The Effect of Hyperglycemia on Nerve Conduction and Structure Is Age Dependent

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The nerve conduction velocity (NCV) of nondiabetic male Wistar rats continues to increase until ~26 weeks of age. Rats made hyperglycemic at 6 weeks of age manifest reduced NCV by 10 weeks of age and show morphological differences in the sciatic tibial nerve after 5 months of hyperglycemia when compared with age-matched controls. Fiber diameter, myelin width, and the number of large myelinated fibers were decreased in the tibial nerves of the hyperglycemic animals. Rats made hyperglycemic at 26 weeks of age had elevated glycosylated hemoglobin and sciatic nerve sorbitol levels but maintained normal NCVs and had little change in morphology after 7 months of hyperglycemia. Thus, animals with maturing peripheral nerve structure and function exposed to chronic hyperglycemia manifest greater pathological alterations than those that occur when more matured nerves are exposed to similarly elevated glucose concentrations for an even greater duration. We suggest that immature animal models commonly used to study diabetic peripheral neuropathy may not be appropriate for understanding a process that commonly develops in humans who become hyperglycemic after maturation of the peripheral nerves. *Diabetes* 45:209–215, 1996

Experimental diabetes in rats has been shown to cause a decrease in nerve conduction similar to that observed in human diabetes (1). The mechanisms for these changes have been sought in an effort to define a method to reverse or prevent this common complication of diabetes. Metabolic factors, such as increased polyol pathway activity, have been observed (2). Increased polyol pathway activity has been linked to a reduction in Na^+-K^+-ATPase activity (3). This reduced activity is believed to impair NCV by altering the transmembrane Na^+-K^+ gradient. Morphological changes such as axon shrinkage and paranodal swelling have also been associated with reduced nerve conduction velocity (NCV) in peripheral nerves of diabetic rats (4).

In studies reporting reduced nerve function in diabetic rats, animals were made hyperglycemic between 6 and 20 weeks of age (5–8). Other studies of peripheral neuropathy used rats that become hyperglycemic spontaneously before 18 weeks of age (4,9). Each of these reports show slower NCVs within weeks of hyperglycemic onset and measurable morphological changes after 4–6 months of hyperglycemia (10). NCVs increase with age in the rat until 26–30 weeks of age (11). Nerve fibers progressively increase in size during this same interval. The increase in nerve axon area and myelin width during functional maturation is impaired by hyperglycemia (10). The following study was designed to determine the effect of hyperglycemia upon the function and structure of immature and mature peripheral nerves. Peripheral nerve function in humans matures to adult levels by late childhood and early adolescence (12). Because most subjects with diabetic peripheral neuropathy have the onset of hyperglycemia after adolescence, it is important to determine whether the most commonly used animal model for diabetic neuropathy is appropriate for the study of human diabetic peripheral neuropathy.

RESEARCH DESIGN AND METHODS

Male Wistar rats were used for this study. Hyperglycemia was induced by intraperitoneal injection of streptozotocin (STZ) into two groups of animals. Group 1 was 6-week-old animals (n = 20) receiving 50 mg/kg STZ (Upjohn, Kalamazoo, MI) in cold buffered sodium citrate (pH 4.5). Group 2 animals (n = 10) received 35 mg/kg STZ at 26 weeks of age. The number of animals evaluated at each time point during the protocol is seen in Table 1. Seven days after STZ injection, tail blood from nonfasting rats was collected, and the glucose content was measured with an Ames Glucometer 2 (Miles, Elkhart, IN). Hyperglycemia was defined as plasma glucose >16.7 mmol/l. Subsequent tests for hyperglycemia were performed every 4 weeks; GHb was determined at the end of each study by affinity chromatography using a Glyc-Affin GHb column (Isolab, Akron, OH). Ultralente insulin (Lilly, Indianapolis, IN), 3–5 U/kg, was injected subcutaneously three times a week at a dose designed to prevent weight loss, but inadequate to produce normal blood glucose levels. Animals were provided free access to food and water. Morphometric and biochemical characteristics were determined at 26 weeks for Group 1 rats and at 54 weeks for group 2 rats (Table 1).

Nerve conduction studies. NCV was measured in each animal 4 weeks after STZ treatment and every 4 weeks thereafter. The animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Rear limb temperature was maintained at 36°C with a radiant heater regulated with a needle thermistor placed subcutaneously in the lower thigh. Stimulating needle electrodes were inserted close to the sciatic nerve at the sciatic notch and the tibial nerve at the ankle. Subcutaneous recording electrodes were placed transversely over the plantar intrinsic foot muscles. Stimuli were supramaximal square-wave pulses at 0.05 ms duration. The impulse wave was stored on an IBM PC model 30. The image was traced with a cursor and entered into a software program, ISC-16 from RC Electronics (Goleta, CA), which calculates the latency. Proximal and distal latencies were measured to the negative peak of the compound muscle action potentials, and NCVs were calculated.

Morphometry. Animals were anesthetized with 100 mg/kg sodium pentobarbital, and the tibial nerve in the leg opposite that used for NCV was exposed and fixed in situ for 20 min with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer. The middle segment of the tibial nerve was then dissected and immersed overnight at 4°C in 2.5% buffered glutaraldehyde. Transverse samples were taken from the nerve and postfixed with 1% chrome osmium tetroxide. Morphometric measurements were performed on 0.75-μm epoxy-embedded sections stained with toluidine

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NCV, nerve conduction velocity; STZ, streptozotocin.

TABLE 1
Study protocol

Age (weeks)	Groups				
	1	1	1	2	2
6	10	10 (S)	10 (S)	10	10
10–26	10N	10N	10N	10N	10N
26	10N, B, M	10N, B, M	10N	10N	10N, (S)
30–50	—	—	10N	10N	10N
54	—	—	10N	10N, B, M	10N, B, M

For the animals aged 10–26 weeks and 30–50 weeks, NCV was performed every 4 weeks. N, NCV; B, blood; M, morphology; S, STZ administered.

blue. Slides were positioned on the stage of an Olympus model BH-2 microscope, and the image was projected through a Panasonic WV 1500-X TV camera to a computer display. Tracings of the outlines of axons and their myelin sheaths were made using a Hipad digitizer under final magnification of 2,200×. These tracings were analyzed for myelinated fiber and axon diameter, axon area, myelin width, and the number of myelinated fibers per nerve. A minimum of 600 myelinated fibers per nerve in regularly spaced frames were analyzed.

Biochemistry. After all physiological measurements were completed, animals were anesthetized with 100 mg/kg sodium pentobarbital. The sciatic nerve was removed from the live animal, and the epineurium was removed. The nerve was immediately frozen in liquid nitrogen and lyophilized to dryness and stored in an atmosphere of nitrogen to prevent auto-oxidation. Sciatic nerves were homogenized in cold (4°C) deionized water with a Polytron PT 3000 homogenizer (Brinkmann, Lucerne, Switzerland). These suspensions (1 ml) were deproteinated by precipitation with 2 ml 0.17 mol/l barium hydroxide and 2 ml 0.17 mol/l zinc sulfate. Sorbitol and *myo*-inositol were measured as acetate derivatives by gas-liquid chromatography (13) with two modifications: *1*) a 30-m capillary column (J&W, Folsom, CA) with a DB-225 liquid phase was used and *2*) the gas-liquid chromatograph was model 5890 (Hewlett Packard, Avondale, PA). The results are expressed as nanomoles per milligram dry weight.

Statistical analysis. Data are presented as means ± SE. Paired data were compared using the Student's *t* test. Peripheral nerve fiber size is not normally distributed; therefore, the Wilcoxon rank-sum test was used to compare mean nerve fiber dimensions in control and diabetic animals. The fiber diameter, axon diameter, axon area, and myelin width were measured for a minimum of 600 nerve fibers in each animal. The fibers representing 1, 5, 10, 25, 50, 75, 90, 95, and 99% for each measurement were selected. The mean value for the 10 animals in each group were used to compare the fiber dimensions used in Figs. 2–5.

RESULTS

NCV. NCVs of 20 group 1 diabetic animals were compared with 30 nondiabetic control subjects for the initial 26 weeks (Fig. 1). After 4 weeks of hyperglycemia, the group 1 diabetic animals had NCVs that were significantly less than those measured in the control animals of the same age. This difference in NCV remained during the next 44 weeks of repeated measurements. At 26 weeks of age, 10 group 2 animals became hyperglycemic after the administration of STZ. After 28 weeks of hyperglycemia confirmed by GHb

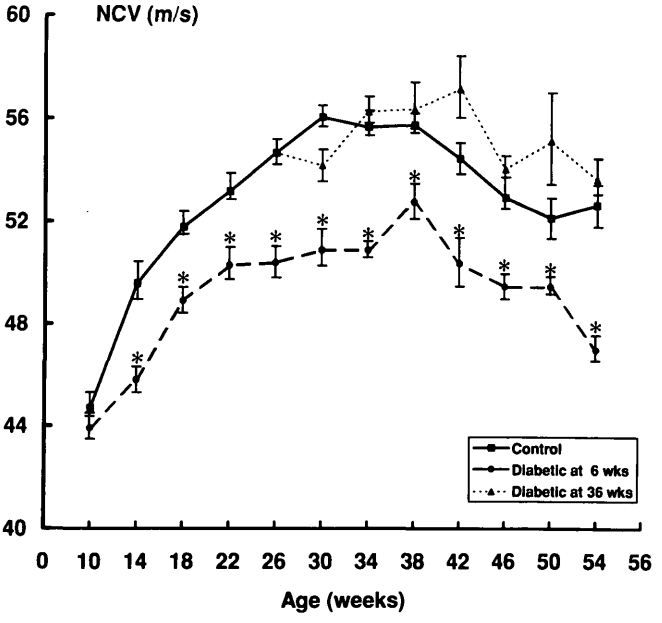

FIG. 1. NCVs performed on male Wistar rats every 4 weeks from 10–56 weeks of age. ■, nondiabetic rats; ●, rats made hyperglycemic with STZ at 6 weeks of age; ▲, rats made hyperglycemic at 26 weeks of age. Data are presented as means ± SE.

levels that were three times the nondiabetic mean (Table 2), the NCVs of the group 2 animals did not differ from the 10 nondiabetic controls of the same age (Fig. 1).

Biochemistry. Group 1 hyperglycemic animals had elevated GHb, elevated sciatic nerve sorbitol, normal nerve inositol levels (Table 2), and reduced NCVs (Fig. 1). Group 2 animals also had elevated GHb and sciatic nerve sorbitol and normal nerve inositol levels (Table 2) after 28 weeks of hyperglycemia (54 weeks old), but the NCVs did not differ from those of age-matched control rats.

Morphometry. Nerve morphometry was altered in the group 1 animals after 20 weeks of hyperglycemia. These animals had smaller nerve fibers with reduced myelin width and unchanged axon area (Table 3). In addition, the group 1 animals had a reduction in the number of large myelinated fibers and a commensurate increase in the number of small myelinated fibers per nerve. The fiber diameter, axon diameter, axon area, and myelin width percentiles were determined for each group of animals evaluated in this protocol (Figs. 2–5). The fiber diameter of nerves found in group 1 diabetic animals was significantly less from the 25th percentile through the 99th percentile when compared with control animals (Fig. 2*A*). Although the axon diameter for group 1 diabetic animals at the 50th and 75th percentile was less (Fig. 3*A*), the major difference in the fibers was the reduced myelin width at every percentile from the 1st through the

TABLE 2
Selected biochemical characteristics of rat sciatic nerve

	Nondiabetic (at 26 weeks)	Group 1 diabetic (at 26 weeks)	Nondiabetic (at 54 weeks)	Group 2 diabetic (at 54 weeks)
Sorbitol (nmol/mg dry wt)	0.47 ± 0.3	4.3 ± 0.15*	0.37 ± 0.07	8.4 ± 0.13†
Inositol (nmol/mg dry wt)	5.5 ± 0.1	5.1 ± 0.5	7.7 ± 0.57	7.4 ± 1.2
GHb	4.9 ± 0.2	12.5 ± 0.5†	5.1 ± 0.4	16.2 ± 2.3†
n	10	10	10	10

Data are means ± SE. *$P < 0.01$. †$P > 0.001$.

TABLE 3
Morphometry of cross-sectioned rat tibial nerves (STZ administered to rats at 6 weeks of age)

Parameter	Nondiabetic (at 26 weeks)	Group 1 diabetic (at 26 weeks)	*P* value
Fiber diameter (μm)	6.44 ± 0.05	5.92 ± 0.10	<0.001
Axon diameter (μm)	4.08 ± 0.05	3.93 ± 0.07	<0.1
Axon area (μm)	14.75 ± 0.38	13.99 ± 0.43	<0.21
Myelin width (μm)	1.18 ± 0.02	0.99 ± 0.03	<0.0001
Number of myelinated fibers >6.5 μm/nerve	2,044 ± 54	1,352 ± 98	<0.0001
Number of myelinated fibers <6.5 μm/nerve	1,544 ± 41	2,101 ± 139	<0.005
GHb (%)	6.3 ± 0.3	16.7 ± 0.3	<0.01
n	10	10	

Data are as means ± SE.

99th when compared with control animals (Fig. 5*A*). In contrast, the fiber diameter found in group 2 diabetic animals at each percentile was no different from that in the control animals (Fig. 2*B*). The group 2 rats showed an increase in axon area over the younger group 1 animals, but the mean axon area and myelin width of the group 2 diabetic animals did not differ from those in the group 2 control animals (Table 4). Myelin width measurements in group 2 animals were somewhat less at the 50th through the 95th percentile (Fig. 5*B*), but the fiber and axon diameters were no different (Figs. 2*B* and 3*B*). There was no difference in nerve function (NCV) when group 2 diabetic animals were compared with control animals (Fig. 1).

Biochemical changes associated with hyperglycemia initiated before maturation of peripheral nerve function were associated with altered nerve morphology and reduced NCVs; these same biochemical changes in animals with functionally mature nerves at the onset of hyperglycemia were associated with normal nerve function and few, if any, morphological changes after 7 months of hyperglycemia.

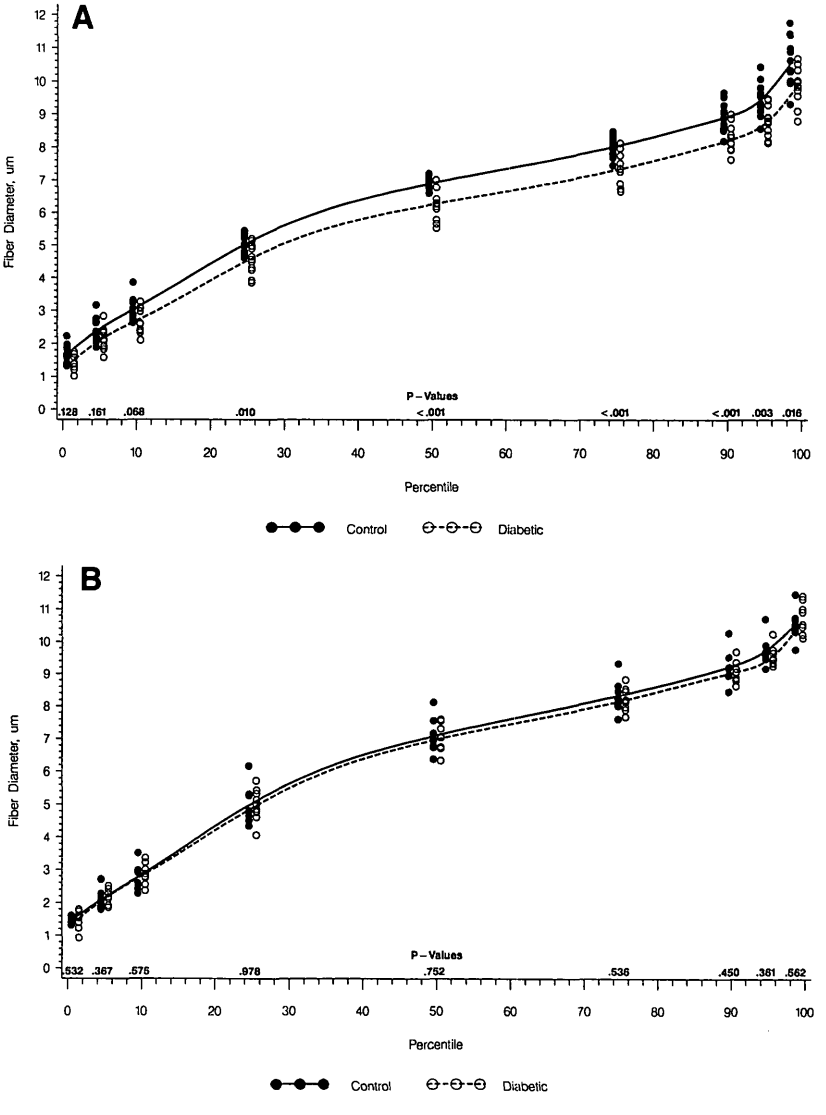

FIG. 2. Percentiles of the fiber diameter distribution for each nerve in 26-week (*A*) and 54-week (*B*) animals. Fiber dimensions were selected by percentile from a minimum of 600 fibers for each animal. The mean for each percentile was determined for diabetic (10) and control (10) animals in groups 1 and 2. The differences at each percentile were tested by Student's *t* test, and the *P* values are shown above the abscissa.

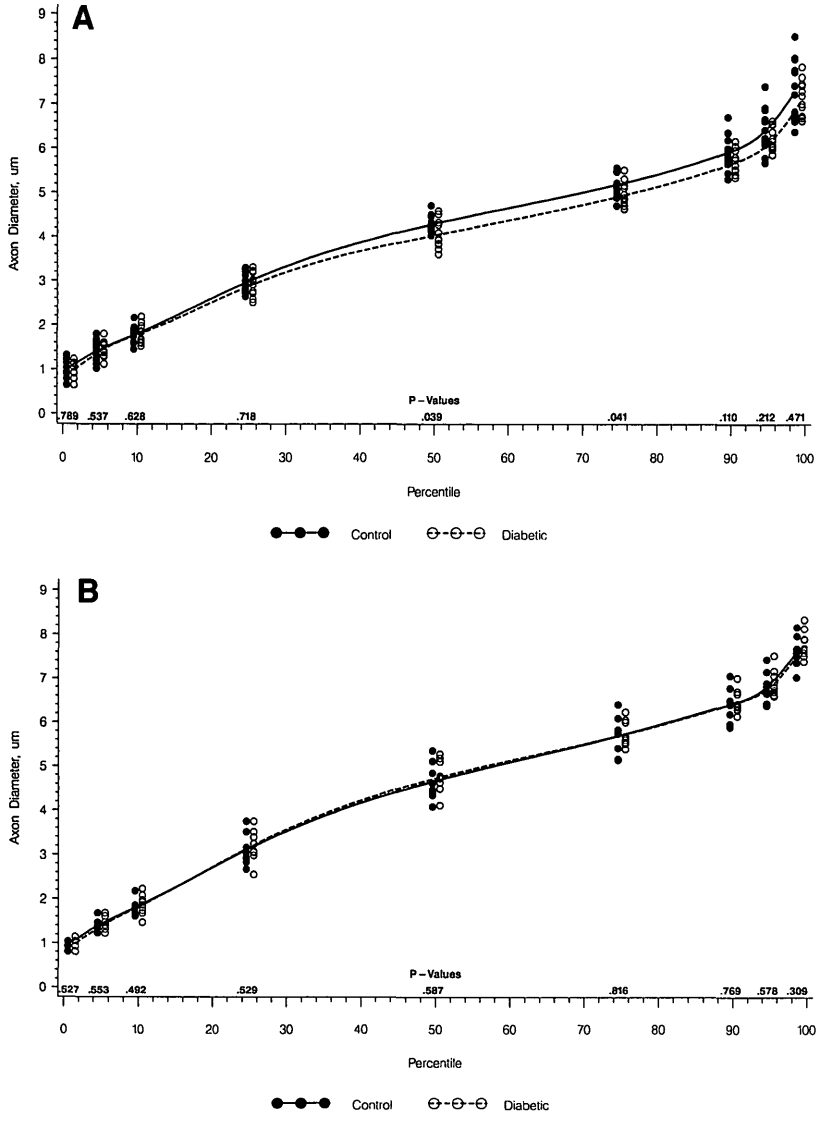

FIG. 3. Percentiles of the axon diameter distribution for each nerve in 26-week (*A*) and 54-week (*B*) animals. Fiber dimensions were selected by percentile from a minimum of 600 fibers for each animal. The mean for each percentile was determined for diabetic (10) and control (10) animals in groups 1 and 2. The differences at each percentile were tested by Student's *t* test, and the *P* values are shown above the abscissa.

DISCUSSION

The longitudinal progression of NCV in male Wistar rats reported in this study indicates that functional maturity occurs between 26 and 30 weeks of age. When hyperglycemia was induced before nerve functional maturity (group 1 animals), the previously reported observations of reduced NCV and structural abnormalities were confirmed. When hyperglycemia was induced after functional maturity of peripheral nerve function (group 2 animals), no reduction of NCV or functionally important structural changes were observed. This extends by 4 months the previous observation of Thomas et al. (14), who reported little or no reduction in NCV 8 weeks after 9-month-old rats were made hyperglycemic with STZ.

The nerve sorbitol levels in both group 1 and group 2 hyperglycemic animals were comparable with those previously reported in diabetic animals (15). The nerve inositol levels in this study, however, were not significantly lower than those found in the age-matched controls. This difference from previous reports could be influenced by the longer duration of diabetes in the current study compared with 2 or 4 weeks of hyperglycemia, previously associated with lower nerve inositol (6,7,15). Support for diabetes duration influencing nerve inositol comes from the observation of elevated sorbitol and unchanged inositol in nerves removed from humans (15,16) with diabetes of sufficient duration to develop clinical neuropathy. This suggests that lower tissue inositol levels may be a consequence of short-term (2–4 weeks) hyperglycemia, which resolves in animals with hyperglycemia of longer (6 months) duration.

It may be that tissue levels of inositol during short-term hyperglycemia (2–4 weeks) reflect a physiological function of osmole regulation (17) rather than a metabolic consequence of increased polyol pathway activity. Thus, the current observation of normal levels of nerve inositol in hyperglycemic animals may indicate a physiological adjustment to chronic hyperglycemia. The present observation of a normal nerve inositol level in association with slowed as well as normal NCVs indicates that the process regulating nerve inositol content is not primary in the pathogenesis of reduced NCVs in subjects with diabetes.

The group 2 animals were exposed to comparable hyperglycemia for a longer period of time (28 vs. 20 weeks) than were the group 1 animals. Nevertheless, the prominent functional and morphological changes evident in the group 1 animals were not found in group 2 animals, suggesting that functionally mature nerves may be more resistant to the pathological effects of hyperglycemia. The observation that

TABLE 4
Morphometry of cross-sectioned rat tibial nerves (STZ administered to rats at 26 weeks of age)

Parameter	Nondiabetic (at 54 weeks)	Group 2 diabetic (at 54 weeks)	*P* value
Fiber diameter (μm)	6.56 ± 0.17	6.52 ± 0.10	<0.83
Axon diameter (μm)	4.39 ± 0.12	4.47 ± 0.08	<0.59
Axon area (μm^2)	17.46 ± 0.94	18.00 ± 0.61	<0.62
Myelin width (μm)	1.09 ± 0.03	1.03 ± 0.01	<0.13
Number of myelinated fibers >6.5 μm/nerve	2,069 ± 87	1,987 ± 47	<0.39
Number of myelinated fibers <6.5 μm/nerve	1,501 ± 124	1,453 ± 101	<0.77
GHb (%)	5.1 ± 0.4	16.2 ± 2.3	<0.01
n	10	10	

Data are means ± SE.

maturing nerves in animals hyperglycemic for 20 weeks have a preponderance of small (<6.5 μm) myelinated fibers while animals with functionally mature nerves exposed to similar hyperglycemia for 28 weeks did not have an increase in smaller myelinated nerve fibers indicates that hyperglycemia impairs growth and maturation of nerves rather than degeneration of functionally mature nerves.

The axon diameter and axon area of the group 1 animals hyperglycemic for 16 weeks and the group 2 animals hyperglycemic for 28 weeks did not differ from their age-matched controls. This indicates that chronic hyperglycemia had no major effect upon axon integrity in these animals but was associated with a significant reduction in NCV in the group 1 animals. The axon shrinkage previously reported in diabetic animals may have been an artifact of hyperosmolar dehydration, as suggested by others (18), since the animals in this experiment gained weight during the course of the experiment, indicating at least adequate hydration. This experiment of chronic hyperglycemia suggests that the major structural change associated with hyperglycemia, as suggested by others (19), occurs in the myelin sheath. Although the mean myelin width of the animals hyperglycemic at 7

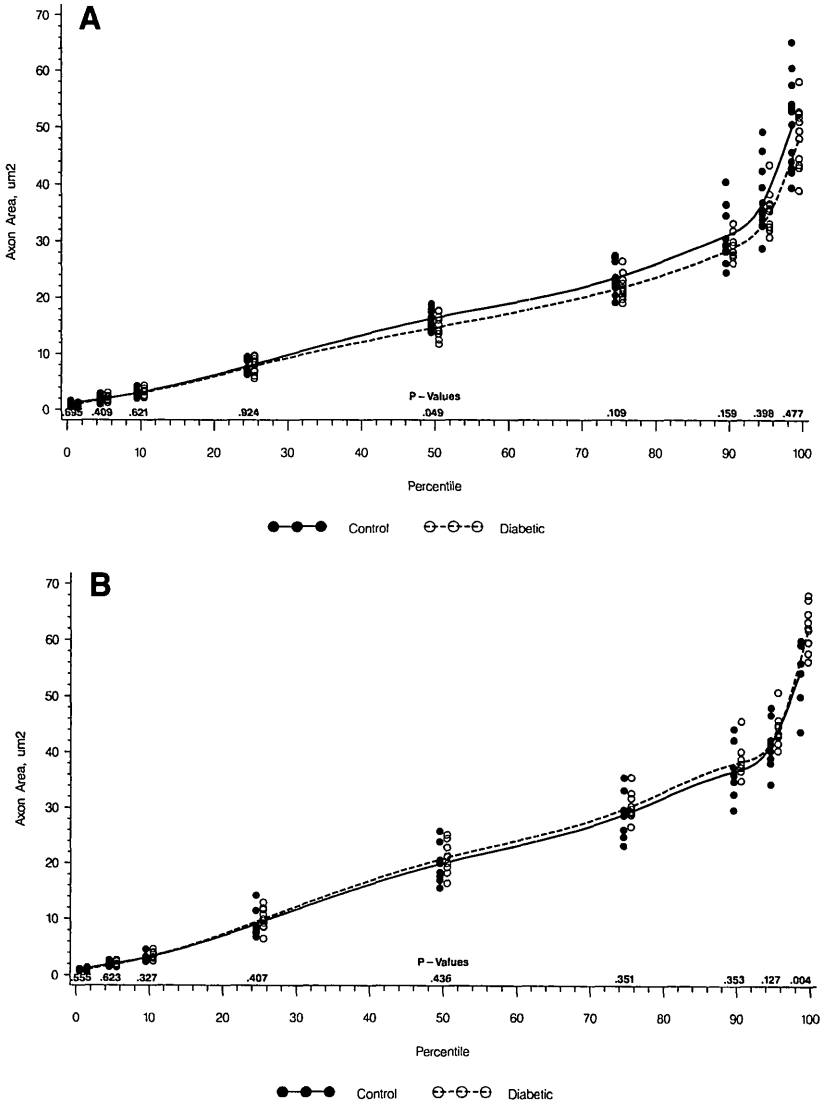

FIG. 4. Percentiles of the axon area distribution for each nerve in 26-week (*A*) and 54-week (*B*) animals. Fiber dimensions were selected by percentile from a minimum of 600 fibers for each animal. The mean for each percentile was determined for diabetic (10) and control (10) animals in groups 1 and 2. The differences at each percentile were tested by Student's *t* test, and the *P* values are shown above the abscissa.

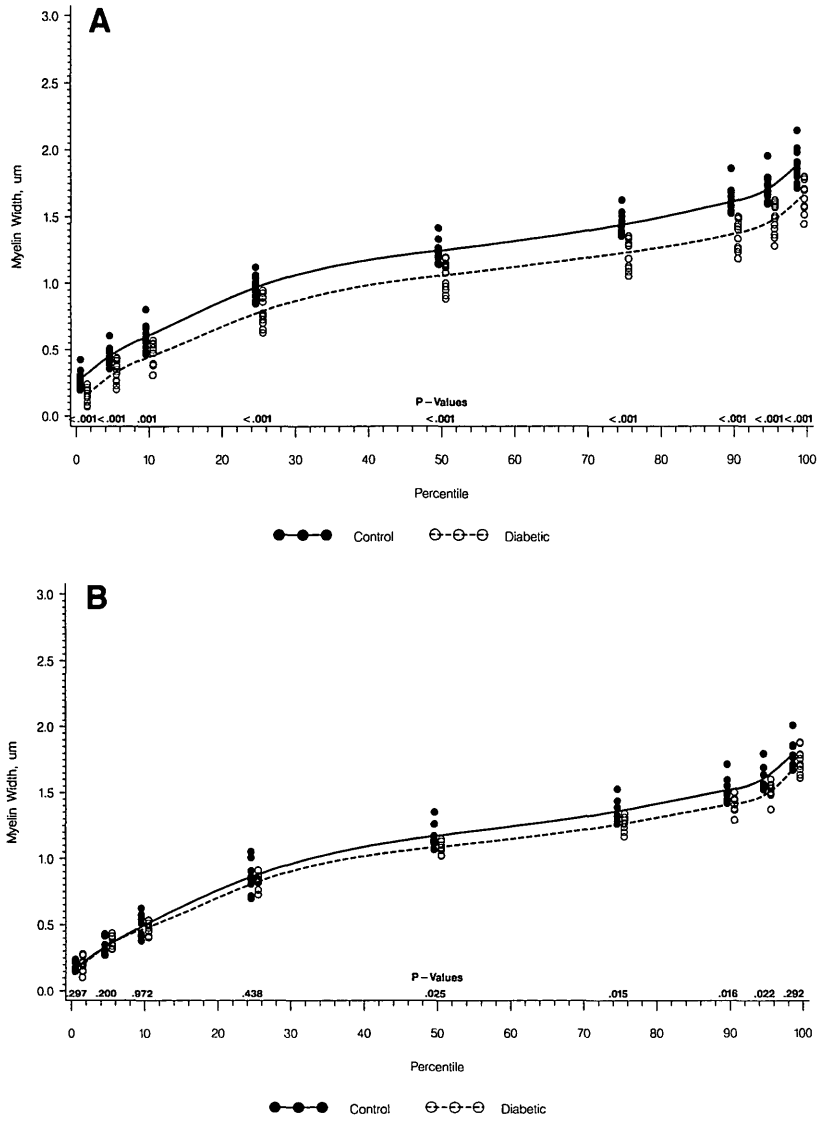

FIG. 5. Percentiles of the myelin width distribution for each nerve in 26-week (*A*) and 54-week (*B*) animals. Fiber dimensions were selected by percentile from a minimum of 600 fibers for each animal. The mean for each percentile was determined for diabetic (10) and control (10) animals in groups 1 and 2. The differences at each percentile were tested by Student's *t* test, and the *P* values are shown above the abscissa.

months did not differ from that in control animals, the reduced myelin widths noted from the 50th through the 95th percentile in the group 2 animals suggest that some insult to myelin integrity may be occurring. This suggests that previous observations of structural and functional defects in maturing rat peripheral nerves exposed to elevated glucose may not occur or that they proceed at a much slower rate in functionally mature nerves even though the biochemical milieu (e.g., increased GHb and polyol pathway activity) is identical. It is possible that more prolonged (>7 months) hyperglycemia may be required in mature nerves to induce the same severity of structural and functional pathological changes observed in less mature nerves. New studies using older animals hyperglycemic for a longer duration (>7 months) are now required to determine if the immature hyperglycemic rat is simply a more sensitive but accurate model of diabetic neuropathy or is instead a model of a neuropathic process that rarely occurs in human diabetes.

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