Original Articles

Abnormal Activity in Diabetic Rat Saphenous Nerve

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This study was conducted to test whether abnormal spontaneous activity similar to that found after peripheral nerve trauma develops in diabetic nerve, and whether duration and/or severity of hyperglycemia affected ongoing activity. We maintained 32 diabetic BB Wistar rats on a euglycemic or hyperglycemic control regimen for 3–15 mo; 22 nondiabetic BB rats served as controls. All animals underwent acute saphenous nerve recordings. Whole nerve conduction velocities in 3- to 6-mo-old euglycemic diabetic rats were not different from controls, but 3- to 6-mo-old hyperglycemic diabetic conduction velocities were slower than in controls ($P < 0.001$) or euglycemic diabetic rats ($P < 0.05$). Compared with controls, 9- to 12-mo-old diabetic nerve conduction velocities were slower under both euglycemic ($P < 0.029$) and hyperglycemic ($P < 0.04$) regimens, but treatment groups did not differ. Combined 3- to 6-mo-old diabetic rats exhibited less resting sympathetic activity than controls under both euglycemic ($P < 0.022$) and hyperglycemic ($P < 0.001$) regimens. Sympathetic activity in 9- to 12-mo-old diabetic rats did not differ from controls. However, less sympathetic activity was found in older controls than in younger ones ($P < 0.028$). In conclusion: *1*) saphenous nerve conduction velocity was slower in diabetic BB rats than in controls; *2*) good glycemic control maintained normal conduction velocity in young adults, but the effect diminished with age; *3*) resting sympathetic activity levels in young adult BB rats were lower than controls; and *4*) sympathetic activity in old BB rats was diminished whether diabetes was present or not.
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DPN, diabetic polyneuropathy; DRG, dorsal root ganglion; ANOVA, analysis of variance; df, degrees of freedom; F_1, offspring from the first generation; F_2, offspring from the second generation

DPN is an axonal neuropathy that involves nerve fiber loss, and probable concurrent degeneration and regeneration within the same peripheral nerve fascicle (1–6). Among the least understood aspects of DPN is the development of positive sensory symptoms in the extremities, which do not respond to painful stimuli. These perceptions of prickling, crawling, thermal sensations, and various qualities and intensities of pain (7) are commonly thought to arise from ectopic generators in injured fibers or sprouts (7–9), or from more central foci (10). Autonomic symptoms also are common, and manifest themselves in the periphery in the loss of sympathetic tone (11) and changes in responses to physiological challenge (12,13). Both clinical (14,15) and laboratory (16–19) studies have linked the onset and severity of DPN with the level of glycemic control achieved. However, the exact mechanisms modulating abnormal sensory and autonomic symptoms remain unknown.

The metabolic damage incurred in DPN shares some symptomatic, histological, and physiological features with traumatic peripheral nerve injury, including chronic pain and dysesthesias. Furthermore, neurophysiological recordings in chronically axotomized rats have demonstrated abnormal spontaneous discharges arising from *1*) the injury site, *2*) in-continuity sensory ganglia, and *3*) postganglionic sympathetic efferents. All these forms of aberrant activity have been implicated in the genesis of neuropathical pain (10,20–23).

In a preliminary study (10), BB rats that had been diabetic 3.25–9 mo exhibited an increase in distally propagating spontaneous activity in saphenous nerve. Antidromic electrical stimulation revealed that most of this activity came from C-fibers . Some of the active units responded to mechanical stimulation of the L5 DRG with immediate changes in firing rate, indicating probable nociceptive sensory neuron involvement. However, be-

cause a wide body of literature describes resting activity in postganglionic sympathetic efferents, their possible contribution could not be excluded. Furthermore, in traumatic nerve injury models, antidromic sensory activity has been recorded from dichotomizing afferents with receptive fields on fascial blood vessels near the recording site (21,24).

The hypotheses of this study were that *1*) abnormal spontaneous activity similar to that seen after traumatic injury develops in diabetic nerve; *2*) duration and/or *3*) severity of hyperglycemia affects the type or amount of activity observed; and *4*) whole nerve conduction velocity slows in nerves exhibiting other diabetes-related changes. The spontaneously diabetic BB rat was chosen for the model because its peripheral nerve consistently exhibits functional and structural abnormalities similar to those found in human diabetic nerve (16,17).

RESEARCH DESIGN AND METHODS

A total of 54 male and female BB Wistar rats (219–544 g) were obtained from the University of Washington colony. These rats were maintained as specific pathogen-free-diabetes prone and diabetes-resistant lines (25,26). Diabetic rats (n = 32) had diabetic durations ranging from 3 to 15 mo. One group of 16 (13 males, 3 females) diabetic rats were maintained on a euglycemic regimen (mean serum glucose 9.17 ± 5.37 mM, values are means ± SD). A second group of 16 (11 males, 5 females) diabetic rats was maintained hyperglycemic (mean serum glucose 16.94 ± 5.52 mM). The remaining 22 rats (14 males, 8 females) were age- and sex-matched diabetes-resistant controls. All animals were maintained in air-filtered cages with ad libitum access to rat chow and water. Body weight and glycosuria (Tes-Tape, Lilly, Indianapolis, IN) were monitored daily, and glycemia was tested weekly via tail vein bleeding. Protamine zinc insulin (Lilly), 0.4–4.8 U/day, was given immediately after detection of glycosuria and each day thereafter to ensure survival. After daily weighing and check of glycosuria, dosages were adjusted accordingly to maintain the chosen glycemic control level.

Statistical analysis. Diabetes was defined by chronic hyperglycemia, which appeared at 60–275 days of age. Data from rats that were diabetic from 3 to 6 mo were combined. This combined group then was compared statistically with 9- to 12-mo-old diabetic rats or their respective age-matched controls. Analyses were by Student's t test (2 tailed) and two-way ANOVA.

Preparation for recording. Acute recording procedures were performed in all animals. The rats were given a general anesthetic (sodium pentobarbital, 60 mg/kg, ip) and a tracheostomy was performed. For drug infusion and blood pressure monitoring, femoral venous and arterial cannulae were inserted contralateral to the limb in which recordings were made. Respiration was controlled with a volume ventilator for a PCO_2 of 35–40 Torr, as measured by intermittent arterial samples. Body temperature was maintained at 38 ± 0.5°C with radiant heat. Supplemental pentobarbital was infused at 0.1 mg/min with a syringe pump. After a partial laminectomy, the cauda equina was completely severed at L3 and a 4–0 silk suture was loosely tied around the L5 dorsal root to facilitate mechanical stimulation of the ganglion. No paralytic agents were used in any of these experiments.

Recording protocol. The recording protocol in all rats was identical. Using a modification of the microfilament recording technique (27), the left saphenous nerve was exposed along the medial thigh, and the area was covered with paraffin oil. The nerve was divided near the knee and one of a pair of silver recording electrodes (R1) was placed near its proximal stump. The second pair was placed on the fascia ~5 mm away.

Bipolar stimulating electrodes were placed under an intact portion of the whole nerve, which had been separated from the fascial 10–15 mm proximal to R1. Whole nerve conduction velocity was then determined when an all-or-nothing action potential response was elicited at a fixed latency after delivery of 0.05 ms biphasic pulses generated by a Grass S88 stimulator with a Grass PSIU6 constant current isolation unit. Then the stimulating electrodes were removed and replaced with a second recording electrode pair (R2) similar to those at R1. After removal of a 2- to 3-mm section of the epi- and perineurium, 30 microfilaments were dissected from each proximal stump and sequentially placed across the R1-active electrode. After the search for and characterization of spontaneous activity was complete, the rats were killed with an overdose of pentobarbital.

Both R1 and R2 were connected to High Z probes (gain = 1, Grass HIP 511E configured for common mode rejection and Grass P511 amplifiers gain = 500–20,000, high filter 30 kHz, low filter 0.1 kHz). Impulses from R2 were fed to a digital delay line attached to an averaging computer (Nicolet 1072). Sampling by the averaging computer was triggered by impulses from the microfilament electrode (R1). The resulting averaged R2 signal was then displayed on a storage oscilloscope (Tektronix 5103) and the conduction velocity was computed by dividing the distance between the two recording electrodes (mm) by the time difference between the R1 action potential and the averaged peak from R2 (ms). This method determines both the axonal conduction velocities of individual fibers and the direction of action potential propagation (27).

The origins of spontaneously firing units were determined by previously described criteria (21). Briefly, those from the area of the DRG typically responded to tugging on the ligature with firing rate change. Resting sympathetic activity appeared as low-amplitude multiunit signals firing in synchronism at 1–2 Hz each. Individual action potentials were often multiphasic, having durations of 3–5 ms, and conduction velocity <1 ms. These units ceased to fire during infusion of the sympathetic ganglion blocker, trimethaphan (1 mg), and resumed within minutes of stopping the infusion. When ganglionic foci of activity could not be ascertained, special care was taken to mechanically stimulate fascia, skin, and joints adjacent to the recording site to test for the presence of branched afferent axons.

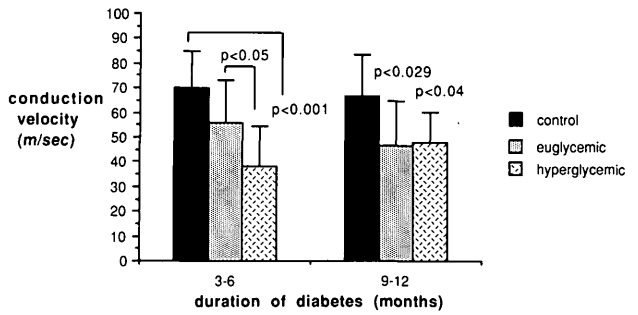

FIG. 1. Saphenous whole nerve conduction velocities in 3- to 6-mo-old euglycemic diabetic rats were not different from controls; however, 3- to 6-mo-old hyperglycemic diabetic conduction velocities were significantly slower than in controls or euglycemic diabetic rats. Conduction velocities also were significantly slower than controls in both groups of 9- to 12-mo-old diabetic rats, but did not differ between treatment regimens. Vertical bars represent SD.

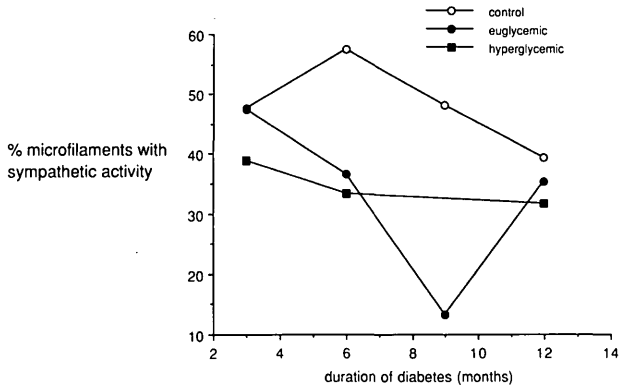

FIG. 2. Chronology of resting sympathetic activity in saphenous nerve is shown as percentage of microfilaments with resting sympathetic activity. Combined 3- to 6-mo-old diabetic rats exhibited significantly less sympathetic activity than controls after either euglycemic ($P < 0.022$) or hyperglycemic ($P < 0.001$) treatment regimen, but the amount of sympathetic activity was not affected by treatment regimen. Sympathetic activity in combined 9- to 12-mo-old diabetic rats did not differ from matched control level, nor did treatment groups differ. However, although conduction velocity did not differ among age-groups, there was significantly less sympathetic activity in older controls than in younger ones ($P < 0.028$).

RESULTS

Whole nerve conduction velocity. Figure 1 (with bars at 2 duration groupings) shows that, in short-term diabetic rats, only the hyperglycemic group conduction velocity was significantly slower than the controls ($t = 4.6$, df = 19, $P < 0.001$). Good glycemic control prevented significant slowing of conduction velocity in 3- to 6-mo-old diabetic rats. In addition, the euglycemic group's conduction velocities were significantly faster than those of the hyperglycemic group ($t = 2.1$, df = 15, $P < 0.05$).

Compared with controls, 9- to 12-mo-old diabetic conduction velocities were significantly slower in both the euglycemic ($t = 2.4$, df = 16, $P < 0.029$) and hyperglycemic ($t = 2.3$, df = 14, $P < 0.04$) groups. However, within the treatment groups, mean conduction velocity did not differ and mean control conduction velocity did not change significantly with advancing age.

Among the 3- to 6-mo-old diabetic rats, conduction velocity measurements in males and females did not differ significantly ($P < 0.8$). Only one female diabetic rat lived long enough to be tested in the 9- to 12-mo-old group. Therefore, late development of a gender difference in DPN sequelae could not be examined.

Resting sympathetic activity. In 54 rats, a total of 1620 microfilaments were examined. Of these, 678 exhibited ongoing activity—671 (41.4% total microfilaments) with sympathetic efferents, 3 (0.19%) antidromic DRG afferents, and 4 (0.25%) dichotomizing afferents with receptive fields on fascial blood vessels near the recording sites.

After 3–6 mo of diabetes, there were fewer microfilaments conducting sympathetic activity in both euglycemic ($t = 2.5$, df = 16 $P < 0.022$) and hyperglycemic ($t = 5.6$, df = 19, $P < 0.001$) groups than in control nerve. Sympathetic activity levels, however, were not affected by treatment regimen.

Sympathetic activity in the 9- to 12-mo-old diabetic rats did not differ significantly from that found in nerves of age-matched controls. Treatment regimen also did not appear to have an effect. However, compared with short-term control values, sympathetic activity was significantly diminished in long-term control nerve ($t = 2.4$, df = 20, $P < 0.028$), indicating that a decrease in sympathetic activity is a component of the aging process in these rats.

Figure 2 shows the chronology of sympathetic activity level decrease. A significant reduction in sympathetic activity from control levels was first found in 3-mo-old hyperglycemic diabetic rats ($t = 2.7$, df = 7, $P < 0.031$), but was not evident until after 6 mo of diabetes in the euglycemic group animals ($t = 2.5$, df = 7, $P < 0.038$). At 9 mo, sympathetic activity was reduced in diabetic rats, but the values were not significantly different from controls. By 12 mo, the values approached unity. Furthermore, control sympathetic activity was decreased 30–40% by 6–9 mo in diabetic rats, and remained at that level at the 12-mo sampling time.

Diabetic male and female sympathetic activity levels did not differ significantly for the 3- to 6-mo-old group ($P < 0.1$). Again, not enough females survived to allow gender comparison among the 9- to 12-mo-old diabetic rats.

Sensory activity. Only ongoing activity was challenged when gentle tugging was applied to the ligature tied to the L5 DRG. After most tugs (98.5%) no change was noted. In three cases (0.45%), conduction velocities were obtained for mechanosensitive ganglion fibers, which were firing spontaneously. These were A-delta fibers with conduction velocities of 3.4 to 3.7 m/s. Sensory ganglion stimulation sometimes (1.05%) caused a transient increase in the number of low-amplitude action potentials, but it could not be determined whether this was due to a firing-rate increase in spontaneously active fibers or recruitment of quiescent units. Of the 11 times this was observed, 10 occurred in diabetic animals; however, the difference was not statistically significant. The first tug frequently recruited high-amplitude action potentials. Successive tugs did not elicit such a response unless they were very strong; this was avoided to prevent unnecessary damage to the ganglion.

Four spontaneously firing branching afferents were identified with receptive fields on fascial blood vessels near the recording site. All of these units were found in control rats ($P < 0.4$, NS). Therefore, their presence appears unrelated to diabetes.

DISCUSSION

The primary features of painful peripheral neuropathies are demyelination and abnormal neural activity. Because demyelination is accompanied by pain in some patients, the metabolic neuropathy found in diabetic rats was examined for abnormal evoked or spontaneous electrical activity. Comparison of our findings with previous reports follows.

Whole nerve conduction velocity. Our data showed that in a predominantly sensory nerve whole nerve conduction velocity slowed as diabetic duration increased, and, in young adult diabetic rats, tight glycemic control prevented significant slowing. However, in older adults, diabetic nerve conduction velocity was slower than controls, regardless of treatment regimen. These data are consistent with previous findings of early onset of sensory neuropathy, and confirmed the presence of DPN in the test animals.

Impaired conduction in peripheral nerve probably is the most frequently recorded finding of distal symmetrical DPN. Most assessments rely heavily on motor nerve conduction velocity. However, both laboratory (16,17) and clinical (14) studies have shown evidence of sensory neuropathy early after onset of diabetes. Sima et al. (18,26,28–31) have used morphometrical and neurophysiological techniques in diabetic BB rats, and found structural and functional damage occurred earlier in sensory than in predominantly motor nerve. Vigorous insulin therapy corrected sensory nerve damage early after the onset of chronic hyperglycemia; but, if therapy was delayed, the damage was permanent.

Sympathetic activity. Our finding of decreased sympathetic output in young adult diabetic cutaneous nerve is in accord with a loss of sympathetic tone in these patients, which results in warm, erythematous feet with abnormally high blood flow and skin temperature (32). The decrease in sympathetic output in older control rats again supports the value of using age-matched controls. Symptoms of autonomic decline (e.g., impaired skin vasoconstriction) in elderly nondiabetic humans are not uncommon (11), and may be exacerbated in a colony of inbred rats. Decreased sympathetic activity in euglycemic diabetic rats may have been caused by insufficient glycemic control or hypoglycemic episodes during insulin treatment. Of primary importance is the progressive nature of efferent diminution during the year after onset of diabetes; the greatest difference from control values occurred in 6-mo-old diabetic rats.

Physiological and morphological evidence for autonomic neuropathy at axonal and somatic levels has been found in the BB rat and other animal models of diabetes (33–41). Lumbar sympathetic chain stimulation has been shown to produce vasoconstriction of peripheral nerve arterioles and closure of capillaries (42). Furthermore, visceral and peripheral symptoms in diabetic patients, which indicate loss of sympathetic tone, are well documented (7). Until now, however, the resting discharge level in sympathetic axons of diabetic rats had not been examined.

The 55% drop in efferent activity we found after 6 mo of moderate to severe hyperglycemia is strikingly similar in magnitude to the 65.5% efferent diminution that developed during the 8 wk after saphenous axotomy in Sprague-Dawley rats (23). An expanding body of evidence indicates that after peripheral nerve injury, regenerating sensory and sympathetic axons interact to produce pain (43–50). Ectopic impulse generation can be enhanced in both myelinated and unmyelinated fibers ending in neuromas by α-adrenergic agents (51–54), or by lumbar sympathetic trunk stimulation (46,51). Furthermore, massive sprouting in the days and weeks after axotomy resulted in increased norepinephrine content near the distal tip of rat neuromas (55), and norepinephrine receptor sites have been demonstrated on peripheral axons (56) and in human skin (57). Finally, recent human studies at this institution have shown that perineuromal injections of α-adrenergic agonists caused excruciating, burning pain in humans (58). Our finding of decreased levels of sympathetic activity in axons ending in neuromas agreed with horseradish peroxidase studies that indicated that approximately two-thirds of the sympathetic fibers ending in a neuroma eventually degenerate (59), and supported the opinion that hyperactivity in sympathetic efferents is not a prerequisite for sympathetically maintained pain (60). The sympathetic activity diminution we found in diabetic nerve parallels the findings of axonal atrophy and may indicate that diabetic neuropathic pain is generated from a limited number of active fibers in peripheral nerve and/or from more central foci.

Sensory activity. Spontaneous antidromic DRG activity or mechanosensitivity appeared unchanged in these experiments. These results differ from what might have been expected after our preliminary study, in which increased distally propagating activity was found. Perhaps the use of a larger number of animals simply facilitated better quantification. This would be exemplified by separating the effect of disease duration from that of glycemic state as factors in the amount of sympathetic activity present. However, two other factors may share responsibility for the unexpected findings.

First, in the earlier experiments, conduction velocities of active units were determined by stimulation of the nerve trunk to recruit the action potential of interest within the microfilament. This technique was problematic; often it was difficult to ascertain with confidence that the recruited action potential was the axon of interest, and stimulation may have induced activity in quiescent fibers. This was particularly true of A-delta and C-fibers, which have high thresholds and typically emerge from a complex compound action potential. Soon after the pilot study was completed, this laboratory applied a modification of the spike-triggered averaging technique to low-amplitude signals from sensory axons. With this method, the same action potential is recorded from two sites

along the nerve and, thus, conduction velocity can be observed directly without relying on electrical stimulation. Although few action potentials are required for large myelinated fibers, determination of C-fiber conduction velocity requires ≥1000 action potentials, which are 1.5 times higher in amplitude than background. Inconveniently, sensory and sympathetic C-fibers share similar amplitudes (50–150 μA). This limitation often prevented separation of resting sympathetic activity from low amplitude sensory nerve action potentials. However, the active fibers that responded to mechanical DRG stimulation, but did not disappear during trimethaphan infusion, were undeniably sensory in origin. Furthermore, spontaneous activity in diabetic sensory ganglia recently has been observed in this laboratory via recordings in the cauda equina, and is under evaluation.

Second, the BB rats used in the original study were provided by Dr. Anders Sima (University of Manitoba, Canada). The University of Washington BB rats used in this study were initially bred and maintained for the genetic and immunological studies of Dr. Ake Lernmark. The possible contribution of genetic factors could not be ignored because sensory neurons and axons in different strains of rats respond to traumatic injury with various levels of spontaneous/ectopic activity (61). However, the two-way ANOVA statistical comparison exposed no difference in sympathetic activity among the parent, F_1, F_2, or backcross generations in either diabetic or control rats. Therefore, it is probable that, as a strain, BB rats exhibit somewhat consistent neurophysiological properties.

Metabolic demyelination undoubtedly is a gradual process compared with abrupt trauma. However, the extent of injury required to produce abnormal activity has not been determined. The possibility remains that DPN could cause enough localized axonal injury to create conditions suitable for ectopic impulse generation. The dramatic differences between spontaneous sensory activity in the rat neuroma and diabetes models may be explained in part by:

1. Size and type of the lesion. DPN is characterized by partial demyelination throughout the periphery. The neuroma model is created by wholesale destruction confined to a specific point of impact.

2. Time frame. The injury process in DPN is progressive. The first significant decrease in the axon-myelin ratio in hyperglycemic diabetic BB rat sural nerve appeared at 4 mo after the onset of diabetes (17). Traumatic nerve injuries are by nature immediate, with conduction completely interrupted.

3. Gallamine was routinely used to paralyze animals in the studies that first defined the electrophysiological properties of experimental neuromas. We found that this potassium channel blocking agent dramatically elevated spontaneous activity levels in both neuromas (20) and their in-continuity DRG (21). Furthermore, in this model, distally propagating DRG activity was rare in unparalyzed animals.

The presence of demyelination in the neuroma and diabetes models implies that the lesions are similar, but not identical. Clearly, additional studies of diabetic nerve, which parallel neuroma studies, are needed to determine whether their structural and functional similarities outweigh their etiological differences.

In conclusion: *1*) whole nerve conduction velocity was slower in diabetic BB rat nerve than in controls; *2*) good glycemic control maintained normal whole nerve conduction velocity in young adults, but the effect diminished with age; *3*) resting sympathetic activity levels in young adult diabetic BB rats were lower than in controls; and *4*) resting sympathetic activity levels in older BB rats were low whether diabetes was present or not.

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