Diabetic Autonomic Neuropathy in BB Rats and Effect of ARI Treatment on Heart-Rate Variability and Vagus Nerve Structure

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The preventive effect of the aldose reductase inhibitor (ARI) ponalrestat on heart-rate variability and the development of autonomic neuropathy in the vagus nerve was investigated in the spontaneously diabetic BB rat. ARI treatment completely prevented the characteristic decrease in heart-rate variability and axonal atrophy of the vagus nerve for 4 mo in hyperglycemic BB rats. After 6 mo of treatment, the preventive effect on heart-rate variability was partial, and the vagus nerve demonstrated an increase in regenerating myelinated and unmyelinated fibers. These data suggest that autonomic neuropathy involving the vagus nerve is metabolically induced by demonstrating that inhibition of the polyol pathway significantly delays the occurrence of functional and structural autonomic neuropathy despite the presence of hyperglycemia. *Diabetes* 39:613–18, 1990

Autonomic nerve dysfunction is a serious and common complication in diabetes mellitus and is responsible for an increased morbidity and mortality in diabetic subjects (1–3). It leads to vagal denervation and sensorimotor and reflex dysfunction of the cardiovascular, urogenital, and gastrointestinal systems (4–8).

We have previously reported on autonomic nerve dysfunction and neuroanatomic abnormalities in various autonomic nerves in spontaneously diabetic BB rats (9–13). From these studies, it appears that functional abnormalities such as decreased heart-rate variability and impaired micturition-reflex function precede ultrastructurally detectable neuroanatomic lesions in the respective autonomic nerves (11,12). This sequence of events suggests an early potentially reversible metabolic defect in autonomic nerves that may initiate and sustain the subsequent development of structural changes similar to the sequence of events occurring in diabetic somatic peripheral nerve (14,15). In the diabetic mixed sensory and motor sciatic nerve, increased ambient glucose concentrations reduce nerve *myo*-inositol by activation of the polyol pathway, which in turn impairs nerve Na^+-K^+-ATPase via decreased activity of protein kinase C (16,17). The Na^+-K^+-ATPase defect has been associated with the early slowing of nerve conduction velocity and the occurrence of structural lesions in somatic nerves (14,15,18,19).

To examine whether activated polyol-pathway and subsequent metabolic abnormalities may underlie autonomic nerve dysfunction and neuropathy, diabetic BB rats were treated with the aldose reductase inhibitor (ARI) ponalrestat from the onset of diabetes. They were examined with respect to heart-rate variability and structural lesions of the vagus nerve after 4 and 6 mo of treatment.

RESEARCH DESIGN AND METHODS

Twenty prediabetic male BB rats and 10 age- and sex-matched non–diabetes-prone BB rats were obtained from the Department of Pathology, University of Massachusetts (Worcester). All animals were maintained in individual air-filtered metabolic cages with ad libitum access to water and rat chow (Wayne Lab Blox F-6, Wayne, Chicago, IL). Body weight, urine volume, ketonuria, and glucosuria (Keto-Diastix, Miles Canada, Etobicoke) were monitored daily, and blood glucose was measured weekly from tail vein blood samples (Glucometer, Ames, Elkhart, IN). Glycosylated hemoglobin was measured from tail vein blood samples every 2nd mo and expressed as percentage of glycosylated hemoglobin (Glyco-Test, Pierce, Rockford, IL). At detection of glucosuria, diabetic rats were started on small daily doses (0.5–3.0 U/day) of protamine zinc insulin (Connaught-Novo, Toronto) designed to maintain an insulin-deficient state with blood glucose levels ranging between 15 and 20 mM. Three

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weeks after onset of diabetes, animals were divided into two groups: *1*) insulin-deficient untreated diabetic and *2*) insulin-deficient diabetic fed ponalrestat-supplemented (ICI, Wilmington, DE) rat chow (80 g ponalrestat/100 kg rat chow), equivalent to a daily dose of 25 mg ponalrestat/kg body wt (actual dose for diabetic rats 32.6 mg/kg body wt). Age- and sex-matched non–diabetes-prone BB rats served as controls. Half of the animals in each experimental group were killed 4 mo after onset of diabetes (5 animals/group) and half at 6 mo postdetection. End-point measurements were performed by investigators unaware of animal identity.

Heart-rate variability was assessed by computed statistic $\overline{R}$ as previously described in detail (11,20). Simultaneous analog movement, ECG, and respiration signals were obtained from a movement monitor and subcutaneous electrodes. The respiration data were amplified and fed to a Wheatstone's bridge apparatus (Barclay Whyte, Winnipeg, Canada), which discriminates differences in transthoracic impedance due to respiration. The ECG signals were fed to an ECG recorder (Cardiofax ECG-6201, Nikon Kohden, Tokyo) for amplification. Movement, ECG, and respiration signals output was converted by an analog-to-digital converter, and input was sent to a microprocessor for circular time wrapping of R-R intervals per respiration cycle. The $\overline{R}$ is expressed as the length of the mean vector of $\overline{R}$ points on a unit circle equal to the length of one respiration cycle arbitrarily set to 1 (11,20). $\overline{R}$ values were collected at 2, 4, and 6 mo of diabetes.

Animals were perfused with 2.5% glutaraldehyde fixative buffered with 0.1 M sodium cacodylate (pH 7.3) under pentobarbital sodium anesthesia (50 mg/kg body wt). The left unifascicular vagus nerve was dissected and immersed for 4 h at 4°C in the same fixative. The specimens were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.3) for 2 h at 4°C. After dehydration through ascending concentrations of ethanol, the tissue was embedded in Epon.

Morphometric abnormalities were assessed by fiber size frequency distributions, density, and occupancy and mean fiber size as previously described in detail (10). These data were obtained from semithin cross sections of the vagus nerve embedded in plastic. From each vagus nerve, all myelinated fibers were digitized with the aid of an HP 9872A digitizer interfaced to an HP 9825A desk computer and plotter (Hewlett-Packard, Fort Collins, CO). Myelinated-fiber occupancy was expressed as percentage of endoneurial area occupied by myelinated fibers.

To assess axonal atrophy, linear regression analyses of the natural logarithm of axonal area and the number of myelin lamellae were examined as previously described (18).

Size frequency distributions of unmyelinated fibers, fiber density, and fiber occupancy were measured from systematic randomly chosen areas of the vagus nerve. These measurements were obtained from electron micrographs with a total magnification of ×24,000, and the calculations were performed in the same way as for myelinated fibers (10).

Data are expressed as means ± SE. Significance of differences between groups was calculated by analysis of variance (ANOVA) and modified *t* test. Linear regression analysis was performed by the least-squares method.

RESULTS

Prediabetic rats developed diabetes at a mean age of 97 ± 5 days. Ponalrestat treatment had no preventive effect on the characteristic decrease in body weight gain seen in untreated diabetic BB rats or on blood glucose or glycosylated hemoglobin levels (Table 1).

After 2 mo of insulin-deficient diabetes, the $\overline{R}$ values were significantly ($P < 0.02$) decreased compared with nondiabetic control values (Table 2). This difference became even more pronounced ($P < 0.001$) after 4 and 6 mo of untreated diabetes. In insulin-deficient diabetic rats, ARI treatment achieved a complete prevention of the defect in heart-rate variability for 4 mo, whereas after 6 mo, there was only a partial preventive effect that amounted to 40% of the deficit (Table 2).

Myelinated fiber size distribution in untreated 4-mo diabetic rats showed a moderate shift toward smaller fibers compared with age-matched nondiabetic controls (Fig. 1*A*). This shift in the frequency distribution of myelinated fibers was prevented by 4 mo of ponalrestat treatment, so that no significant difference could be demonstrated compared with the fiber size distribution in nondiabetic controls (Fig. 1*B*). No differences were demonstrated in myelinated fiber density or occupancy between any of the groups at 4 mo.

Six months of ponalrestat treatment failed to prevent the characteristic myelinated fiber atrophy in diabetic rats. Instead, myelinated fiber size was significantly less ($P < 0.05$) in ponalrestat-treated diabetic than untreated diabetic rats

TABLE 1
Effect of ponalrestat treatment on body weight and hyperglycemia at 4 and 6 mo of diabetes

Group	Body weight (g)	Blood glucose (mM)	Glycosylated hemoglobin (%)
4 mo			
Control	460 ± 12	5.7 ± 0.5	3.9 ± 0.4
	$P < 0.001$	$P < 0.001$	$P < 0.001$
Diabetic	353 ± 10	17.8 ± 3.0	8.5 ± 0.4
	$P < 0.001$	$P < 0.002$	$P < 0.001$
Ponalrestat-treated diabetic	363 ± 17	17.3 ± 1.2	8.2 ± 0.6
6 mo			
Control	500 ± 21	3.4 ± 0.6	4.4 ± 0.5
	$P < 0.002$	$P < 0.001$	$P < 0.002$
Diabetic	383 ± 12	20.8 ± 2.1	8.4 ± 0.9
	$P < 0.001$	$P < 0.001$	$P < 0.001$
Ponalrestat-treated diabetic	363 ± 17	24.3 ± 0.7	7.7 ± 0.5

$n = 5$ for each group. $P < 0.005$ for body weight, blood glucose, and glycosylated hemoglobin by analysis of variance. All other comparisons not significant.

TABLE 2
$\bar{R}$ values ($\times 10^3$) in control, untreated diabetic, and ponalrestat-treated diabetic BB rats

Group	Diabetes duration		
	2 mo*	4 mo†	6 mo†
Control	27.6 ± 2.0	24.5 ± 2.1	21.1 ± 1.3
	$P < 0.02$	$P < 0.001$	$P < 0.001$
Diabetic	20.0 ± 0.9	11.8 ± 0.5	11.1 ± 0.4
	$P < 0.02$	$P < 0.001$	$P < 0.02$ $P < 0.002$
Ponalrestat-treated diabetic	27.1 ± 2.3	26.7 ± 1.5	15.1 ± 1.0

For definition of $\bar{R}$, see RESEARCH DESIGN AND METHODS and refs. 11 and 20 for review. $n = 5$ for each group.
*$P < 0.05$ by analysis of variance (ANOVA).
†$P < 0.005$ by ANOVA. All other comparisons not significant.

(Table 3; Fig. 2). In contrast, ponalrestat treatment appeared to have a small nonsignificant protective effect on fiber number as reflected by fiber density but did not prevent the diminished ($P < 0.02$) fiber occupancy demonstrated in untreated diabetic BB rats (Table 3). Microscopic examination of semithin cross sections of the vagus nerve embedded in plastic revealed no interstitial inflammation, endoneurial granulomata, or exacerbation of axonal degeneration in ponalrestat-treated rats.

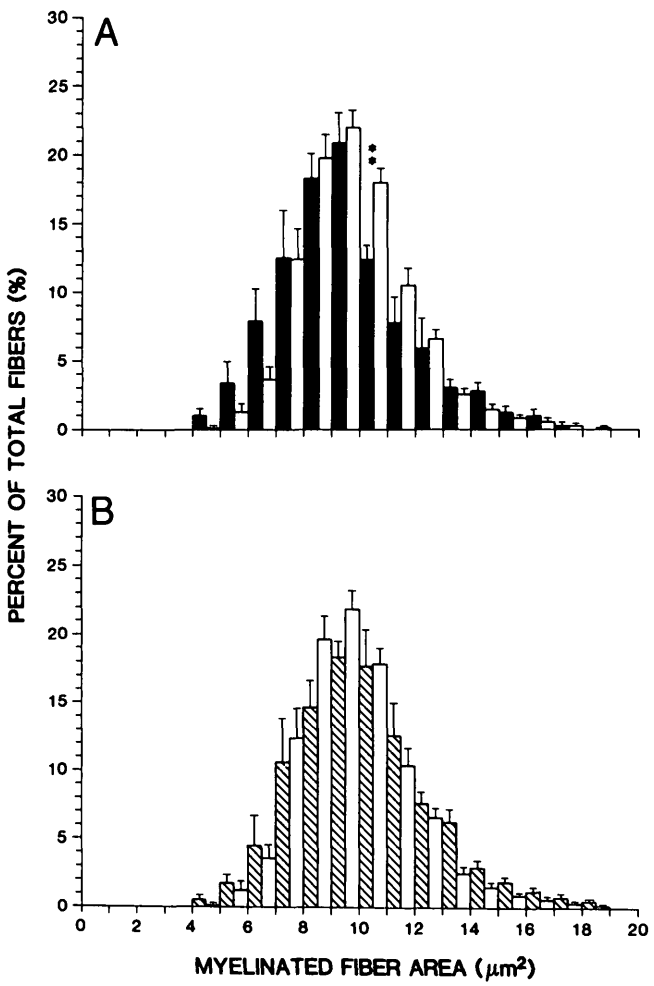

FIG. 1. *A*: myelinated fiber size frequency distributions in 4-mo untreated diabetic (*solid bars*; $n = 5$) and control (*open bars*; $n = 5$) rats. Diabetic rats show fiber atrophy with shift to smaller size frequencies. *B*: 4 mo of ponalrestat treatment (*hatched bars*; $n = 5$) prevented this shift of myelinated fiber size frequency. *Open bars*, control ($n = 5$). **$P < 0.01$.

Four months of ARI treatment completely prevented the characteristic axonal atrophy of myelinated fibers reflected by an unchanged axon-myelin ratio compared with nondiabetic controls (Fig. 3, *top*). However, this protective effect was not sustained at 6 mo, when the axon-myelin ratio in ponalrestat-treated diabetic rats did not differ significantly from that in untreated insulin-deficient rats (Fig. 3, *bottom*).

Diabetes untreated for 4 mo resulted in a marked shift in unmyelinated fiber size distribution toward smaller fiber sizes (Fig. 4*A*). This shift, reflecting axonal atrophy, was markedly but not completely prevented by ponalrestat treatment (Fig. 4*B*). Unmyelinated fiber density was not affected by 4 mo of diabetes.

After 6 mo of treatment, no effect could be demonstrated on mean unmyelinated fiber size, whereas fiber density was markedly increased compared with both untreated diabetic rats ($P < 0.002$) and nondiabetic controls ($P < 0.001$) (Table 3). Furthermore, ponalrestat treatment had a protective effect ($P < 0.02$) on unmyelinated fiber occupancy compared with untreated diabetic rats (Table 3).

DISCUSSION

Diabetic autonomic neuropathy affecting the vagus nerve in the spontaneously diabetic BB rat is characterized by progressive axonal atrophy of myelinated and unmyelinated fibers (10), which is preceded and accompanied by vagal dysfunction substantiated by impaired heart-rate variability (11). Our findings in untreated diabetic BB rats confirm the previous findings and suggest a metabolic pathogenesis for vagal dysfunction and neuropathy. Complete prevention by the ARI ponalrestat of decrease in heart-rate variability for 4 mo in hyperglycemic animals supports the notion that hyperglycemia-induced activation of the polyol pathway plays an important inciting role in the development of autonomic neuropathy. However, this beneficial effect was only partial at 6 mo of diabetes, suggesting that inhibition of the polyol pathway in the presence of severe continuous hyperglycemia cannot be sustained by ponalrestat. These findings appear to concur with a beneficial effect of ARI treatment on autonomic nerve dysfunction in diabetic patients (21). On the other hand, it is conceivable that nonenzymatic glycosylation of long-lived axonal proteins (22) unaffected by aldose reductase inhibition may account for incomplete prevention of axonal atrophy in the persistently hyperglycemic

TABLE 3
Effect of ponalrestat treatment on myelinated and unmyelinated fiber morphometry at 6 mo

Group	Myelinated fiber morphometry			Unmyelinated fiber morphometry		
	Mean fiber size (μm^2)*	Fiber density (n/mm^2)†	Fiber occupancy (%)‡	Mean fiber size (μm^2)*	Fiber density ($\times 1000/mm^2$)†	Fiber occupancy (%)‡
Control	7.03 ± 0.15	10,525 ± 979	7.40 ± 0.68	0.96 ± 0.05	339 ± 15	32.37 ± 0.91
	$P < 0.05$	$P < 0.02$	$P < 0.02$	$P < 0.05$	$P < 0.002$	$P < 0.02$
Diabetic	5.92 ± 0.48	7328 ± 759	4.82 ± 0.57	0.82 ± 0.04	358 ± 10	29.37 ± 0.79
	$P < 0.05$		$P < 0.001$	$P < 0.02$	$P < 0.001$	
Ponalrestat-treated diabetic	4.65 ± 0.26	9429 ± 523	4.67 ± 0.43	0.80 ± 0.03	445 ± 20	35.91 ± 2.46

*$P < 0.05$ by analysis of variance (ANOVA).
†$P < 0.01$ by ANOVA.
‡$P < 0.005$ by ANOVA. All other comparisons not significant.

ARI-treated rats at 6 mo. Alternatively, some investigators have suggested that axonal atrophy in murine diabetes may be consequent to maturational and/or nutritional factors in the generally leaner diabetic animals (23). However, this is an unlikely explanation for the residual axonal atrophy that occurred at 6 mo of ARI treatment, because at 4 mo, axonal atrophy was completely prevented in ARI-treated rats, being as stunted as in untreated diabetic rats in whom significant axonal atrophy was present.

In the vagus nerve, the myelinated fiber population represents visceral afferents, and most of the unmyelinated fibers are presynaptic parasympathetic fibers (24). The preventive effect of ponalrestat treatment on axonal atrophy of myelinated fibers, as demonstrated by the axon-myelin ratio after 4 mo of treatment, is analogous to the effect of ARI treatment on somatic afferents in diabetic BB rats (25). Because axonal atrophy in diabetic neuropathy is believed to be a consequence of slowed axonal transport secondary to the Na^+-K^+-ATPase defect (26,27), it is expected that correction of the metabolic defect responsible for the impaired Na^+-K^+-ATPase activity would ameliorate the axonal transport defect and axonal atrophy. This construct is consistent with the normalization of axonal transport after ARI treatment reported by Tomlinson et al. (28–30).

However, after 6 mo of ARI treatment of hyperglycemic rats, this beneficial effect on axonal atrophy was not sustained. Instead, mean myelinated fiber size was markedly reduced even compared with untreated diabetic rats, which is most likely accounted for by the addition of small regenerating myelinated fibers reflected in a nonsignificant 29% increase in myelinated fiber density.

A similar effect of ponalrestat treatment was demonstrated on the perhaps functionally more relevant unmyelinated fiber population of the vagus nerve. The significant shift of fibers toward smaller sizes in untreated diabetic rats was largely ameliorated after 4 mo of ponalrestat treatment, indicating a protective effect on unmyelinated fiber atrophy.

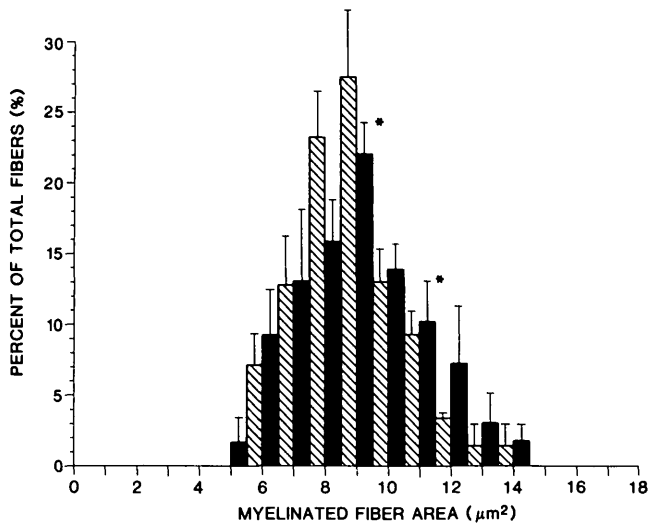

FIG. 2. Myelinated fiber size distribution in 6-mo ponalrestat-treated diabetic (*hatched bars*; $n = 5$) versus untreated diabetic (*solid bars*; $n = 5$) rats. Paradoxical shift to smaller myelinated fiber sizes in treated rats probably represents addition of small regenerated fibers. *$P < 0.05$.

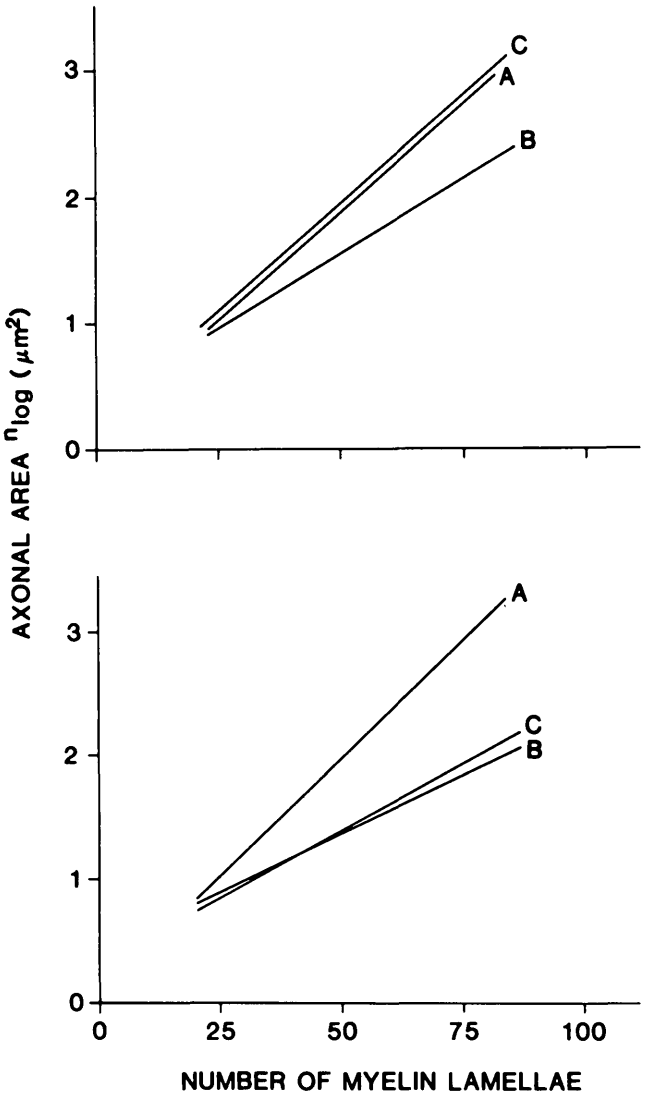

FIG. 3. ***Top*****: axon-myelin ratio at 4 mo shows complete prevention of characteristic axonal atrophy as signified by decreased slope of regression (*b*) in untreated diabetic rats. A, control (*n* = 5): *a* = 0.20 ± 0.07, *b* = 0.034 ± 0.004 (*P* < 0.05 by analysis of variance [ANOVA]); B, diabetic (*n* = 5): *a* = 0.36 ± 0.05, *b* = 0.024 ± 0.001; C, ponalrestat-treated diabetic (*n* = 5): *a* = 0.24 ± 0.09, *b* = 0.035 ± 0.004. For *b*, *P* < 0.05, A vs. B and B vs. C.** ***Bottom*****: preventive effect above was not sustained at 6 mo. A (*n* = 5): *a* = 0.07 ± 0.09, *b* = 0.038 ± 0.004 (*P* < 0.005 by ANOVA); B (*n* = 5): *a* = 0.42 ± 0.06, *b* = 0.019 ± 0.003; C (*n* = 5): *a* = 0.28 ± 0.10, *b* = 0.002 ± 0.003. For *b*, *P* < 0.002 A vs. B and *P* < 0.01 A vs. C.**

Similar to the effect on myelinated fibers, the effect on unmyelinated parasympathetic fibers was not sustained at 6 mo of treatment. Instead, mean unmyelinated fiber size was not significantly different from that of untreated rats, whereas both unmyelinated fiber density and occupancy were increased >20% compared with untreated diabetic BB rats, suggesting a significant addition of small regenerating fibers.

These findings are similar to those reported in the sural nerve of the BB rat after long-term prevention with an ARI (25) and after intervention therapy with the ARI sorbinil in human diabetic neuropathy (31), demonstrating a substantial regeneration of myelinated fibers. Unlike the data reported by Sharma et al. (32), we were not able to demonstrate exacerbation of axonal degeneration after combined ponalrestat-insulin treatment in the vagus or the surval nerve (25). These data suggest that the metabolic perturbations in diabetic nerve not only cause nerve degeneration but also impede the normal regenerative capacity of peripheral nerve. It is possible that the same abnormality of the polyol pathway and subsequent *myo*-inositol–related protein kinase C–signaling mechanism suggested for the Na^+-K^+-ATPase defect may have an inhibitory effect on the responsiveness of neurotrophic factors necessary for nerve fiber regeneration (31).

In summary, ponalrestat treatment of the hyperglycemic BB rat has a significant effect on autonomic neuropathy by delaying the characteristic decrease in heart-rate variability and the neuroanatomic abnormalities of the vagus nerve. In addition, the same treatment regimen appears to markedly promote nerve fiber regeneration in the vagus nerve via an unknown mechanism.

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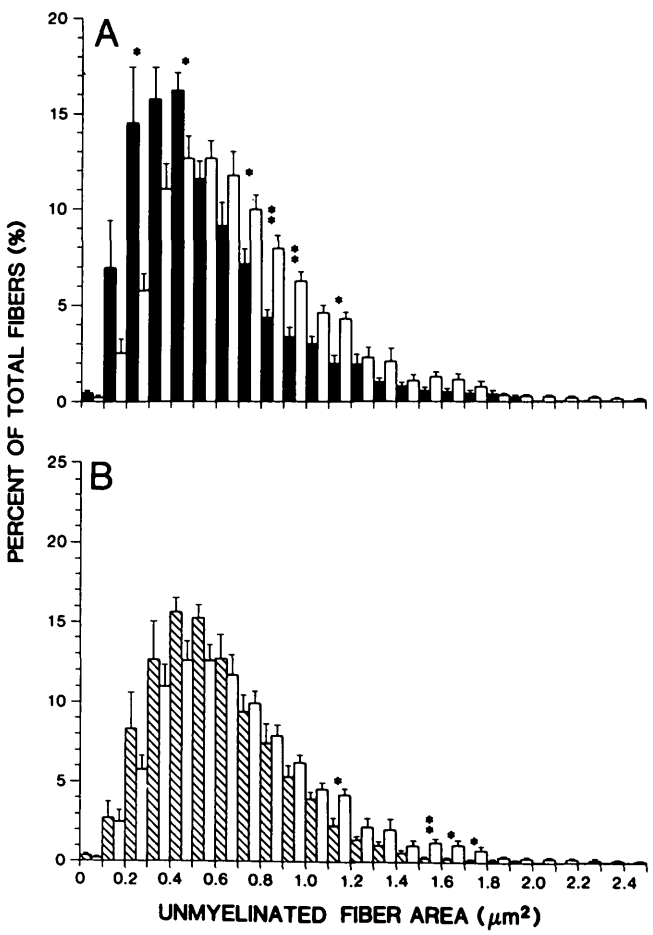

FIG. 4. ***A*****: unmyelinated fiber size frequency distribution in diabetic rats at 4 mo is significantly shifted to smaller fiber sizes, indicating unmyelinated fiber atrophy.** ***Open bars*****, control (*n* = 5);** ***solid bars*****, untreated diabetic (*n* = 5).** ***B*****: shift in fiber size is largely prevented by ponalrestat treatment.** ***Open bars*****, control (*n* = 5);** ***hatched bars*****, ponalrestat-treated diabetic (*n* = 5). **P* < 0.05; ***P* < 0.01.**

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