Insulin-Induced Hypoglycemic Peripheral Motor Neuropathy in Spontaneously Diabetic WBN/Kob Rats

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Intensive insulin therapy can lead to hypoglycemia, with patients sometimes developing hypoglycemic neuropathy. Spontaneously diabetic Wistar Bonn Kobori (WBN/Kob) rats develop diabetic peripheral motor neuropathy characterized by segmental demyelination and axonal degeneration. We examined the short-term effects of hypoglycemia on neuropathic changes in these rats. Spontaneous diabetic WBN/Kob rats received insulin implants for 40 d and were divided into 3 groups based on blood glucose levels: group N, normoglycemic to slightly hyperglycemic (150 to 250 mg/dL); group H, hypoglycemic to slightly hyperglycemic (50 to 200 mg/dL); and group D, nontreated spontaneously diabetic (350 to 420 mg/dL). Conduction velocity was measured in sciatic–tibial motor nerves; these nerves also underwent qualitative and quantitative histomorphologic analysis. Conduction velocity was not significantly different in N, D, and H groups. Morphologic analysis of the sciatic nerves of H rats showed severe changes, including axonal degeneration, myelin distention, and endoneurial fibrosis, that tended to occur in large, myelinated fibers. N and D rats showed relatively mild changes. The degree and distribution of degenerated nerve fibers in H rats were significantly higher than in N and D rats. These results suggest that hypoglycemia of less than 50 mg/dL induced severe peripheral neuropathy. Hypoglycemic lesions differed from the hyperglycemic lesions in diabetic WBN/Kob rats. This rat strain is an appropriate model for investigating the hypoglycemic peripheral neuropathy that can be associated with a diabetic condition.

Peripheral neuropathy is a leading complication of diabetes mellitus. Although its exact pathogenesis is not fully understood, chronic hyperglycemia and resultant microenvironmental changes in peripheral nerve tissue contribute to the development of neuropathy.[5] Therefore, intensive insulin therapy is needed to prevent such complications in patients with type 1 diabetes. However, intensive insulin therapy can lead to hypoglycemia, with patients sometimes developing hypoglycemic peripheral neuropathy.[8]

Although experimental hypoglycemic peripheral neuropathy has been studied by using animal models of type 1 diabetes, few studies have included morphologic analyses.[4,7,14,15] These studies showed that hypoglycemia causes axonopathy involving both degenerative and regenerative events. However, hyperglycemic peripheral neuropathy characterized by axonal atrophy has also been induced in diabetic animal models, such that the hyperglycemic changes in these models were similar to hypoglycemic changes. Diabetic WBN/Kob rats spontaneously develop diabetic peripheral motor neuropathy characterized by segmental demyelination and secondary axonal degeneration.[12,13,19] Morphologic changes in diabetic peripheral motor neuropathy are characterized by various degenerative and regenerative changes in myelin sheath, demyelination, and a shift toward axons of smaller di-

ameter. Therefore, WBN/Kob rats may be useful for distinguishing hyperglycemic from hypoglycemic changes. In addition, the threshold of hypoglycemia that induces the morphologic and clinical changes characteristic of peripheral neuropathy in diabetic animals remains unclear.[14] In the present study, we investigated the effects of short-term hypoglycemia on peripheral neuropathic changes in diabetic WBN/Kob rats.

Materials and Methods

Male SPF Wistar Bonn Kobori (WBN/Kob) rats (Japan SLC, Shizuoka, Japan) were reared in a barrier animal room maintained at 24 ± 2 °C and relative humidity of 60% ± 20%, with 12:12-h light:dark cycles and at least 12 changes of sterilized fresh air hourly. All rats were housed and reared in aluminum mesh cages. These animals were free of common rodent viruses, bacteria, and parasites (*Pseudomonas aeruginosa, Salmonella* spp., *Salmonella typhimurium, Pasteurella pneumotropica, Bordetella bronchiseptica, Streptococcus pneumoniae, Corynebacterium kutscheri, Clostridium piliforme, Mycoplasma pulmonis,* Sialodacryoadenitis virus, Sendai virus, Hantavirus, Pneumonia virus of mice, Murine adenovirus, *Giardia muris, Spironucleus muris, Syphacia* spp., and *Aspiculuris tetraptera).* To prevent ascending bacterial infection, cages were changed at least once each week. Rats were given a pelleted diet (CRF1, Oriental Yeast, Tokyo, Japan) and chlorinated water ad libitum. All procedures for animal handling and experimental treatments were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Committee for Animal Ex-

periments of Setsunan University and the Japanese Association for Laboratory Animal Science.

All rats had ongoing hyperglycemia (greater than 200 mg/dL) and glucosuria (greater than 250 mg/dL) from 40 to 45 wk of age. We used a total of 30 male 75-wk-old spontaneous diabetic WBN/Kob rats, 20 of which were treated for 40 d by receiving 1 or 2 sustained-release insulin implants (Linshin Canada, Toronto, Canada) by subcutaneous injection. After insulin treatment, the rats' blood glucose levels dropped sharply to 50 mg/dL and were maintained at 50 to 200 mg/dL (hypoglycemic to slightly hyperglycemic; group H rats) or 150 to 210 mg/dL, reaching 250 mg/dL by the end of the experimental period (normoglycemic to slightly hyperglycemic; group N rats). The remaining 10 spontaneous diabetic rats were maintained without insulin treatment (blood glucose, 350 to 420 mg/dL; group D).

Blood samples from the tail vein were obtained, and blood glucose and plasma insulin levels were measured by the glucose oxidase method (Glutest E, Sanwakagaku, Aichi, Japan) and the sandwich ELISA method (Rat Insulin Kit, Morinaga, Yokohama, Japan), respectively. Samples were collected from 1300 to 1600. The blood glucose and insulin levels of insulin-treated rats (groups N and H) were measured once each week, whereas untreated rats (group D) were evaluated only at the start and end of the experiment.

At the end of the experiment (40 d after initiation of treatment), rats were anesthetized with ketamine (40 mg/kg IM; Ketalar, Tokyo, Sankyo) and xylazine (2.0 mg/kg IM; Seractal, Bayer, Tokyo, Japan). The right sciatic nerve was exposed by incisions in the regions of the great trochanter and ankle, and the distance between incisions was measured. Bipolar stimulating electrodes were placed on the nerves through the incisions. Bipolar recording electrodes were inserted percutaneously into either the interossei or lumbricalis muscle. The muscle potentials and conduction velocity were recorded by using an electromyography system (Polygraph 360 System, Nippon-denki-sanei, Tokyo, Japan, and BioSignal Processing Program, Nihonsanteku, Osaka, Japan). The hindlimb skin temperature was maintained at 37 °C.

Five rats from each group were anesthetized 40 d after initiation of the experiment. Sciatic and tibial nerves were removed and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Samples were trimmed, dehydrated in an automated processor, and embedded in paraffin. Sections (4-μm thick) were immunohistochemically stained with antineurofilament mouse monoclonal antibody (diluted 1:100; catalog no. M0762, Dako, Glostrup, Denmark) and stained with Luxol fast blue or Masson trichrome.

Samples of single teased nerve fibers from sciatic and tibial nerves were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide solution (pH 7.4) for 2 h. After fixation, nerve samples were rinsed briefly in 70% alcohol and immersed in glycerin. Single nerve fibers were teased apart.

Another 5 animals from each group were anesthetized 40 d after dosing, perfused with physiologic saline, and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After perfusion, the left sciatic and tibial nerves were removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After fixation, tissue samples were postfixed in 1% osmium tetroxide solution (pH 7.4) for 2 h and processed into epoxy resin. Semithin (1 μm) sections were cut and stained with to-
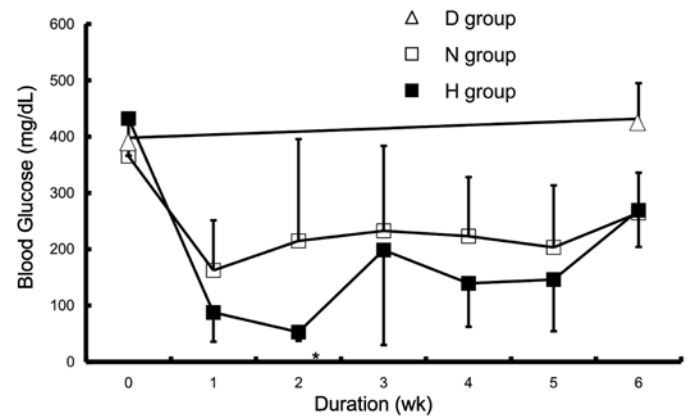
Figure 1. Blood glucose levels (mean ± 1 SD) in diabetic WBN/Kob rats with or without insulin treatment for 6 wk. Rats in group D maintained hyperglycemia (366 to 455 mg/dL) throughout the experimental period. The average blood glucose level of N rats dropped to 150 mg/dL and gradually increased to 250 mg/dL by the end of the experimental period. Average blood glucose in H rats sharply dropped to 50 mg/dL and remained at 50 to 200 mg/dL. *, $P < 0.05$.

luidine blue. For morphometric analysis, semithin cross-sections of a distal portion of tibial nerve were used. Digital images (20× objective lens, 3900 × 3090 pixels) were captured by using a digital camera (DC500, Leica Microsystems, Wetzlar, Germany) attached to a light microscope (DM5500, Leica Microsystems). The sections were analyzed morphometrically by using image processing and analysis software (Ultimage Pro version 2.6.1, Graftek, Austin, France). Morphometric parameters analyzed were total fascicular area; number and size (cross-sectional area) of myelinated nerve fibers, myelin, and axons; and mean fiber, axon, and myelin size (cross-sectional area). Fiber occupancy (nerve fiber area/fascicular area) was calculated by dividing the total area of myelinated fibers into the total fascicular area. Fiber density (number of fibers/mm^2) was calculated by dividing the total number of myelinated fibers into the total fascicular area.

Data are presented as mean ± 1 SD. One-way ANOVA was used with the Tukey multiple comparisons test to determine whether values differed. A P value less than 0.05 was considered statistically significant. Statistical analyses were performed by using the StatMate III program (ATMS, Tokyo, Japan)

Results

Blood glucose and plasma insulin levels. Rats in group D were hyperglycemic (366 to 455 mg/dL) throughout the 40-d experimental period. After insulin treatment began, the average blood glucose level of group N rats dropped to 150 to 210 mg/dL and returned to values of around 250 mg/dL by the end of the experimental period (Figure 1). Although glucose levels varied considerably among individual N rats, no single glucose measurement fell below 50 mg/dL. In contrast, the lowest level of each group H rat fell below 50 mg/dL at least once during the experimental period. In response to insulin treatment, the average blood glucose of group H rats dropped sharply to 50 mg/dL and then was maintained between 50 and 200 mg/dL.

Plasma insulin levels were significantly ($P < 0.05$) higher for the insulin treated groups N and H than for the untreated group D; plasma insulin concentration did not differ between the insulin treatment groups (data not shown). The mean body weights of

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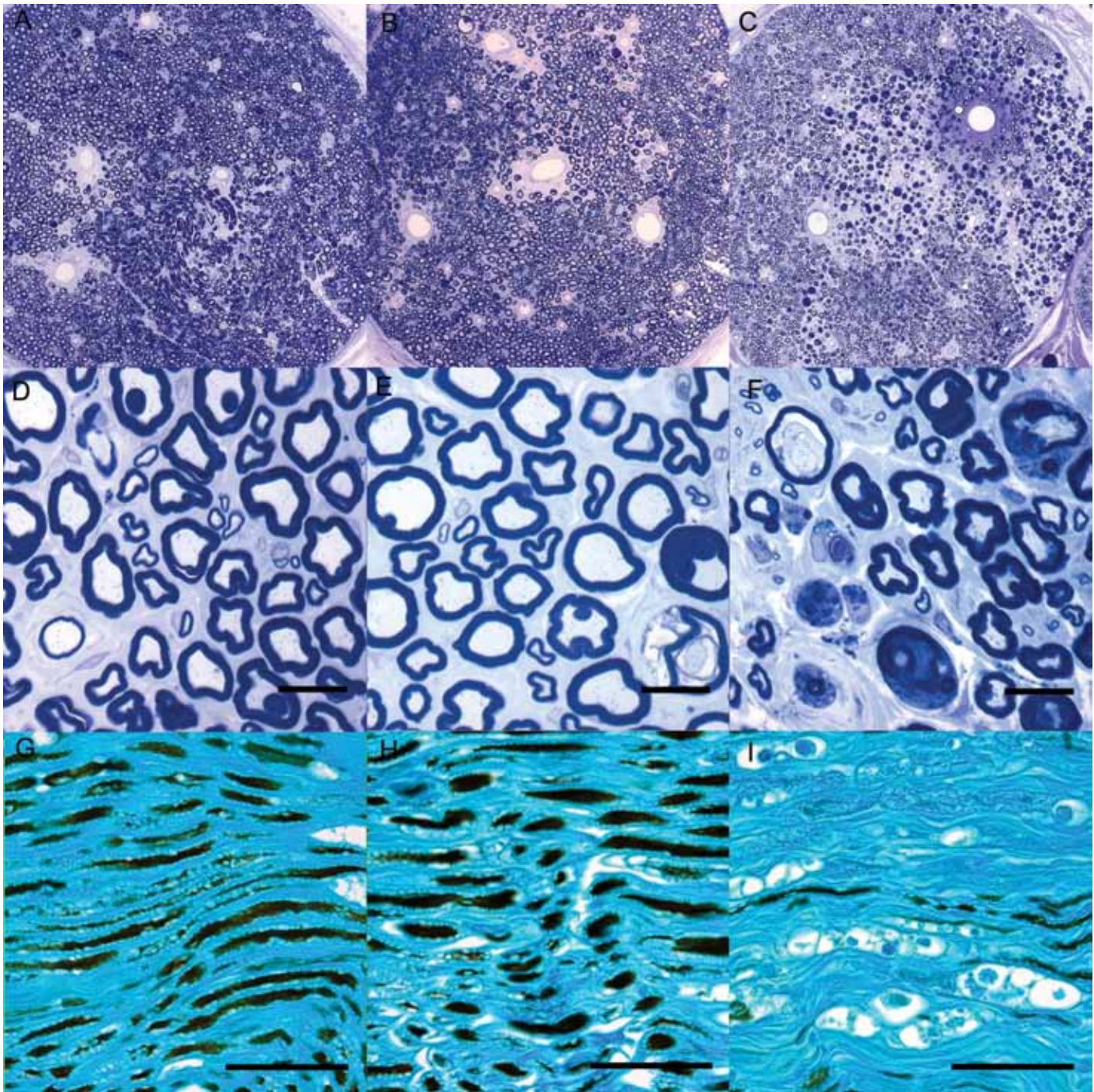
Figure 2. Representative sections of rat sciatic nerve. Nontreated spontaneously diabetic rats (group D; A, D) and normoglycemic to slightly hyperglycemic rats (group N; B, E) show only mild changes including slight myelin distention and endoneurial fibrosis around vessels. Small to large axons are uniformly positive for neurofilament in group D (G) and group N (H) rats. In contrast, hypoglycemic to slightly hyperglycemic rats (group H) had many degenerate fibers with endoneurial fibrosis (C); degenerate fibers were characterized by axonal atrophy with myelin distention (F). Longitudinal sections of nerves from group H rats contained many myelin ovoid formations and loss of immunopositivity for neurofilaments (I). Toluidine blue stain, A through F; Luxol fast blue and neurofilament immunostaining, G through I. Bar, 100 μm (D through F), 50 μm (G through I).

the insulin treatment groups did not differ significantly from that of D group (data not shown). None of the rats in any group exhibited any clinical signs of neurologic disturbance.

Motor nerve conduction velocity. The conduction velocity of group N rats (47.0 ± 9.3 m/s) did not differ significantly from that of either group D (36.8 ± 6.4 m/s) or H (34.3 ± 3.2 m/s) rats.

Morphology. Teased sciatic nerve fibers of group H rats often showed myelin fragmentation, beading, myelin bubble formation, and demyelination in the internodal area; all of these changes were characteristic of this group and were present throughout single fibers. All 3 groups of rats sometimes also showed segmental demyelination and mild myelin distention

Histopathologic analysis of the sciatic nerves of group H rats revealed severe changes in myelinated nerve fibers (Figure 2). The affected fibers were characterized by axonal loss or degeneration with myelin distention (Figure 2 F). The myelin sheath of atrophic axon collapsed, folded, and distended to form myelin ovoids (Figure 3). Immunostaining of neurofilament counterstained with Luxol fast blue confirmed both axonal loss and degeneration and myelin degeneration (Figure 2 I). Grossly, large myelinated fibers tended to be affected more often than were small and medium-sized fibers. The affected fibers were often Schwann cells with abundant myelin debris and vacuoles within the cytoplasm (Figure 3 A). Several thinly myelinated small fibers were surrounded by the abundant cytoplasm of a single Schwann cell (Figure 3 A). Schwann cells occasionally encompassed small, thinly myelinated and medium fibers together with those showing myelin distention or ovoids (Figure 3 B). Endoneurial fibrosis was prominent around the degenerated fibers, but no vascular changes in the endoneurial small vessels were seen (Figure 2 F). The changes in nerve fibers were localized in the central part of nerve fascicles, with no predilection for either perivascular or poorly perfused areas (Figure 2 F). Although a few nerve fibers in rats of groups N and D had mild changes (for example, minor myelin distention and endoneurial fibrosis; Figure 2), the degree and distribution of degenerated nerve fibers in group H were higher than those in groups N and D. Tibial nerve fibers showed similar changes albeit less prominently and frequently those in sciatic nerves. The number of myelinated nerve fibers, fiber occupancy, fiber density, and proportions of small nerves were not significantly different in group H as compared with groups N and D (Table 1).

Discussion

The results of the present study showed that insulin administration could result in severe hypoglycemic peripheral nerve lesions in diabetic WBN/Kob rats. Experimental insulin-induced hypoglycemic peripheral neuropathy is defined as axonal degeneration and secondary breakdown of the myelin sheath.[4,6-11,14-16,20,21] Segmental de- and remyelination are not specific to hypoglycemic neuropathy,[4,6-11,14-16] as these lesions also occur in age-dependent neuropathy.[3,18] The axonal atrophy of hypoglycemic neuropathy tends to affect large myelinated nerve fibers preferentially, leading to an increased number of small fibers.[4,9,10,15,16] The predominant changes in our hypoglycemic rats were axonal degeneration with myelin ovoid formation similar to Wallerian degeneration. Morphologic evidence of severe degeneration or loss of axons strongly suggested that the primary change in affected nerve fibers was axonopathy. The presence of a few small thinly myelinated fibers and myelin debris in the enlarged cytoplasm of a single Schwann cell is consistent with the concurrent events of nerve fiber sprouting with early remyelination and myelin breakdown due to axonal degeneration or loss.[12]

Morphometric analysis did not reveal a significant reduction in cross-sectional area of fibers, axons, and myelin in our hypoglycemic rats. In previous reports, morphologic effects, like primary axonal change and secondary myelin degeneration, preferentially
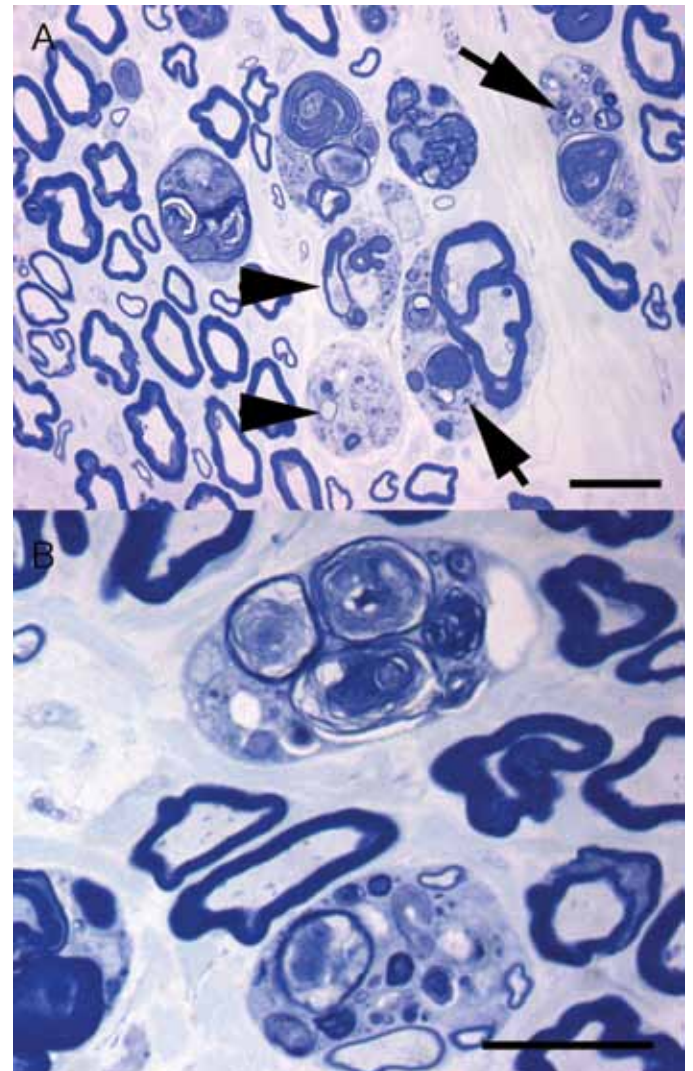
Figure 3. Representative sections of sciatic nerves of hypoglycemic to slightly hyperglycemic rats (group H). (A) These affected fibers are bound together with hypertrophic Schwann cells containing myelin debri and vacuoles (arrows). Several thinly myelinated fibers are surrounded by a single hypertrophic Schwann cell (arrowheads). (C) A single hypertrophic Schwann cell surrounds both small, thinly myelinated fibers and medium fibers with myelin distention. Toluidine blue stain; bar, 100 μm.

affected large fibers and led to higher density of small axons.[6-11,14-16] The peripheral neuropathy of diabetic WBN/Kob rats manifested as myelin distention due to segmental demyelination in predominantly motor neurons that subsequently was accompanied by axonal changes.[12,13,19] In the present study, spontaneous diabetic rats without insulin treatment (group D) showed slight myelin distention without axonal change, suggesting an early spontaneous diabetic change in these rats. These findings suggest that the hypoglycemia of diabetic WBN/Kob rats could lead to a primary axonal change.

Hypoglycemic neuropathy has been investigated by using nondiabetic rats given exogenous insulin.[20,21] Axonal degeneration was the characteristic fiber alteration in these animals, and it predominated in central fascicular distributions of the distal

Table 1. Morphometric analysis of tibial nerves

	Total fascicular area (µm²)	Fiber occupancy (%)	No. of fibers	Fiber density (no. of fibers/mm²)	Mean fiber size (µm²)	Mean axon size (µm²)	Mean myelin size (µm²)
Nontreated, spontaneously diabetic rats (group D)	182427.48 ± 16824.13	46.92 ± 3.71	1545.75 ± 192.12	8489.21 ± 905.11	55.11 ± 31.68	13.32 ± 9.65	41.79 ± 24.49
Normoglycemic to slightly hyperglycemic rats (group N)	199452.36 ± 28641.69	46.90 ± 6.96	1604.60 ± 184.72	8154.89 ± 1325.15	56.30 ± 38.08	14.16 ± 12.62	42.17 ± 28.74
Hypoglycemic to slightly hyperglycemic rats (group H)	193477.61 ± 29124.96	40.50 ± 8.40	1440.60 ± 372.37	7605.16 ± 1872.92	54.99 ± 79.95	13.50 ± 10.81	39.58 ± 26.05

sciatic, proximal tibial, and proximal peroneal nerves. A generalized deficiency of energy substrate can induce fiber degeneration at the 'watershed zones' of poorest perfusion.[21] Nondiabetic rats subjected to hypoglycemia due to insulin treatment reportedly show nerve fiber degeneration characterized by irregular myelin sheaths and axonal atrophy.[17] Insulin-induced hypoglycemia in aged B6C3F1 mice caused segmental axonal degeneration and remyelination.[3] These changes were much milder than those of the hypoglycemic neuropathy that was induced in diabetic animals and were similar to those in spontaneous peripheral neuropathy in aged rats and mice. Therefore, the sustained hypoglycemia induced by insulin treatment of nondiabetic rats may accelerate the progression of spontaneous lesions.[3,17] The hypoglycemic neuropathies of diabetic streptozotocin-treated and BB/Wor rats receiving insulin were reported to be primary axonopathies followed by myelinopathies that were morphologically similar to hyperglycemic lesions.[4,14,15] Similarly, the hypoglycemic lesions of the WBN/Kob rats in the present study were due to primary axonopathy followed by severe breakdown of myelin sheath and differed completely from hyperglycemic lesions, which are characterized by segmental demyelination in this strain of rats.[12,13] Therefore, the hypoglycemic lesions of WBN/Kob rats can be differentiated readily from their hyperglycemic lesions.

Both fluctuations in blood glucose levels and the duration and severity of hypoglycemia induce deterioration of neurologic lesions.[4,14,15] In our hypoglycemic rats, hypoglycemia below 50 mg/dL decreased conduction velocity in motor nerves and induced severe hypoglycemic neuropathy. In contrast, when average blood glucose levels were maintained between 150 and 250 mg/dL, the reduced conduction velocity and morphologic abnormalities were prevented. The individual blood glucose levels of these rats varied but never fell below 50 mg/dL, indicating that hypoglycemia below 50 mg/dL induced severe peripheral neuropathy in diabetic WBN/Kob rats.

Rats housed on wire-mesh cage floors can develop a pressure-induced neuropathy of the plantar and tibial nerves.[1,2] These lesions are characterized by demyelination, and their incidence increases with age. However, the hypoglycemic neuropathy we describe here was more severe in the sciatic nerve than in the tibial nerve. Therefore, pressure-induced neuropathy is unlikely to have influenced the hypoglycemic and hyperglycemic neuropathies in our study rats.

In conclusion, our results suggest that hypoglycemia maintained below 50 mg/dL by treatment with insulin implants induced severe hypoglycemic neuropathy characterized by a primary axonopathy in diabetic WBN/Kob rats. The resulting hypoglycemic lesions of peripheral nerves were distinct from hyperglycemic lesions, which were characterized by segmental demyelination in WBN/Kob rats, making these rats a suitable model for investigating hypoglycemic peripheral neuropathy.

Acknowledgment

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