We evaluated the effects of chronic hyperglycemia on L5 dorsal root ganglion (DRG) neurons using immunohistochemical and electrophysiologic techniques for evidence of oxidative injury. Experimental diabetic neuropathy was induced by streptozocin. To evaluate the pathogenesis of the neuropathy, we studied peripheral nerve after 1, 3, and 12 months of diabetes. Electrophysiologic abnormalities were present from the first month and persisted over 12 months. 8-Hydroxy-2'-deoxyguanosine labeling was significantly increased at all time points in DRG neurons, indicating oxidative injury. Caspase-3 labeling was significantly increased at all three time points, indicating commitment to the effenter limb of the apoptotic pathway. Apoptosis was confirmed by a significant increase in the percentage of neurons undergoing apoptosis at 1 month (8%), 3 months (7%), and 12 months (11%). These findings support the concept that oxidative stress leads to oxidative injury of DRG neurons, with mitochondrion as a specific target, leading to impaired mitochondrial function and apoptosis, manifested clinically as a predominantly sensory neuropathy. 

**Oxidative Injury and Apoptosis of Dorsal Root Ganglion Neurons in Chronic Experimental Diabetic Neuropathy**

Ann M. Schmeichel, James D. Schmelzer, and Phillip A. Low

**RESEARCH DESIGN AND METHODS**

**Animals.** Male Sprague-Dawley rats that weighed 250 ± 5 g at the beginning of the study were used. Experimental diabetes was produced by the intraperitoneal injection of streptozotocin (STZ) in 0.05 mol/l citrate buffer at pH 4.5 (65 mg/ml; dose 1.32 ml/kg). The control group received an intraperitoneal injection of citrate buffer alone. Control and diabetic rats had free access to Purina Rodent Laboratory Chow and water. They did not receive insulin treatment. The rats were accepted as diabetic when their fasting blood glucose exceeded 16.7 mmol/l 3 days after injection of STZ and remained >16.7 mmol/l at the time when they were killed. Several groups of rats were used to accomplish these studies.

Duration of diabetes was different for three groups:

1. EDN, duration 1 month (n = 8); age- and gender-matched control littermates (n = 8)
2. EDN, 3 months (n = 8); age- and gender-matched control littermates (n = 8)
3. EDN, 12 months (n = 9); age- and gender-matched control littermates (n = 9)
4. EDN, 12 months (n = 5); age- and gender-matched control littermates (n = 5).

Nerve conduction studies were done on groups 1–4 and one EDN and one control from group 4. Groups 1–3 were used for immunohistochemical studies (8-hydroxy-2'-deoxyguanosine [8-OHdG] and caspase-3) and for TUNEL. Group 4 was used for histochemical studies of cytochrome oxidase reactivity. 

**Immunohistochemistry.** We used methods that are standard in our laboratory. Nerve was fixed in situ for 30 min, flash frozen, and cut into 10-μm sections. Endogenous peroxidase, avidin, and biotin were blocked appropriately, and a 0.05% Tween-20 in phosphate buffer was used. Sections were incubated in the primary antibody for 1 h. After washing, specific immunoreactive activity was visualized using a Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) with diaminobenzidine as the chromagen. Negative controls were generated by omission of the primary antibodies and the use of a blocking peptide. Specificity for 8-OHdG was additionally tested by a pretreatment with an RNase solution that did not reduce staining and a DNase-1 (Boehringer Mannheim) treatment that reduced staining to background staining intensity. We used the following antibodies: anti-8-OHdG (mouse monoclonal, 1:40; Genox, Baltimore, MD) and anti-caspase-3 (rabbit polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Staining for cytochrome oxidase was performed using methods from Seligman et al. (12). A TUNEL labeling kit (Onecogene, Boston, MA) was used for labeling apoptotic nuclei. Positive and negative controls were generated per kit protocol. An additional positive control was also used by use of DNase-1 to “nick” the DNA. Three levels of DRG were assessed per animal at 100-μm intervals. An average of 100 neurons/1 month, 104 neurons/3 months, and 125 neurons/12 months were graded per animal.

**Evaluation.** For 8-OHdG and caspase-3, we used a semiquantitative grading system, because cells varied by the severity and presence of staining. To a first approximation, the grades were as follows: 0, light staining and only affecting ≤5% of neurons; 1, staining affecting 5–10% of neurons; 2, staining affecting 10–20% of neurons; 3, staining affecting >20% of neurons. In the evaluation of TUNEL positivity, neurons were considered positive when the nuclei stained positively and showed chromatin clumping. The number of neurons that fulfilled the criteria was expressed as a percentage.
**TABLE 1**
Electrophysiology on diabetic (EDN) and aged-matched control (CON) rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CV (m/s)</th>
<th>Ampl (µV)</th>
<th>CV (m/s)</th>
<th>Ampl (µV)</th>
<th>CV (m/s)</th>
<th>Ampl (µV)</th>
<th>CV (m/s)</th>
<th>Ampl (µV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON 1 mo</td>
<td>8</td>
<td>42.8 ± 0.7</td>
<td>19.5 ± 1.3</td>
<td>43.3 ± 1.6</td>
<td>10.9 ± 0.2</td>
<td>45.7 ± 1.0</td>
<td>78 ± 4</td>
<td>31.5 ± 1.1</td>
<td>9.5 ± 0.4</td>
</tr>
<tr>
<td>EDN 1 mo</td>
<td>8</td>
<td>36.4 ± 0.8</td>
<td>23.0 ± 2.1</td>
<td>39.8 ± 1.3</td>
<td>8.8 ± 0.8</td>
<td>38.9 ± 1.1</td>
<td>89 ± 5</td>
<td>27.2 ± 1.6</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>CON 3 mo</td>
<td>8</td>
<td>53.1 ± 1.1</td>
<td>46.1 ± 2.7</td>
<td>49.4 ± 1.0</td>
<td>9.9 ± 0.4</td>
<td>53.6 ± 1.0</td>
<td>142 ± 5</td>
<td>37.5 ± 1.1</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>EDN 3 mo</td>
<td>8</td>
<td>45.2 ± 0.9</td>
<td>55.1 ± 5.1</td>
<td>41.9 ± 0.9</td>
<td>7.3 ± 0.7</td>
<td>45.5 ± 0.9</td>
<td>140 ± 9</td>
<td>35.6 ± 1.0</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>CON 12 mo</td>
<td>9</td>
<td>47.1 ± 2.0</td>
<td>24.5 ± 2.2</td>
<td>51.1 ± 1.2</td>
<td>5.0 ± 1.0</td>
<td>63.5 ± 0.9</td>
<td>188 ± 8</td>
<td>46.4 ± 0.3</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>EDN 12 mo</td>
<td>9</td>
<td>40.4 ± 1.4</td>
<td>33.0 ± 2.1</td>
<td>46.0 ± 1.1</td>
<td>6.1 ± 1.1</td>
<td>49.1 ± 1.1</td>
<td>201 ± 13</td>
<td>40.7 ± 1.8</td>
<td>5.1 ± 0.9</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001; †P < 0.05; ‡P < 0.01. Ampl, amplitude; CMAP, compound muscle action potential; CV, conduction velocity; NAP, nerve action potential.

**Electrophysiology.** We used techniques that are standard for our laboratory (13,14). Sensory nerve conduction velocity of digital and caudal nerves was measured using fine stainless steel near-nerve–stimulating and –recording electrodes. Motor nerve conduction velocity was measured in the sciatic-tibial and caudal nerves. The compound muscle action potentials were recorded from the pair of fine stainless steel electrodes in the dorsum of hind paw while stimulated by another pair of electrodes at the level of the sciatic notch and ankle. Recordings were made at 35°C; amplified 1,000 times, stored on computer disks, and analyzed off-line using a Nicolet 4094 digital oscilloscope (Nicolet Instruments, Madison, WI) with associated stimulators and stimulus isolation units.

**Statistical methods.** Means of continuous variables were compared between groups using ungrouped two-tailed t test. Nonparametric relationships were examined with Mann-Whitney U tests. Univariate associations between variables were performed using Spearman rank correlations. Data were expressed as mean ± SE unless otherwise stated. P < 0.05 was considered significant.

**RESULTS**

**Animals.** Final weights for control rats at 1, 3, and 12 months were 401 ± 7, 455 ± 10, and 595 ± 12 g, respectively, and were significantly (P < 0.001) greater than those of diabetic rats with corresponding values of 243 ± 12, 214 ± 15, and 294 ± 26 g. Blood glucose values for control rats at 1, 3, and 12 months were 9.9 ± 0.3, 8.7 ± 0.4, and 8.3 ± 0.4 mmol/l, respectively, and were significantly (P < 0.001) lower than values in diabetic rats of 39.4 ± 1.4, 33.3 ± 1.6, and 30.9 ± 1.2 mmol/l. Glycosylated hemoglobin values in control rats at these time points were 3.5 ± 0.2, 3.5 ± 0.1, and 5.0 ± 0.1%, significantly (P < 0.001) lower than corresponding values in diabetic rats of 15.4 ± 0.5, 15.8 ± 0.4, and 15.2 ± 0.5%.

**Electrophysiology.** At 1 month, sensory conduction velocities were consistently and highly significantly increased (digital P < 0.001; caudal P < 0.01), whereas corresponding motor conduction velocities were less significant (sciatic-tibial P = 0.02) or marginal (caudal P = 0.045) in controls. At 3 months, both motor and sensory conduction velocities were highly significantly reduced for the limb nerves of EDN (digital sensory P < 0.001; sciatic-tibial motor P < 0.001). However, greater involvement of sensory conduction continued to be seen for caudal nerve, where sensory deficit was highly significant (P < 0.0001) and motor fibers were not significantly reduced in EDN. At 12 months, differences between diabetic and control rats had lessened for limb nerves but persisted in caudal nerve. Both limb and caudal nerves had reduced motor and sensory conduction velocities. For all three time points, there was a more variable reduction in amplitudes (Table 1).

**Histochemistry.** Patchy areas of cytochrome oxidase staining loss, indicating a loss of enzyme activity, were seen (Fig. 1). Succinic dehydrogenase and trichrome staining did not show major differences between diabetic and control rats (data not shown).

**8-OHdG.** Excess labeling with 8-OHdG was seen at all three time points evaluated (Fig. 2). Immunolabeling was nuclear in a granular multifocal distribution. Labeling index was evaluated semiquantitatively on the basis of the number and intensity of immunolabeling (Fig. 3). Immunolabeling was significantly increased (Mann-Whitney U test) at 1 month (EDN 0.81 ± 0.19 vs. controls 0.19 ± 0.13; P < 0.001), 3 months (EDN 1.20 ± 0.24 vs. controls 0.00 ± 0.00; P < 0.001), and 12 months (EDN 1.72 ± 0.29 vs. controls 0.57 ± 0.20; P < 0.001). There was a progressive increase with duration of diabetes.

**Caspase-3.** An increased number of DRG neurons stained positive for caspase-3 at all three time points evaluated (Fig. 4). Labeling index was evaluated semiquantitatively (Fig. 5) and was significantly increased (Mann-Whitney U test) at 1 month (EDN 1.50 ± 0.19 vs. controls 0.19 ± 0.09;
P < 0.001), 3 months (EDN 1.21 ± 0.57 vs. controls 0.07 ± 0.19; P < 0.001), and 12 months (EDN 1.40 ± 0.10 vs. controls 0.60 ± 0.20; P < 0.001).

TUNEL. The number of TUNEL-positive DRG neurons was increased at all three time points evaluated (Fig. 6). TUNEL positivity expressed as a percentage of positive DRG neurons was significantly increased (unpaired t test, two-tailed) at 1 month (EDN 8.00 ± 1.00% vs controls 2.00 ± 0.00; P < 0.001), 3 months (EDN 7.00 ± 1.00 vs. controls 1.00 ± 0.00; P < 0.001), and 12 months (EDN 11.00 ± 2.00 vs. controls 1.00 ± 0.00; P < 0.001). We compared the percentage of small versus large neurons, as previously defined (15), affected by apoptosis. The percentage of small versus large apoptotic DRG neurons at 1 month was 7% large and 10% small, (difference between large and small, P = 0.05). Corresponding percentages at 3 months were 6% large and 8% small (NS), and at 12 months were 11% large and 12% small (NS).

Correlations. The associations between the index of oxidative stress (8-OHdG) and apoptosis (TUNEL, caspase-3) were evaluated using Spearman correlation statistics. At 1 month, 8-OHdG significantly correlated with caspase-3 (R = 0.71; P < 0.01) and TUNEL (R = 0.80; P < 0.001). At 3 months, the significant association persisted between 8-OHdG, caspase-3 (R = 0.93; P < 0.001), and TUNEL (R = 0.74; P < 0.01). However, by 12 months, the association was not significant for either caspase-3 (R = 0.34; NS) or TUNEL (R = 0.41; NS). For the relationship between caspase-3 and TUNEL, correlation was highly significant at 1 month (R = 0.80; P < 0.001) and 3 months (R = 0.64; P = 0.01) but was no longer significant by 12 months (R = 0.45; NS).

DISCUSSION
There are several findings of this study. First, there is early development of neuropathy electrophysiologically, with more pronounced involvement of sensory fibers followed by a combined sensorimotor neuropathy. In the chronic model (12 months), mitochondrial function may be impaired (cytochrome oxidase stain). Pathophysiologically, there is oxidative injury to DRG neurons early with persistence and some accentuation with increasing age. Oxidative DNA injury is associated with commitment to
apoptosis as indicated by caspase-3 expression and TUNEL positivity.

Although motor and sensory conduction abnormalities are present in EDN, the most consistent abnormalities in chronic EDN are sensory, and it is sometimes the only abnormality in chronic EDN (13). Early electrophysiologic studies have focused on motor conduction abnormalities (16,17), because sensory recordings are technically more demanding and require miniaturized recording electrodes. The present study demonstrates more severe involvement of sensory conduction at the earlier time point of 1 month with involvement of both motor and sensory conduction at later time points. The presence of combined motor and sensory deficits and their reversibility with treatment (5,6,9,13,18) and the close correlation between the deficits in conduction and perfusion suggest that one component of the neuropathy, especially short term, is related to microvascular pathology. We suggest that the chronic distal sensory deficits seen in chronic progressive EDN might be more closely related to DRG pathology demonstrated in this study and in Kishi et al. (15).

There is vacuolar and pigmentary degeneration of DRG neurons in very chronic EDN (14), in association with florid pathological changes of dorsal and ventral roots. Previously, demyelination of ventral root had been described in aged rats, but the sensory roots were unaffected (19). Lipid peroxidation effects are greater in nerve root and DRG because the blood-nerve and perineurial barriers are lower at these sites (20) and may contribute to the development of a radicular myelopathy (14). Other investigators have confirmed these findings of mitochondrial pathology, including ballooning of mitochondria and disruption of the internal cristae as a result of the neurotoxic effects of glucose (2,21–23).

Oxidative stress depends on a balance among free radical generation, pro-oxidant status, and free radical defenses (24). Changes in all three areas occur in EDN (1). Peripheral nerve antioxidant defenses are very low compared with brain and liver (25,26); normal nerve reduced glutathione (GSH) and glutathione containing enzymatic scavengers (glutathione peroxidase and reductase) are only ~10% that of brain (25,26). Increased generation of reduced oxygen species occurs in both human and EDN
Diabetic peripheral nerve has increased conjugated dienes (9,28), hydroperoxides, and GSH (9). These changes are more consistent in lumbar dorsal root ganglia and superior cervical ganglion than in nerve (9). The most sensitive index of ongoing oxidative stress is a reduction in GSH (9,13). Pro-oxidant status is altered: diabetic sciatic nerve has increased lipolysis (18) and altered reducing equivalents (29). The diabetic state results in reduced superoxide dismutase (28) and a further reduction in GSH (9) and glutathione peroxidase, the limiting antioxidant enzymatic scavenger in the diabetic mouse (30). These earlier studies demonstrating footprints of oxidative stress have recently been supplanted by immunocytochemical evidence of DNA damage and cellular localization (31,32). Reduced oxygen species cause irreversible DNA damage to specific proteins. In recent years, antibodies have been generated against modified structures specific for reduced oxygen species–induced damage (31,33). The epitopes include 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins (34). Urinary 8-hydroxy-2'-deoxyguanosine has been reported to be increased in human diabetes (35,36). 8-OHdG staining in the present study confirms oxidative injury to DRG neurons, which, with their large complement of mitochondria and high oxidative metabolism, compose a susceptible target to oxidative injury. The vacuoles observed in our study may be related to dysfunctional mitochondria (2; current study). Mitochondrial DNA is unusually susceptible to oxidative damage (37), and dysfunctional mitochondria have increased reduced oxygen species leakage (38). A vicious cycle of oxidative damage to inner membrane proteins (of mitochondria), leading to imbalances in the electron transport chain, resulting in increased superoxide and hydrogen peroxide production, which in turn further damages membrane proteins, has been suggested (39) as a major pathogenetic mechanism in other states such as aging (40).

The highly significant association between 8-OHdG and caspase-3 immunoreactivity and TUNEL positivity at 1 and 3 months support the notion that oxidative injury is responsible for apoptosis. Support for the importance of apoptosis derives from the close correlation between caspase-3 and TUNEL positivity. The loss of the association between oxidative injury and indexes of apoptosis at
12 months and the progressive loosening of the relationship between TUNEL and caspase-3 immunoreactivity are of interest. The declining correlations with chronicity of diabetes suggest the evolution of more complex interactions. It has been suggested that apoptosis in certain neurological disorders can be much delayed (apoptosis lente) (41). At short time points, every neuron that enters DNA fragmentation, and hence there is a highly significant correlation between TUNEL positivity and caspase-3 immunoreactivity. With increasing duration of disease, because of apoptosis lente, there is less concordance between caspase-3 immunoreactivity and TUNEL positivity as a result of the variable rate at which TUNEL-positive neurons disappear.

We have previously reported a selective loss of the largest DRG neurons (15). The present study lends some support to that observation. Normally large neurons compose 28% (42) or 29% (15), whereas small neurons compose >70% of neurons. When we separately analyzed the percentage of large versus small DRG neurons affected by apoptosis, large were, especially with more chronic diabetes, as frequently affected as small, so the larger neurons do seem to be particularly vulnerable.

Reduced cytochrome oxidase activity, although preliminary, supplements the morphological changes in providing additional evidence of dysfunctional mitochondrium. These findings interdigitate well with the in vitro and in vivo studies of recent-onset diabetes by the Michigan group. They demonstrate the reduction of mitochondrial membrane potential, leakage of cytochrome c, and caspase-3 activation (2,21–23). Our study links these changes with oxidative injury and extends the changes from 1 to 12 months of diabetes.

Caspase-3 activation commits the neuron to the effenter limb of the apoptotic pathway. TUNEL positivity at all three time points indicates that a small population of sensory neurons is committed to apoptosis. These findings agree with our recent observations of a loss of DRG neurons in EDN, affecting primarily the largest neurons (15). In that study, small DRG neurons were unaffected, but there was a 41% attrition of the largest DRG neurons.

These findings now permit a reasonable pathogenetic model of sensory neuropathy of EDN (Fig. 7). Hyperglycemia results in oxidative stress by numerous pathways described in the introduction. Of particular importance to mitochondrial function is the reduction in GSH (43), reduction in NADPH, and increase in NADH (22). The generation of reduced oxygen species, especially superoxide, and peroxynitrite results in single-strand break of DNA (44). Pivotal to the pathogenesis of apoptosis in DRG neurons is the release of cytochrome c from the outer mitochondrial membrane. The egress of cytochrome c requires the opening of mitochondrial pores (44). There is good evidence for this event. Mitochondrial membrane potential falls in hyperglycemic mitochondrium (45) associated with swelling of the organelle. The precise mechanism of pore opening is not known. Most likely, mechanisms are a combination of an alteration in the balance between the protective and proapoptotic components of the bcl family and a reduction in GSH (33). Cytochrome c release has been demonstrated (45), and this binds to Apaf 1, which then recruits procaspase-9 (apoptosome complex) (46). This oligomerization and autoactivation of procaspase-9 to caspase-9 is followed by proteolytic cleavage of procaspase-3 (and other executioner procaspases) activating the executioner caspase-3. It is consistently increased in experimental diabetes (2,21). The net effect is apoptosis of DRG neurons (Fig. 8).

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (NS22352, NS32352, NS39722, and MO1 RR00585 to P.A.L.), the Juvenile Diabetes Research Foundation, and Mayo Funds.

REFERENCES

6. Cameron NE, Cotter MA, Jack AM, Basso MD, Holman TC: Protein kinase C effects on nerve function, perfusion, Na(+), K(+)−ATPase activity and glutathione content in diabetic rats. Diabetologia 42:1120–1139, 1999


