The Effect of Aldose Reductase Inhibition on Motor Nerve Conduction Velocity in Diabetic Rats

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SUMMARY

This study examined the effects of an aldose reductase inhibitor (CP 45634, Sorbinil, Pfizer, New York, New York) on the neuropathy of streptozotocin-induced diabetic rats. Sorbinil treatment for 4 wk reduced sciatic nerve sorbitol concentration and improved motor nerve conduction velocity in diabetes of 2–9 mo duration. It remains to be determined whether Sorbinil can prevent chronic diabetic neuropathy.

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Peripheral neuropathy remains a common and serious complication of diabetes. It is typically a distal sensorimotor neuropathy occurring in patients with long-standing diabetes, especially in those who are poorly controlled.[1–3] A reduction of motor nerve conduction velocity (MCV) can be demonstrated in patients with diabetes of short duration before the onset of clinical symptoms.[4,5] It is not known whether this early electrophysiologic abnormality is a forerunner of the later chronic neuropathy.

The etiology of diabetic neuropathy is not well understood. Although Schwann cell abnormalities with subsequent demyelination have been postulated as a possible cause, defective axonal function has also been suggested as the primary disturbance.[1,2,6,7] Biochemical changes demonstrable in diabetic nerves are numerous and include sorbitol accumulation,[1,2,6] *myo*-inositol deficiency,[1,2,8] increased nonenzymatic glycosylation of proteins, and abnormal composition of myelin.[9,10] A causal relationship between these parameters and the acute electrophysiologic changes or chronic diabetic neuropathy has not been definitely established. Insulin treatment improves nerve function in diabetes.[1,4,5] However, insulin reverses many of the metabolic abnormalities found in diabetic nerves, and it has not been possible to determine which is the primary biochemical abnormality.

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One abnormality that may be an important metabolic disturbance in diabetic neuropathy is the regulation of nerve sorbitol. The formation of sorbitol from glucose in the peripheral nerve is catalyzed by the enzyme aldose reductase.[1,2,6] If sorbitol accumulation is detrimental to nerve function in diabetes, the inhibition of aldose reductase should lead to a fall in sorbitol concentration and an improvement of neuropathy. The use of an aldose reductase inhibitor (Alrestatin) in the treatment of diabetic patients with neuropathy has been reported previously.[11,12] Little beneficial effect on either MCV or symptoms could be attributed directly to the pharmacologic action of this agent. However, the importance of sorbitol accumulation is not excluded by these findings. In such a study in man it may not be possible to administer aldose reductase inhibitor in sufficient dosage to reduce the nerve sorbitol level. Furthermore, in long-standing diabetes, the neuropathy may already be present in an irreversible form. In this investigation the effect of an aldose reductase inhibitor (CP 45634, Sorbinil, Pfizer) on nerve sorbitol concentration and MCV was assessed in rats with diabetes of 2–9 mo duration. In such a study the efficacy of aldose reductase inhibition in normalizing nerve sorbitol and conduction velocity could be verified in diabetes of well-defined duration.

MATERIALS AND METHODS

Animals. Inbred female Wistar rats were obtained from the Australian Atomic Energy Commission (Lucas Heights). Diabetes was induced by the intravenous injection of streptozotocin (70 mg/kg body wt) and confirmed by blood glucose levels (Glucometer, Ames Division, Miles Laboratories, Inc., Elkhart, Indiana) of greater than 20 mmol/L at 3 days after injection. Animals with blood glucose levels below 20 mmol/L were rejected (about 10% of animals). The mean weight of the rats was 193 ± 28 g at the time of induction of diabetes. Noninjected littermates were used as controls.

Sciatic nerve sorbitol concentration was measured in 12 rats with diabetes of 2 mo duration, 16 normal rats, and 11 diabetic rats treated with 3–6 U/day of subcutaneous insulin for 2 wk. Rats were killed by an overdose of halothane. Both

sciatic nerves were immediately removed and stored at −80°C for later determination of sorbitol concentration.

Three groups of animals with diabetes of 2, 5, and 9 mo duration (N = 7, 6, 8) and their respective littermate controls (N = 7, 12, 9) were used to study the effects of Sorbinil on sciatic nerve sorbitol concentration and MCV. Before the commencement of Sorbinil, animals were fed standard laboratory chow (Allied Feed, Concord, NSW, Australia; 21% protein; 3.3 cal/g). For the purpose of drug administration, the chow was powdered and Sorbinil was added to a concentration of 0.15 mg/g. A small amount of distilled water was added; the mixture was dried in an oven at 37°C. Both diabetic and control animals were fed Sorbinil-enriched chow ad libitum, and the average Sorbinil consumption was 5 mg/kg body wt/day. Sorbinil was given for 4 wk and MCV was measured before commencement and after completion of the treatment period. Immediately after the final measurements the rats were killed, sciatic nerves were removed, and blood was obtained by cardiac puncture for glucose and glycosylated hemoglobin determinations. The results of nerve sorbitol measurement were pooled since there were no age- or weight-related changes in nerve sorbitol concentration (normal: weight 160–301 g, age 8 wk–12 mo, N = 23, r = 0.1; diabetic: weight 150–248 g, age 8 wk–12 mo, N = 15, r = 0.1). The results of MCV of each group are presented separately since there was an age-related rise in nerve conduction.

MCV was measured in another 123 rats with body weight ranging from 167 to 307 g to determine the relationship between body weight and MCV. MCV was also measured in 20 littermate rats before and after half of the group was treated with Sorbinil for 1 mo.

Motor nerve conduction velocity. Rats were anesthetized with ketamine (Ketalar) intraperitoneally (140 mg/kg body wt). MCV was determined by stimulating supramaximally the sciatic-tibial nerve at the sciatic notch and medial malleolus with paired stimulating electrodes and recording the electromyogram of the interosseous muscles of the hindpaw. The stimulus was a square wave of 0.10 ms duration derived from a Grass SD9 stimulator. The proximal recording electrode was inserted subcutaneously at the ankle and the distal electrode at the web between the third and fourth toes. Body temperature was measured with a rectal thermistor and animal temperature was controlled by laying the animal on a copper heating coil under external radiant heat. The mean rectal temperature was 37.3 ± 0.8°C for normal rats and 37.0 ± 1.1°C for diabetic rats (range 35.5–39.0°C) during the MCV estimation. The recording electrodes were connected to a capacitor coupled differential input preamplifier, and the output was displayed on a Tetronix 5113 Dual Beam Storage Oscilloscope. Latencies were measured on a photographic record with a micrometer; each latency was estimated from the start of the stimulus artifact to the onset of the first negative deflection of the muscle action potential. The distance between the stimulating electrodes was measured with the leg straightened but not stretched.

MCV was measured twice in each leg with all electrodes being withdrawn and reinserted for each measurement. The mean of the four readings was taken as the MCV. Our control studies showed that multiple measurements performed in this manner gave the least intermeasurement variation (8.3%).

Measurement of nerve sorbitol concentration. The method of sciatic nerve sorbitol measurement was adapted from Sherman and Stewart.[13] Both sciatic nerves were weighed and then frozen in liquid nitrogen before being crushed finely with a mortar and a pestle. The nerve sorbitol was extracted with a solution containing EDTA (1 mmol/L) and perchloric acid (3 mol/L) in a ratio of 25:6 (vol/vol). This solution was added to the crushed nerves (0.075 ml/mg of nerve), and extraction allowed to proceed for 30 min at 4°C while being shaken. After centrifuging at 3000 *g* for 10 min, a 2-ml aliquot was brought to pH 7.5–8.0 by adding 0.8 ml of potassium carbonate (4 mol/L) and 50 μl of Tris base (2 mol/L). The complete mixture was centrifuged for 10 min at 3000 *g* at 4°C. The supernatant was made up to 4.4 ml with glycine buffer (GB: 40 mmol/L, pH 9.6) and used in the sorbitol assay.

Sorbitol was measured by an enzymatic method using sorbitol dehydrogenase purchased from Boehringer Mannheim (E.C.1.1.1. 14 Batch 1281123). Each nerve extract was measured in two dilutions in duplicate. The incubation mixture was made up of 1 ml of extract, 0.5 ml NAD^+ (1.6 mmol/L, Boehringer Mannheim), 0.1 ml sorbitol dehydrogenase (10 U/ml), and 0.4 ml GB. In the standard curve the extract was replaced by an equal volume of a solution of sorbitol (2.5–20 μmol/L). All solutions were made up in GB except sorbitol dehydrogenase, which was reconstituted with distilled water. Incubation was allowed to proceed for 30 min at room temperature and immediately read in a fluorometer at an excitation wavelength of 360 nm and recording wavelength of 460 nm. In each assay pools of nerve extract with high, medium, and low sorbitol content were included as controls. Control experiments showed that glucose, fructose, *myo*-inositol, and galactose (20 mmol/L) did not interfere with the sorbitol assay. Sorbinil at fivefold therapeutic level (100 μg/ml) also had no effect on the assay.

Assessment of severity of diabetes. Before each MCV measurement, tail vein blood glucose levels were measured with a glucometer (Ames). Samples obtained by cardiac puncture at death were used to measure blood glucose levels by an autoanalyzer (glucose-oxidase method). Using the same samples, the glycosylated hemoglobins were measured by an aminophenylboronic acid affinity chromatography method.[14] Each animal was weighed to the nearest gram before induction of diabetes, before commencement of Sorbinil therapy, and before each MCV measurement.

Statistical methods. Comparison of MCV of the normal and diabetic animals at each time interval was by unpaired Student's *t* test (two-sided). Comparison of MCV of normal or diabetic animals before and after Sorbinil treatment was by paired Student's *t* test (two-sided), data from the 2-, 5-, and 9-mo groups were pooled. MCV was shown to be normally distributed by the Kolmogorov-Smirnov test. Sorbitol results were not normally distributed and were tested by the Wilcoxon rank-sum test. Linear regression was calculated by the least-squares method. Results were expressed as mean ± SD.

RESULTS

Sciatic nerve sorbitol concentration. The standard curve of the sorbitol assay was linear with sorbitol concentrations up to 10 μmol/L; all estimations fell within the linear part of

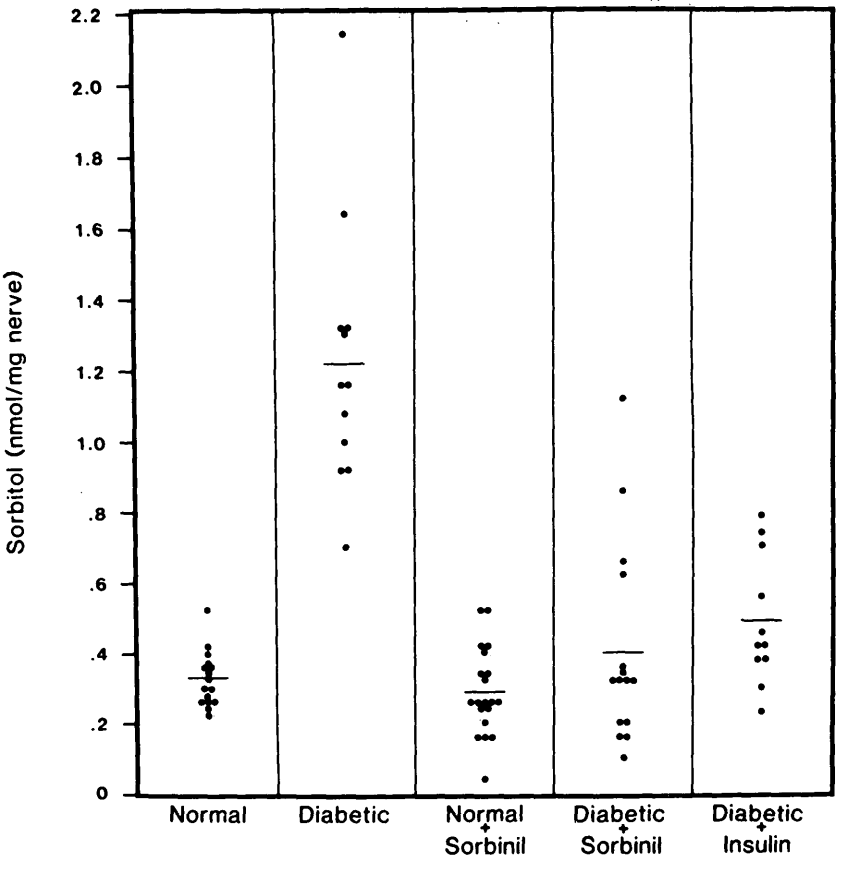

FIGURE 1. Sciatic nerve sorbitol concentration in the different groups of experimental animals. The diabetic group ($P < 0.001$) and insulin-treated diabetic group ($P < 0.01$) are significantly different from normal.

the curve. The recovery of sorbitol added to the extraction mixture was 100% (two experiments), and the mean interassay coefficient of variation was 17%, 23%, and 17% for the high, medium, and low controls, respectively (six experiments). The mean sciatic nerve sorbitol concentration in normal rats (0.34 ± 0.08 nmol/mg nerve) was not affected by Sorbinil treatment (0.28 ± 0.12 nmol/mg nerve). In rats with diabetes of 8 wk duration the mean sciatic nerve sorbitol concentration was increased to 1.22 ± 0.38 nmol/mg nerve (significantly different from normal control $P < 0.001$ and each of the 2-, 5-, and 9-mo Sorbinil-treated groups). Insulin treatment reduced sciatic nerve sorbitol to 0.48 ± 0.18 nmol/mg nerve; Sorbinil alone (no insulin) reduced nerve sorbitol to 0.40 ± 0.28 nmol/mg nerve. The mean sciatic nerve sorbitol of insulin-treated ($P < 0.01$) but not Sorbinil-treated diabetic rats remained significantly above nondiabetic littermates. Results are summarized in Figure 1.

Motor nerve conduction velocity. The MCVs in the three groups of rats treated with Sorbinil are shown in Table 1. Before commencement of Sorbinil there was a significantly lower MCV in all groups of diabetic animals in comparison with age-matched controls. After 4 wk of Sorbinil treatment the diabetic animals showed an increase in MCV ($P < 0.001$), whereas in the controls MCV remained unchanged. The differences in MCV between normal and diabetic animals became insignificant. The average increase in MCV during the month of Sorbinil treatment for the animals with diabetes of 2, 5, and 9 mo duration were 7.9, 6.4, and 3.6 m/s, respectively. Without Sorbinil treatment the average monthly increase in MCV of diabetic animals was only 1.6–2.1 m/s.

There were fewer animals studied at 4 wk since some animals had died during the intervening period. The mean initial MCV in the diabetic rats that died was not significantly

TABLE 1
Motor nerve conduction velocity before and after Sorbinil treatment

	Motor nerve conduction velocity (m/s)					
	2 mo		5 mo		9 mo	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Before Sorbinil treatment	50.1 ± 4.5 (N = 7)	39.8 ± 5.7* (N = 7)	52.2 ± 3.3 (N = 12)	46.1 ± 4.9* (N = 6)	60.9 ± 4.8 (N = 9)	52.8 ± 5.4* (N = 8)
After Sorbinil treatment	49.7 ± 1.2 (N = 5)	47.7 ± 6.7† (N = 4)	57.3 ± 4.9 (N = 10)	52.5 ± 3.8† (N = 5)	58.5 ± 4.3 (N = 5)	56.4 ± 2.3† (N = 6)

* Significantly different from normal before Sorbinil treatment ($0.001 < P < 0.01$).
† Significantly different from diabetic before Sorbinil treatment ($P < 0.001$).

TABLE 2
The severity of diabetes

	2 mo		5 mo		9 mo	
	Normal	Diabetic*	Normal	Diabetic*	Normal	Diabetic*
Blood glucose (mmol/L)†	5.7 ± 1.5	33.3 ± 4.2	6.5 ± 0.9	33.1 ± 0.7	4.8 ± 0.9	24.3 ± 11.8
Glycosylated hemoglobin (%)†	—	—	14.7 ± 3.3	23.8 ± 4.2	15.5 ± 1.7	24.1 ± 4.9
Weight before Sorbinil treatment (g)	244 ± 11	197 ± 21	259 ± 15	215 ± 24	279 ± 17	231 ± 16
Weight after Sorbinil treatment (g)	238 ± 7	192 ± 36	259 ± 14	218 ± 28	262 ± 21	231 ± 12

* Blood glucose, glycosylated hemoglobin, and weight of diabetic animals significantly different from normal ($P < 0.05$).
† Taken at time of death.

different from that of the animals that survived (43.3, 48.4, 53.4 m/s for the 2-, 5-, and 9-mo groups, respectively). If only animals that were studied on both occasions were considered, the differences of initial MCV between diabetic and normal animals remained.

The MCV of Sorbinil-treated normal rats did not differ from untreated littermates (53.0 ± 2.3 m/s and 53.9 ± 2.9 m/s, respectively).

The severity of diabetes in experimental animals. The blood glucose in all diabetic rats (glucometer, Ames) remained above 20 mmol/L before and after Sorbinil treatment. The blood glucose and glycosylated hemoglobin levels at death were significantly higher in the Sorbinil-treated diabetics than in the Sorbinil-treated controls. The mean weight of the diabetic rats did not change during the study period and was on average 20% below that of controls. These results are summarized in Table 2.

The effect of body weight on MCV. In the 123 rats studied, the relationship between MCV (Y) and body weight (X) was described by the equation: $Y = 0.068X + 36.3$ ($r = 0.44$, $P < 0.0001$, standard errors Y/X = 4.4). When adjusted for weight, the 2-mo diabetic group (but not the 5- and 9-mo groups) remained less than normal.

DISCUSSION

Diabetic neuropathy is a common and distressing complication of diabetes. The pathogenesis of this complication is not completely understood.[1–11,15] Biochemical alterations in diabetic nerves include excess sorbitol, deficiency of *myo*-inositol, increased glycosylation of protein, and changes in the composition of the myelin lipid.[1,2,6,8–10] Slowing of axoplasmic transport[16,17] and increased permeability of neuronal vessels have been reported.[18] Morphologic changes observed include segmental demyelination, endoneurial edema, and distal axonal degeneration.[19–21]

Sorbitol accumulation via the polyol pathway has been implicated as a mechanism for the neuronal dysfunction in diabetic neuropathy.[1,2,6] It has been postulated that the accumulation of sorbitol increases the osmotic pressure within Schwann cells and thereby affects the synthesis and function of myelin.[1,2,6] Alternatively, excess sorbitol has been suggested to cause fructose accumulation in the endoneurial space, accounting for the endoneurial edema shown to occur in diabetic nerves by Jakobsen.[20] The edema, in turn, leads to defective conduction of electrical impulses. Many investigations have confirmed the presence of excess sorbitol in diabetic peripheral nerves both in man and animals.[1,2,6] However, a direct causal role for excess sorbitol in the slowing of MCV observed in diabetes remains unproved.[1] Treatment by insulin is known to reduce nerve sorbitol and improve MCV.[1,4] However, insulin treatment also normalizes hyperglycemia and reverses many other metabolic derangements that occur in diabetes. Thus it cannot be certain that insulin exerts its beneficial effect by reducing sorbitol concentration. The use of an aldose reductase inhibitor (Alrestatin) in the treatment of diabetic neuropathy has been reported previously.[11] There was no change in MCV, and although some patients showed subjective improvements in symptoms, it was likely that placebo action played a major role. However, Alrestatin has a short circulating half-life (1 h in rats) and weak pharmacologic action, and does not normalize nerve sorbitol concentration.[22] Thus it is unknown whether the negative results obtained in the human study are due to a lack of pharmacologic efficacy of this agent or that sorbitol accumulation does not play a major role in the pathogenesis of diabetic neuropathy.

Sorbinil is an aldose reductase inhibitor that has a long circulating half-life[23,24] (4 h in rats); in the present study it has been shown to be effective in normalizing nerve sorbitol concentration in most diabetic animals. Its use has been reported in 31 human diabetic subjects treated for a 9-wk period when an improvement, but not complete normalization, in MCV was observed in one of the three nerves studied.[25] In the present study the reduction of sorbitol accumulation was associated with a rise in MCV in diabetic animals. Although MCV remained slightly less than control values, the difference was no longer statistically significant. It was not determined in the current investigation whether Sorbinil treatment instituted earlier and for longer periods might have completely prevented or abolished the abnormal nerve conduction of diabetic animals. However, since all groups of diabetic animals responded favorably to Sorbinil treatment, it appeared that aldose-reductase inhibition is effective in reversing motor nerve dysfunction in rats in which diabetes had been present for 2–9 mo.

Other possible explanations for the improvement in MCV may include an improvement in diabetes (Sorbinil-induced or spontaneous) and death of diabetic animals with more severe nerve dysfunction between the two MCV measurements. It appears unlikely that there is a Sorbinil-induced

improvement in glucose tolerance. When human diabetic patients were treated with Sorbinil there was no change in the glycosylated hemoglobin levels.[25] Although Alrestatin was reported to increase insulin secretion in rats,[26] similar action in humans could not be shown.[27] In this study all diabetic animals remained severely hyperglycemic throughout. The body weight of the diabetic animals did not increase during the treatment period and remained below that of the littermate nondiabetic controls. At the time of death, the blood glucose levels and glycosylated hemoglobin concentrations in the Sorbinil-treated diabetic rats were clearly greater than those of the Sorbinil-treated controls. Although some animals died shortly after the initial MCV measurement, their MCV was not different from the rest of the animals. Thus, their removal was not responsible for the rise in nerve conduction.

It would have been preferable to include untreated normal and diabetic rats as additional controls in the 2-, 5-, and 9-mo experimental groups. This would allow even more precise evaluation of the effect of the Sorbinil treatment. However, the limited numbers of littermates available and the high mortality rate of diabetic animals make this impractical in such long-term studies. The absence of these control groups does not invalidate our findings. The improvement in MCV in the diabetic animals could not be attributed to spontaneous improvement. In the absence of Sorbinil treatment, the difference in MCV in diabetic and age-matched controls remained significant even after 9 mo of established diabetes, and the increase in MCV during Sorbinil treatment was greater than that observed in untreated diabetic rats. The MCV of Sorbinil-treated normal rats was not different from that of untreated littermates. Thus it is unlikely that the absence of a difference in MCV in the normal and diabetic rats after Sorbinil treatment is due to a neurotoxic effect of the drug on normal rats. Since there was no demonstrable effect of body weight on nerve sorbitol concentration, it was justifiable to pool the nerve sorbitol results for statistical evaluation. The efficacy of Sorbinil in inhibiting aldose reductase was confirmed by the lower nerve sorbitol concentration when only the 2-mo diabetic group was compared with rats with diabetes of the same duration (nonlittermate).

The results in this study are consistent with the hypothesis that aldose-reductase inhibition reduces sorbitol accumulation in diabetic nerves and improves MCV. However, the possibility that Sorbinil may have additional pharmacologic action and exert its beneficial effect by mechanisms other than suppression of aldose reductase cannot be excluded.

The abnormality of MCV in streptozotocin-induced diabetes is similar to the slowing of MCV observed in human diabetes of recent onset.[4,5] Motor nerve abnormalities occur within a short time after the onset of diabetes before clinical manifestations of diabetic neuropathy. Both can be improved by insulin treatment and each can be partially corrected by inhibition of aldose reductase. This reduction in MCV is present in the 2-mo diabetic group (but not in the 5- and 9-mo groups) after adjustment for body weight. It is not clear whether this is due to spontaneous improvement of the neuropathy with time or selection of less severely affected animals in the 5- and 9-mo groups. The relationship of these early electrophysiologic abnormalities to established diabetic peripheral neuropathy is uncertain. A long-term clinical study in humans using a potent aldose reductase inhibitor with few toxic effects will be necessary to answer this question. The precise mechanism of the detrimental effect of sorbitol also remains unclear. The rise in osmotic pressure due to sorbitol accumulation is small and seems unlikely to be enough to explain the profound functional and structural changes of diabetic neuropathy unless the increase is strongly compartmentalized. Increased polyol pathway activity may also act by altering the NAD^+/NADH or $NADP^+$/NADPH ratio.[28] Even if sorbitol accumulation can be proven to be detrimental to nerve function, other factors need to be explored, since diabetic neuropathy may have a multifactorial etiology. Treatment regimens consisting of an aldose-reductase inhibitor in combination with insulin or *myo*-inositol supplementation may help to determine the relative importance of various metabolic factors in the pathogenesis of diabetic neuropathy and the optimal treatment for this condition.

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