Red Blood Cell Sorbitol as an Indicator of Polyol Pathway Activity

Inhibition by Sorbinil in Insulin-dependent Diabetic Subjects

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SUMMARY

In a double-blind crossover study of 15 diabetic patients, elevated red blood cell (RBC) sorbitol levels were reduced by oral doses of the potent aldose reductase inhibitor, sorbinil (250 mg o.d.), to near-normal ranges. In diabetic rats with severe hyperglycemia, oral sorbinil (5 mg/kg) dramatically reduced (80–90%) sorbitol levels in tissues without affecting blood glucose; the RBC dose-response curve was similar to that in lens and sciatic nerve. In streptozotocin-treated rats with varying degrees of diabetes sorbitol levels in the lens, sciatic nerve, and RBC were elevated in proportion to the degree of hyperglycemia. RBC sorbitol levels in these animals were positively correlated with the levels in lens and sciatic nerve. These results establish that orally administered sorbinil is effective in lowering elevated sorbitol levels, and strongly suggest that the reduction seen in RBC sorbitol levels in human diabetic subjects is likely to reflect comparable effects of the drug in less accessible tissues associated with the long-term complications of diabetes. DIABETES 33:45–49, January 1984.

Intracellular accumulation of sorbitol as a result of increased flux of glucose through the polyol pathway (Figure 1) has been implicated in the pathogenesis of some of the long-term complications of diabetes.[1,2] This pathway consists of two enzymes that convert glucose first to the sugar alcohol sorbitol and then to the keto sugar fructose (see Figure 1).

Aldose reductase, the first enzyme in the pathway, is characterized by a low affinity for glucose (K_m = 37–180 mM)[3,4] and a broad substrate specificity for many sugar aldehydes including galactose. The large intracellular pool of glucose and galactose characteristic of diabetes and galactosemia results in excess flux through the polyol pathway with the accumulation of poorly metabolized and slowly diffusible products inside cells, which leads to hypertonicity and swelling in the case of the lens fibrils. The polyol pathway is present in several tissues that accumulate sorbitol in proportion to the extracellular concentrations of glucose; these tissues, notably the lens, peripheral nerve, aorta, retina, and renal papilla,[1,2] are particularly susceptible to diabetic complications.

Evidence for a causal relationship between increased flux through the polyol pathway and diabetic complications has come from studies on cataractogenesis and impaired peripheral nerve function in diabetic and galactosemic animals.[1,2] The development of lenticular cataracts and defects in nerve conduction velocity are associated with increases in lens,[2,5–9] and peripheral nerve[1,10–14] sorbitol (or galactitol) and fructose levels. Cataractogenesis results from tissue hypertonicity resulting from accumulation of polyol pathway products. Macroaggregation of lenticular proteins due to the loss of osmotic integrity may cause the final lens opacity.[9] While the role of the polyol pathway in the cataractogenic process is fairly well characterized, the biochemical abnormalities leading to diabetic neuropathy are only beginning to be elucidated.[15] Impairment in nerve conduction velocity correlates well with increases in tissue sorbitol and fructose levels,[1,10–14] which have been implicated in the etiology of neuropathy.[15–17] Loss of peripheral nerve *myo*-inositol has also been implicated in the pathogenesis of diabetic neuropathy,[14,17] and a relationship has been established between elevated polyol pathway activity and loss of lens inositol in experimental diabetes.[19] Whether this phenomenon also applies to peripheral nerves remains to be determined.

The most compelling evidence linking flux through the polyol pathway with diabetic complications has come from studies with inhibitors of aldose reductase. A number of compounds, including the potent aldose reductase inhibitor sorbinil (d-6-fluoro-spiro-[chroman-4,4′-imidazolidine]-2′,5′-dione), reduce elevated lens and nerve polyol pathway products while delaying or preventing cataract development[5–7,20]

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$$\text{D-glucose (galactose)} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{aldose}} \text{Sorbitol (galactitol)} + \text{NADP}^+$$

$$\text{Sorbitol} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{sorbitol}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

FIGURE 1. The polyol pathway.

and reversing nerve-conduction-velocity defects.[21] In addition, sorbinil has been shown to improve nerve conduction velocities in diabetic patients.[22] These observations suggest that aldose reductase inhibitors could have potential in preventing or treating cataracts, neuropathy, and possibly other complications of clinical diabetes.

The ability to monitor tissue levels of polyol pathway products would clearly be advantageous during therapy directed at normalizing these levels in patients with diabetes. Sorbitol has been shown to accumulate in response to extracellular glucose concentrations in human red blood cells, both in vitro and in vivo.[23–25] The nature of the dependency of sorbitol accumulation upon media glucose concentrations and the in vitro inhibition of such accumulation by a specific aldose reductase inhibitor indicate that sorbitol production in the red cell may be mediated by the polyol pathway.[23] This report examines the relationship between sorbitol accumulation in red blood cells, lenses, and sciatic nerves of experimentally diabetic rats and evaluates the relative sensitivities of sorbitol accumulation in these tissues to inhibition by sorbinil. In addition, the effectiveness of sorbinil in reducing red cell sorbitol in patients with diabetes mellitus is examined.

METHODS

Animal studies. Male Sprague-Dawley rats (150–240 g) from Charles River Laboratories (Wilmington, Massachusetts) were fed ad libitum (Laboratory Rat Pellets, Ralston Purina Co., St. Louis, Missouri). Hyperglycemia was induced by intravenous injection of streptozotocin (Upjohn Chemical Company, Kalamazoo, Michigan) at two concentrations in a 0.01 M citrate buffer, pH 4.5. The low dose of streptozotocin (35 mg/kg) given to rats fasted for 18 h produced varying degrees of fed glycemia (110–400 mg glucose/dl) between rats. These levels were generally stable in individual rats during the period before they were killed. The high dose of streptozotocin (85 mg/kg) given to fed rats produced uniformly severe diabetes with blood glucose levels in the fed state ranging between 400 and 550 mg/dl. Eight days after injection with the low dose of streptozotocin, blood samples were obtained from fed rats for measurements of glucose; sciatic nerves and lenses were then removed (under pentobarbital anesthesia) for sorbitol analyses. A second blood sample for the determination of red cell sorbitol and hemoglobin was obtained at this time. The same procedure was followed at 2 wk and 22 days after induction of diabetes in rats receiving the high dose of streptozotocin. The animals not killed at 2 wk received sorbinil or distilled water daily by gastric lavage for the next 8 days until the tissue samples were collected. Sciatic nerves, lenses, and washed red blood cells were extracted and sorbitol measured by a modification[20] of a coupled fluorometric assay.[4] Hemoglobin was assayed with a Coulter Hemoglobinometer (Coulter Diagnostics) and whole blood glucose was measured by the ferricyanide method (Technicon Autoanalyzer, Tarrytown, New York).

Human studies. Insulin-dependent diabetic men ranging from 18 to 40 yr with clinical diabetes of more than 5 yr duration participated in the study. The potential risks and benefits of the study protocol were explained in detail to each patient. Informed consent was obtained under guidelines of the Committee of Associates on Human Experimentation at the University of South Florida. Two of eighteen individuals initially enrolled were dropped after the first week because they found the protocol too rigorous; therefore, 16 subjects participated in the 10-wk study. During the first and last 2 wk, subjects had clinical and laboratory evaluation of their degree of metabolic control and no medication was administered other than their usual insulin dose. Over the

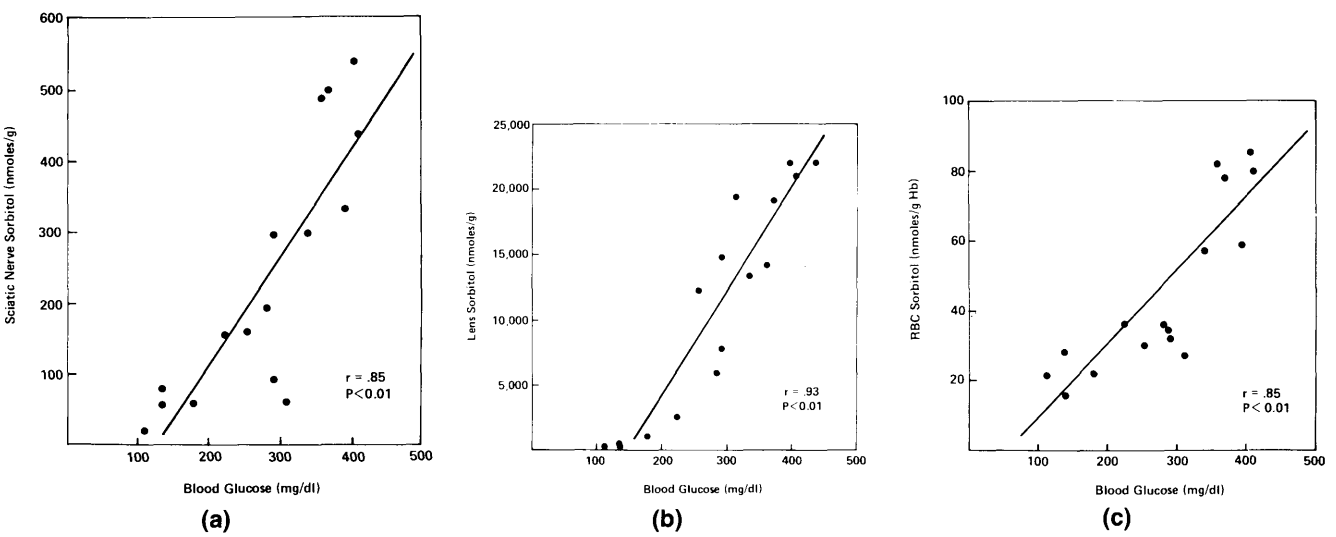

FIGURE 2. The relationships between blood glucose concentrations and nerve, lens, and red blood cell sorbitol. Rats starved 18 h were given the low dose (35 mg/kg) of streptozotocin (see METHODS), which produced rats with varying degrees of glycemia. Eight-day postinjection rats were killed and tissue sorbitol and plasma glucose were assayed as described in METHODS. Data were analyzed by least-squares regression. (a) Sciatic nerve sorbitol versus blood glucose; (b) lens sorbitol versus blood glucose; and (c) red blood cell sorbitol versus blood glucose.

six intervening weeks, each individual was instructed to take two unmarked capsules per day. During two consecutive weeks of the six, the capsules contained 250 mg of the aldose reductase inhibitor, sorbinil (CP-45, 634). The weeks of drug and placebo administration were not known by either the subjects or the investigators until the conclusion of the study. A blood sample was collected at the beginning of the study and weekly thereafter for the determination of sorbinil levels, clinical chemistry parameters, and red blood cell sorbitol. During the data analysis, one subject was excluded because no measurable levels of sorbinil were detected in any of the samples collected during the study period. Hemoglobin A_{1c} was measured by HPLC.[26] Red cell sorbitol was measured by a modification[23] of a coupled fluorometric assay previously described.[4] Selected high sorbitol values were confirmed by GLC of acetate derivatives.[23] Data are expressed as the mean ± SEM and are analyzed using Student's distribution of *t* unless otherwise noted.

RESULTS

Sorbitol levels and the degree of hyperglycemia. Treatment with the low dose of streptozotocin produced rats with varying degrees of glycemia, although the blood glucose level within each animal was relatively constant. Sorbitol levels in each tissue increased in proportion to the degree of hyperglycemia. Figure 2 illustrates the highly significant correlation between the level of blood glucose and sorbitol levels in sciatic nerves [Figure 2(a)], lenses [Figure 2(b)], and red cells [Figure 2(c)]. The relationship between red cell sorbitol and blood glucose levels [Figure 2(c)] supports the findings of Malone et al.,[23] who reported a similar correlation in diabetic children. In addition, Figures 3(a) and 3(b) show that there are also significant correlations between sorbitol levels in red cells and the coincident sorbitol levels present in both the sciatic nerve and the lens.

Effects of sorbinil on tissue sorbitol in severely streptozotocin-diabetic rats. Two weeks after induction of diabetes with the high dose of streptozotocin (85 mg/kg), rats had blood glucose concentrations of 400–550 mg/dl, which were not affected by subsequent therapy with sorbinil (Table 1). Sorbitol levels in red cells, sciatic nerves, and lenses of animals not treated with sorbinil were elevated approximately 6-, 10-, and 100-fold, respectively, above levels measured in tissues from nondiabetic animals. The greater concentration of sorbitol in lens compared with nerve has been observed previously and is thought to be due to the low ratio of hexokinase to aldose reductase found in the lens.[2] Diabetic rats treated for 8 days with a range (0–5 mg/kg/day) of sorbinil doses had marked reductions in sorbitol levels in all three tissues (Table 1). The correlation of red cell to nerve ($r = 0.81$, $P < 0.001$) and lens ($r = 0.64$, $P < 0.01$) sorbitol in these animals was highly significant. The daily oral doses required to reduce sorbitol levels by approximately 50% was 0.5–1.0 mg/kg for red cells and sciatic nerve and 1.0–2.5 mg/kg for lens (Table 1). A daily sorbinil dose of 5 mg/kg returned nerve and red cell sorbitol levels to normal while lens sorbitol, although not normalized, was decreased by 80% in these severely diabetic animals.

Effects of sorbinil therapy on red blood cell sorbitol, fasting plasma glucose, and hemoglobin A_{1c} in insulin-dependent diabetic patients. In 15 insulin-dependent diabetic patients, red blood cell sorbitol levels averaged 28.8 ± 2.7 nmol/g Hb (Figure 4) during 4 wk of placebo therapy, a value almost three times that reported for nondiabetic individuals (10.1 ± 1.3 nmol/g Hb).[23] During the two consecutive weeks of sorbinil therapy, red cell sorbitol was 13.8 ± 2.4 nmol/g Hb, a highly significant reduction from the placebo period ($P < 0.01$) (Figure 4). The effect of sorbinil was apparent within 1 wk of therapy. Sorbinil treatment had no effect on either fasting plasma glucose or hemoglobin A_{1c} levels (Figure 4). No adverse physical or biochemical reactions were noted in the patients during sorbinil treatment. The fever and rash previously reported in a small number of patients treated with sorbinil[22] was not observed in this group.

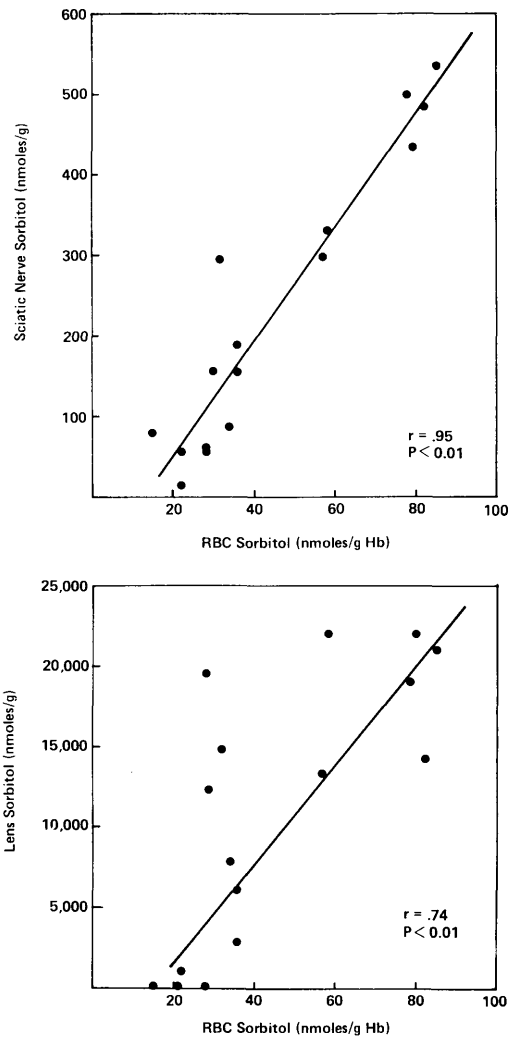

FIGURE 3. Correlations between red blood cell sorbitol and tissue sorbitol levels. Data from each individual animal in Figure 2 were replotted as (a) red cell sorbitol versus nerve and (b) lens sorbitol.

DISCUSSION

Red cell sorbitol levels in diabetic rats were found to increase in proportion to the prevailing glucose concentration [Figure 2(c)]. Furthermore, red cell sorbitol levels reflect the coincident sorbitol content of both nerve and lens tissue; this was the case both with varying degrees of hyperglycemia [Figure 3(a) and (b)] and after treatment with sorbinil (Ta-

TABLE 1
Reduction of elevated sorbitol levels in tissues of chronically diabetic rats by sorbinil

	Experimental group	Days of treatment 1	Days of treatment 8	Percent reversal
Blood glucose (mg/dl)	Nondiabetic	105 ± 1.2 (15)		
	Untreated diabetic I	447 ± 18	—	—
	Untreated diabetic II	449 ± 22	456 ± 14	—
	Sorbinil 0.5 mg/kg/day	453 ± 9	481 ± 12	0
	1.0	479 ± 16	483 ± 22	0
	2.5	509 ± 34	470 ± 16 (4)	0
	5.0	468 ± 27	447 ± 14	2
RBC sorbitol (nmol/g Hb)	Nondiabetic	16.1 ± 4.2	—	
	Untreated diabetic I	105.99 ± 8.7	—	—
	Untreated diabetic II		92.6 ± 8.5	—
	Sorbinil 0.5 mg/kg/day		48.2 ± 6.2*	48
	1.0		37.4 ± 5.7†	60
	2.5		19.9 ± 4.6 (4)†	79
	5.0		17.7 ± 4.5†	81
Sciatic nerve sorbitol (nmol/g)	Nondiabetic	73 ± 17 (9)	—	—
	Untreated diabetic I	868 ± 170	—	—
	Untreated diabetic II		1,076 ± 105	—
	Sorbinil 0.5 mg/kg/day		685 ± 48 (4)†	36
	1.0		434 ± 78†	60
	2.5		172 ± 22†	84
	5.0		96 ± 20†	91
Lens sorbitol (μmol/g)	Nondiabetic	0.15 ± 0.03 (10)		
	Untreated diabetic I	18.8 ± 1.3	—	—
	Untreated diabetic II		22.5 ± 2.3	—
	Sorbinil 0.5 mg/kg/day		23.3 ± 2.8	0
	1.0		13.6 ± 2.2*	40
	2.5		8.2 ± 2.2†	64
	5.0		4.4 ± 0.8†	80

Streptozotocin (85 mg/kg) was administered as in METHODS. Rats remained untreated for 2 wk and the untreated diabetic group I was killed. At this time sorbinil was administered orally to diabetics at the doses shown except for an untreated diabetic group II, which received only water during the dosing period. Treatment was continued for 8 days. On the eighth day, rats were killed 3 h postdose. N = 5 except where noted in parentheses. Values for nondiabetic rats in the fed state are historical and were not obtained during experiments with diabetic rats.
*$P < 0.05$ compared with untreated diabetic group II.
†$P < 0.01$ compared with untreated diabetic group II.

ble 1). The oral dose of sorbinil required to reduce red cell sorbitol by 50% in diabetic rats (0.5 mg/kg/day) was similar to that required for a 50% reduction in lens (1.0–2.5 mg/kg/day) and nerve (0.5–1.0 mg/kg/day). The dose required to restore both nerve and red cell sorbitol to nondiabetic levels was 5.0 mg/kg/day; this dose also produced an 80% reduction in the pretreatment levels of sorbitol in the lens.

Several observations suggest that the red blood cell may be a useful tissue to monitor polyol pathway activity in man. Morrison et al.[24] demonstrated the presence of sorbitol in red blood cells from normal subjects and diabetic patients and found it to be linearly related to plasma glucose levels. Human red cells incubated in vitro show an increase in both intracellular sorbitol and fructose as a function of the media glucose concentration.[24] Malone et al.[23] observed a positive correlation between plasma glucose levels and red cell sorbitol in a large series of insulin-dependent diabetic children. In that study, it was also shown that sorbitol accumulation in human red cells was completely inhibited by the prototype aldose reductase inhibitor tetramethylene glutaric acid (TMG); in a similar fashion sorbinil was also effective in inhibiting sorbitol accumulation in the red cell (unpublished observation). Studies aimed at characterizing aldose reducing activities in red cells have been few and thus far have not identified an enzyme with kinetic properties identical to aldose reductase isolated from other tissues. One enzyme isolated from human erythrocytes has been identified as L-hexonate dehydrogenase.[27] While L-hexonate dehydrogenase has kinetic properties similar to aldose reductase, its low affinity for glucose (0.5–2.0 M) and insensitivity to TMG suggest that this enzyme is not responsible for the sorbitol accumulation noted in the red cell. A second enzyme isolated from human red cells is also reported to have kinetic properties similar to aldose reductase;[28] this enzyme, however, does not reduce glucose and, therefore, cannot account for red cell sorbitol accumulation noted in response to extracellular glucose concentrations. While further work will be necessary to define the nature of the red cell enzyme responsible for sorbitol production, the present study in diabetic rats demonstrates that red cell sorbitol is a useful marker for polyol pathway activity in lens and nerve and suggests the presence in red cells of aldose reductase, or a kinetically similar enzyme.

The finding that oral sorbinil therapy reduces the elevated red cell sorbitol levels of diabetic patients (Figure 4) is consistent with the presence of aldose reductase or a similar enzyme in human red cells, and demonstrates for the first time the ability of an aldose reductase inhibitor to alter sor-

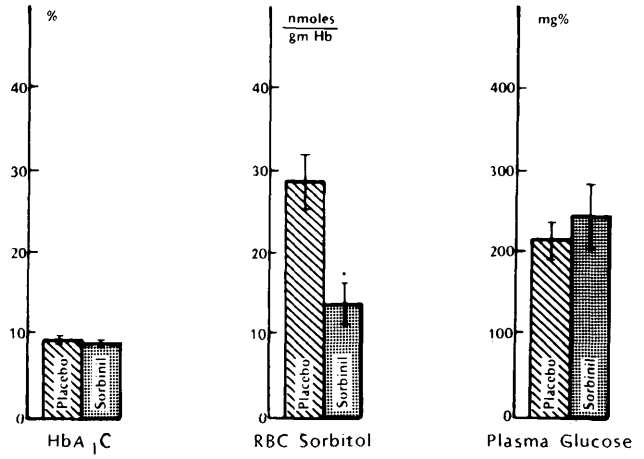

FIGURE 4. Changes in blood chemistry parameters associated with placebo (4 wk) and sorbinil (2 wk) treatment in insulin-dependent diabetic patients. Data are from 15 insulin-dependent diabetic men who participated in the 6-wk double-blind placebo trial as described in METHODS. The asterisk (*) indicates a significant difference ($P < 0.01$) for placebo versus sorbinil (250 mg) as determined by paired *t* testing where the average of four placebo assessments were compared with the average of two measurements during sorbinil treatment.

bitol levels in man. Sorbinil (250 mg/day) normalized red blood cell sorbitol in the majority of patients. Compared with normal human values for red cell sorbitol (e.g., 10.1 ± 1.3 nmol/g Hb),[23] 80% (12/15) of the patients had elevated sorbitol levels during the double-blind placebo period, which contrasted with 27% (4/15) during sorbinil therapy. The mean red cell sorbitol level for all patients during sorbinil therapy (13.8 ± 2.4 nmol/mg Hb) was not appreciably different from normal values. That this effect of sorbinil was not a consequence of altered blood glucose control was indicated by the lack of drug effect on fasting blood glucose and hemoglobin A_{1c} levels (Figure 4). Thus, if the red blood cell response to sorbinil in man is predictive of the sorbitol response in lens and peripheral nerves, as is the case in the rat, sorbinil at doses similar to those employed in the present study may prove beneficial in the treatment of cataracts and peripheral neuropathy in diabetes. The finding that sorbinil (250 mg/day) improved nerve conduction velocity in diabetic patients[22] underscores this possibility.

In summary, this study has demonstrated that the aldose reductase inhibitor sorbinil is effective in reducing red cell sorbitol in diabetic patients and that the red cell may be a useful index of sorbitol levels in other less accessible tissues in which diabetic complications occur. Measurements of red cell sorbitol in clinical studies may therefore prove valuable in elucidating the role of the polyol pathway in the evolution of diabetic complications as well as in monitoring the success of therapy aimed at blocking the activity of this pathway.

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