcl-xl. Furthermore, activation of FAS and the death-receptor NGF-p75R as well as increased expression of cleaved PARP reported in hippocampus could not be demonstrated in DRGs. The apoptotic phenomena seen in hippocampus were associated with significant neuronal loss (11), which therefore suggest that diabetes induces a more severe apoptotic stress in central neuronal populations than in peripheral DRG neurons.

Instead, DRG neurons showed atrophy and vacuolar cytoplasmic degeneration, characteristic of hydropic degeneration. The frequency of nodules of Nageotte, signifying ganglion cell loss, was not increased in diabetic rats. The mechanisms underlying these changes are not known. However, it is likely that impaired synthesis of neuroskel-

etetal proteins previously demonstrated in this model (33) may account for not only axonal atrophy but also for the atrophy of neuronal perikaria as demonstrated here (24). The vacuolar changes are likely to reflect impaired neuronal energy metabolism and perturbed sodium handling possibly via impaired Na⁺/K⁺-ATPase activity and/or decreased DRG blood flow (34) and may therefore be analogous to nodal axonal swellings seen in peripheral nerve fibers in DPN (35). These constructs are consistent with impaired insulin/C-peptide action affecting Na⁺/K⁺-ATPase activity and resulting in downregulation of NGF and suppression of its high-affinity TrkA receptor as demonstrated here (18,19,36–38).

Abnormalities in insulin and NGF signaling but not in that of IGF-1 may account for the greater susceptibility of C-fibers and their parent NGF-dependent neurons in DRGs. This is supported by the findings in the isohyperglycemic and hyperinsulinemic type 2 counterpart to this type 1 diabetes model, in which perturbations of NGF and its high-affinity receptor are milder and associated with less severe C-fiber pathology (19).

Increased hyperalgesia, as reported here and previously (18,19,21), has been associated with increased spontaneous firing of nociceptive neurons (39) due to upregulation of B₃ and tetrodotoxin-resistant voltage-gated Na⁺ channels in small nociceptive and Aδ DRG neurons (40,41). This will inevitably result in increased Na⁺ influx as demonstrated by increased Na⁺ current densities and a shift in steady-state inactivation in diabetic dorsal root ganglia (41). Coupled with perturbed Na⁺/K⁺-ATPase activity, these changes may not only account for the hyperexcitability of nociceptive DRGs but also for the observed progressive hydropic degenerative changes. Thermal hyperalgesia is observed in humans and STZ-D rats with DPN.

FIG. 5. Expressions of HSPs and neurotrophic factor receptors in DRGs. A: HSP27. B: HSP70. C: HSC70. D: HSP40. E: TrkA. F: IGF-1 receptor α. G: Insulin receptor α (n = 4). H: NGF content in sciatic nerve (SN) (n = 5). Control rats; BB/Wor rats. Results are means ± SD. *P < 0.05 vs. control rats.

FIG. 6. Quantitative analyses of neurons showing HSPs stainability in DRGs. A: HSP27 (n = 3); arrows show axonal staining and asterisks show positive neuronal staining. B: HSP70 (n = 3); arrows show Schwann cell staining and asterisks show positive small neurons. Results are means ± SD. *P < 0.05 vs. control rats. Bar = 50 μm.
In STZ-D rats, this is proceeded by a short period of hyperalgesia (42–45) analogous to a transient period of hyperalgesia in BB/Wor rats (18,19). The transient hyperalgesia most likely correlates to anatomically intact hyperexitable C-fibers, whereas the ensuing hypoalgesia probably reflects the progressive denervation by C-fibers due to the dying-back process.

In summary, the present study we could not confirm previous reports on high apoptotic activity and cell death in DRGs. Instead, the present data are consistent with those reported by Cheng and Zochodne (5), who failed to show loss of DRG neurons in 12-month STZ-D rats. The data are also in keeping with those reported by Burnand et al. (46), who failed to demonstrate the induction of apoptosis-related genes in acutely (8 weeks) STZ-D rats. The axonal loss and DRG neuronal atrophy reported here are consistent with an axonal dying-back phenomenon due to impaired cytoskeletal protein synthesis (6,7,33) secondary to insulin/C-peptide deficiencies and deprived neurotrophic support.

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