Ascorbic Acid Metabolism and Polyol Pathway in Diabetes

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It has been reported previously that the plasma concentration of ascorbic acid (AA) is reduced in streptozocin-induced diabetic rats and can be normalized by treatment with the aldose reductase inhibitor tolrestat. This study was designed to investigate further the relationship between the polyol pathway and AA metabolism in diabetic rats. Disturbance of AA metabolism was demonstrable after 1 wk of diabetes. Dietary *myo*-inositol supplementation was effective in normalizing plasma AA levels, as was treatment with tolrestat. In untreated diabetes, despite low plasma AA concentration, there was increased urinary excretion of AA that was reversed by treatment with either tolrestat or *myo*-inositol. In contrast, AA supplementation normalized plasma AA concentrations while further increasing urinary AA excretion. The abnormality of AA metabolism was less severe in galactose-fed rats, which had normal plasma AA levels and only minor increases in urinary AA excretion. These studies demonstrated a disturbance in the regulation of plasma and urinary AA concentration in experimental diabetes and confirmed the relationship of AA with the polyol pathway. Because AA has many important biological functions, abnormalities of AA metabolism could be important in the pathogenesis of some diabetic complications. The interaction of the polyol and AA pathways suggests that this could be another site of action for aldose reductase inhibitors.
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Plasma and tissue concentrations of ascorbic acid (AA) are decreased in both diabetic animals and humans (1–4). AA plays an important role in the synthesis and posttranslational modifications of collagen. It is also necessary for the regulation of many cellular biochemical processes, including the scavenging of free radicals (5–8). Moreover, AA and glucose share structural similarities and may compete for membrane transport (3). The disturbance of AA metabolism in diabetes is therefore of great interest and may be important in the pathogenesis of some diabetic complications.

The mechanism for the disturbance of AA metabolism in diabetes is not well understood. In a previous study (4), we demonstrated an interaction of AA and polyol pathways by showing that the decreased plasma AA concentration in diabetes can be normalized by treatment with the aldose reductase inhibitor tolrestat (Ay-27,773, Ayerst, Princeton, NJ). In this study, we further investigated this phenomenon by examining the plasma and urinary AA concentration during treatment of diabetic animals with tolrestat, *myo*-inositol, and AA. Galactose-fed rats were also studied as another model of disturbance in the polyol pathway.

MATERIALS AND METHODS

Animals. Female Wistar rats (180–200 g body wt) were used for this study. A separate group of animals (~30) was used for each experiment; each group was divided into treatment subgroups (i.e., normal, diabetic, and treated diabetic) of approximately equal number. Diabetes was induced by an intravenous injection of streptozocin (65 mg/kg; Calbiochem, San Diego, CA), and only animals with tail blood glucose levels >20 mM were used. All experiments were approved by the Animal Experiment Ethics Review Committee of the University of Sydney.

Treatment of animals. Tolrestat was prepared fresh each day and given by gavage at a dosage of 5 mg/kg body wt. *myo*-Inositol (Calbiochem) was given in the chow at a concentration of 3% (wt/wt), and galactose (Ajax, Sydney, Australia) was given similarly at a concentration of 50% (wt/wt). AA (Ajax) was given in drinking water (1 g/L) prepared fresh each day, representing a consumption of 40–60 mg/day. All animals were fed ad libitum with chow that contained no AA

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detectable by high-performance liquid chromatography (HPLC).

All treatments began with the induction of diabetes and continued for 4 wk, when the animals were killed. Untreated normal and diabetic rats were used as controls.

AA measurement. AA was measured by HPLC with a uBondapak C_{18} column (Waters, Milford, MA). For the measurement of urinary AA, the animals were individually housed in metabolic cages during the 4th wk of treatment, and urine was collected for 24 h in a container spiked with 20% trichloroacetic acid (TCA; 1.2 ml for normal rats and 9.4 ml for diabetic rats). Blood was obtained from rats by cardiac puncture after death by an overdose of ketamine (200 mg/kg; Parke-Davis, Sydney, Australia). The blood sample was immediately centrifuged, and plasma was deproteinized by the addition of 40% TCA to a final concentration of 5% TCA. After another centrifugation, the supernatant was stored at −80°C and used for AA assay within 2 wk. Control experiments showed that there was <2% degradation of AA collected under these conditions. AA was monitored by absorbance at 254 nm, its concentration determined by the area under the peak, and read against a standard of AA

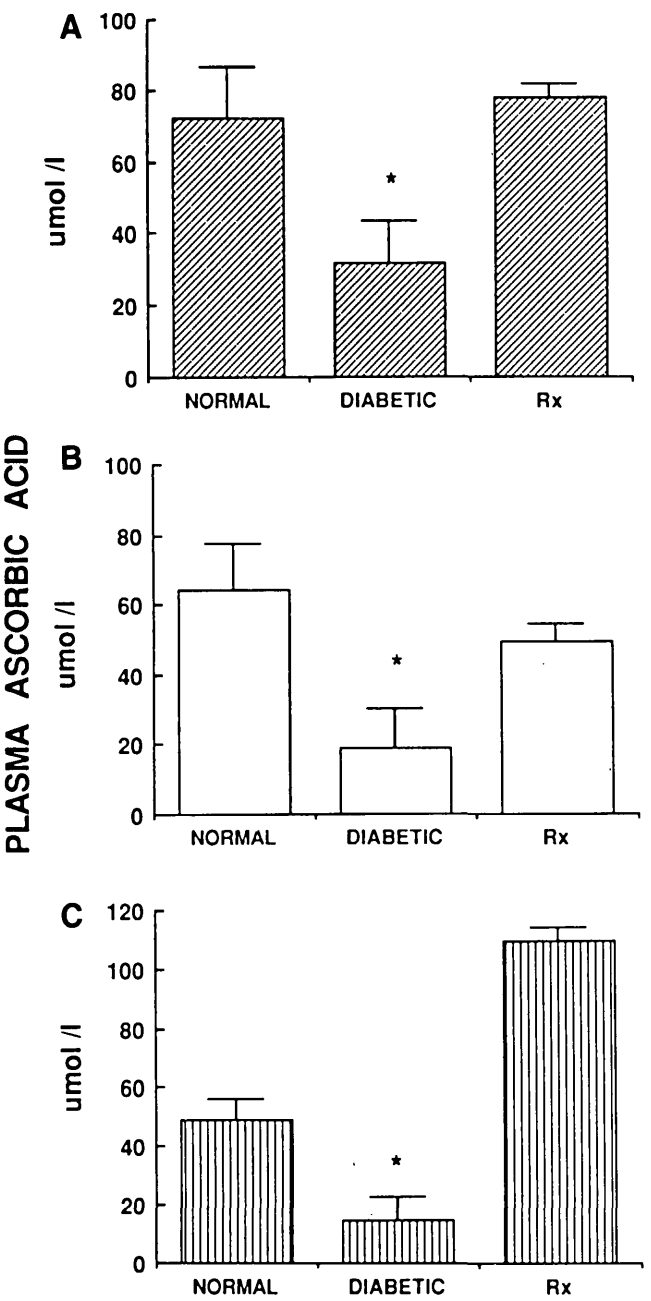

FIG. 1. Plasma ascorbic acid in normal and diabetic rats and diabetic rats treated with tolrestat (*A*), *myo*-inositol (*B*), or ascorbic acid (*C*). *Significantly different from normal and treated diabetic rats at $P < .05$.

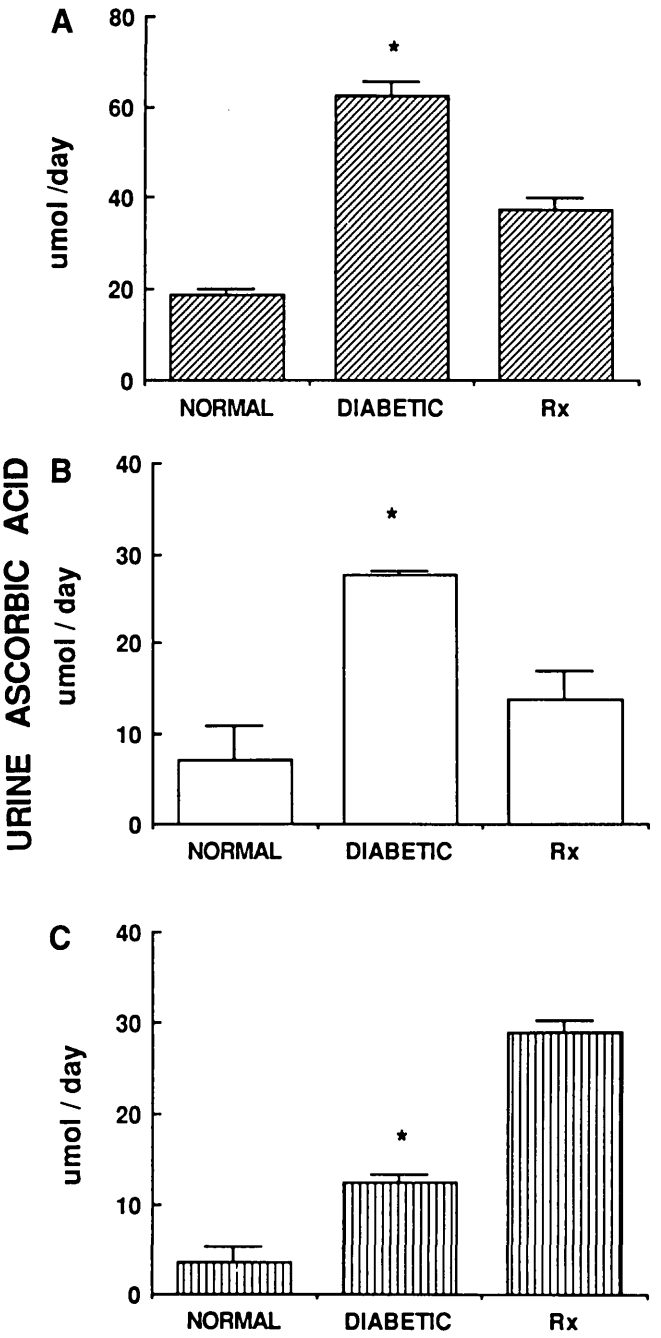

FIG. 2. Urinary excretion of ascorbic acid over 24 h in normal and diabetic rats and diabetic rats treated with tolrestat (*A*), *myo*-inositol (*B*), or ascorbic acid (*C*). *Significantly different from normal and treated diabetic rats at $P < .05$.

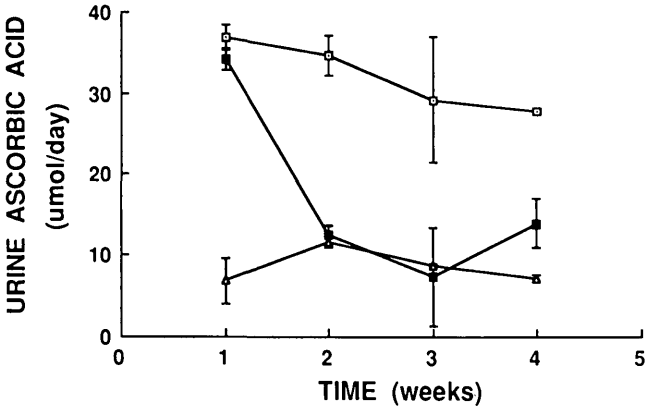

FIG. 3. Urinary excretion of ascorbic acid over 24 h after induction of diabetes in normal (■) and diabetic (⊡) rats and diabetic rats treated with *myo*-inositol (△).

prepared fresh each day from a stock solution. Urinary AA was expressed as micromoles excreted per day and plasma AA as micromoles per liter.

Sorbitol and *myo*-inositol measurement. The concentration of sorbitol and *myo*-inositol in the cortex and medulla of normal and diabetic kidney was also measured. Sorbitol was measured by sorbitol dehydrogenase and monitored by absorbance at 340 nm (9). *myo*-Inositol was measured fluorometrically with *myo*-inositol dehydrogenase by excitation at 345 nm and emission at 460 nm (10).

Statistical methods. Results were examined by analysis of variance with post hoc comparison by Duncan's multiple-range test. Results are expressed as means ± SD.

RESULTS

Plasma AA concentration. Plasma AA concentrations of diabetic animals during treatment with tolrestat, *myo*-inositol, and AA supplementation are shown in Fig. 1. Plasma AA was reduced in each group of diabetic animals and increased by all three treatment modalities, reaching supraphysiologic levels in the AA-treated diabetic animals. Tolrestat and *myo*-inositol treatment had no effect on plasma AA concentration in normal rats.

Urinary AA excretion. The results of urinary AA excretion are shown in Fig. 2. In all three groups of untreated diabetic animals the daily excretion of AA was higher than in the normal controls. Tolrestat and *myo*-inositol treatment of diabetic animals caused a significant reduction in AA excretion, although in each case it remained slightly elevated in comparison with the controls. In contrast, urinary AA was further increased in diabetic animals treated with AA.

The time course for the development of increased AA excretion in diabetes and its normalization by *myo*-inositol treatment is shown in Fig. 3. After 1 wk of diabetes, urinary AA excretion was already increased fivefold. This was not normalized until the 2nd wk of *myo*-inositol treatment.

Diabetes control. Tolrestat, *myo*-inositol, or AA treatment did not affect the metabolic control of diabetes. There was no difference in the plasma glucose level and body weight between the treated and untreated diabetic animals.

Sorbitol and *myo*-inositol levels in kidney. The level of sorbitol increased in both the cortex and medulla of diabetic kidney and normalized with tolrestat treatment (Table 1). The level of *myo*-inositol decreased in the cortex but not medulla of diabetic kidney but was not affected by *myo*-inositol supplementation.

Galactose-fed rats. Plasma AA concentration of the galactose-fed rats (64.9 ± 19.8 μM) was not significantly different from that of the normal rats (62.7 ± 17.6 μM). Urinary excretion of AA was significantly higher ($P < .001$) in galactose-fed rats (17.8 ± 1.7 μmol/day) than in normal rats (7.1 ± 3.8 μmol/day), but the magnitude of the increase was less than that observed in diabetes. This increase was also normalized by tolrestat treatment (7.6 ± 3.9 μmol/day).

DISCUSSION

Metabolism of AA is abnormal in diabetes, and reduced plasma and tissue concentrations of this vitamin have been reported (1–4). AA has many important functions and is essential for the maintenance of health. It is a cofactor regulating the activity of proline hydroxylase (EC 1.14.11.2), which catalyzes the formation of hydroxyproline, an amino acid specific for collagen and required for its structural stability (6). AA deficiency may therefore be responsible for some of the collagen abnormalities in diabetes, e.g., impaired wound healing (11), decreased production of granulation tissue (12), and reduced proline hydroxylase activity (13). We reported previously that decreased proline hydroxylase activity in the granulation tissue of diabetic animals can be normalized by dietary supplementation of AA (4). AA is also important in regulating the intracellular redox state and scavenging free radicals (5). Taking these factors into consideration, the deficiency of AA in diabetes is potentially of great relevance in the pathogenesis of some diabetic complications.

The mechanism of AA deficiency in diabetes is not com-

TABLE 1
Sorbitol and *myo*-inositol concentrations in normal and diabetic kidney

Kidney	Sorbitol (nmol/g)		*myo*-Inositol (μmol/g)	
	Medulla	Cortex	Medulla	Cortex
Normal	87.8 ± 16.0	65.6 ± 12.2	0.47 ± 0.08	0.37 ± 0.04
Diabetic	103.9 ± 16.9*	101.5 ± 14.9*	0.44 ± 0.10	0.17 ± 0.04*
Diabetic with tolrestat	68.4 ± 8.0	83.2 ± 10.9	0.42 ± 0.04	0.15 ± 0.03*
Diabetic with *myo*-inositol	97.9 ± 9.3	86.0 ± 10.8	0.45 ± 0.11	0.16 ± 0.08*

*Significantly different from normal at $P < .05$.

pletely understood. This study confirmed the ability of the aldose reductase inhibitor tolrestat to normalize plasma AA level. The observation that treatment with *myo*-inositol is also effective in this regard is further evidence that the polyol pathway is involved in the abnormal metabolism of AA in diabetes (14). *myo*-Inositol supplementation can reverse some of the polyol-related functional derangements in diabetes, apparently by preventing the depletion of a small intracellular pool of *myo*-inositol (14). This has the secondary effect of preventing decreased activity of Na^+-K^+-ATPase, an enzyme required for many cellular functions, e.g., generation of transmembrane electrical potential. One site where aldose reductase inhibitor and *myo*-inositol may act to affect AA metabolism is the renal tubule, which reabsorbs AA by a Na^+-gradient–dependent mechanism (15). Supporting this hypothesis is the observation that in diabetes urinary excretion of AA greatly increases but is substantially reduced by treatment with tolrestat or *myo*-inositol, with simultaneous restoration of plasma AA concentration. This pattern is distinct from that seen in animals treated with AA, in which the normalization of plasma AA is achieved with a further increase in urinary AA excretion. The ability of tolrestat to normalize sorbitol level in the renal medulla of diabetic animals is consistent with the possibility that its action on AA metabolism is at least partly mediated at the renal tubular level. Although our data do not demonstrate decreased *myo*-inositol concentration in the medulla of diabetic kidney, this does not exclude *myo*-inositol deficiency in the renal tubular cells. This may occur because a subpool of rapidly turned-over intracellular *myo*-inositol is depleted in diabetes (14). The contrasting response of *myo*-inositol concentration in the renal cortex and medulla to the development of diabetes is not surprising. More and more, tissues are reported to have different patterns of change in *myo*-inositol metabolism in diabetes, a reflection of the unique biochemical features of each tissue (16).

This interpretation of the action of aldose reductase inhibitor and *myo*-inositol in raising plasma and decreasing urinary AA is not necessarily the only possibility. Because there was no AA in the rat chow used in this study, the increased urinary AA in untreated diabetic animals clearly reflects increased synthesis, perhaps a compensatory attempt to normalize plasma AA concentration. The primary action of aldose reductase inhibitor and *myo*-inositol may therefore be to normalize plasma AA and thereby reduce the stimulus for increased AA production, which in turn leads to a reduction in urinary output of AA. The amelioration of glomerular hyperfiltration in experimental diabetes by dietary *myo*-inositol and aldose reductase inhibitor may also have contributed to the reduced excretion of AA (17).

These studies also confirm the close relationship between the polyol pathway and AA metabolism in diabetes. The interaction is not a simple one: the disturbance of AA metabolism in galactose-fed rats was much less severe than in diabetes, and we found normal plasma AA concentration and only modestly increased urinary AA, which again was normalized by aldose reductase inhibition. Although galactose feeding has been used extensively as a model to simulate the polyol disturbance in diabetes, increasing differences of this model from diabetes have been reported (18). The aldose reductase inhibitors are being investigated extensively for their possible role in the treatment and prevention of diabetic complications. Initially, interest was focused on their action in preventing sorbitol accumulation. Subsequently, attention was turned to their ability to normalize tissue *myo*-inositol level. Their action on AA metabolism can now be added to this list and may turn out to be important as a result of the ubiquitous nature and the great biological significance of AA.

The relevance of these findings to the situation in human diabetes remains to be explored. Because rats synthesize AA and do not rely on dietary AA intake, their metabolism of this vitamin may be quite different from humans. However, diabetic patients also have lower plasma AA concentrations. Our unpublished observations have shown that diabetic patients excrete AA in amounts inversely proportional to their glycosylated hemoglobin level, suggesting tissue depletion of this vitamin with increasing hyperglycemia. Because humans cannot synthesize AA, a change in production rate cannot be a factor but other disturbances in the metabolism of AA are possible. These may include a shift in its equilibrium with dehydroascorbate, alteration of renal excretion, change in half-life, and competition for cellular uptake during high glucose level. Further studies to characterize the nature of AA disturbance and its functional significance in humans would be of great interest.

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