Rapid Publications

Deficiency of Ascorbic Acid in Experimental Diabetes

Relationship With Collagen and Polyol Pathway Abnormalities

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The plasma and tissue concentration of ascorbic acid (AA) is reduced in diabetes. This study was designed to investigate the mechanism and significance of this phenomenon. The low plasma AA concentration of diabetic rats can be normalized by dietary AA supplement (20–40 mg/day), a dosage approximately equal to the maximal synthetic rate of this substance in the rats. Treatment of diabetic rats with this regime prevented the decrease in activity of granulation tissue prolyl hydroxylase (PRLase), an AA-dependent enzyme required for maintaining the normal properties of collagen. The decreased plasma AA concentration and granulation tissue PRLase activity in diabetes can also be normalized by the aldose reductase inhibitor tolrestat. We conclude that in diabetic animals there is a true deficiency of AA that may be responsible for some of the changes of collagen observed in diabetes. Treatment with AA or an aldose reductase inhibitor may prevent some of the diabetic complications with underlying collagen abnormalities. *Diabetes* 37:359–61, 1988.

The plasma and tissue concentration of ascorbic acid (AA) has been reported to be decreased in diabetic animals and humans (1–3). The mechanism of this reduction in AA level in diabetes is not well understood, and its functional significance is equally uncertain. AA regulates the biosynthesis and posttranslational modifications of collagen, actions substantially mediated by its ability to maintain the activity of the enzyme prolyl hydroxylase (PRLase; EC 1.14.11.2) (4–6). Because collagen is quantitatively the most important structural protein, any disturbance of AA metabolism in diabetes can lead to tissue damage. Investigations into the mechanism and functional effects of the AA abnormalities in diabetes may provide valuable information on the pathogenesis of some diabetic complications.

In this study, the effects of AA supplementation on plasma AA concentration and granulation tissue PRLase activity of diabetic animals were investigated. Because the polyol pathway is implicated in many biochemical changes in diabetes, we also examined the effects of aldose reductase inhibition by tolrestat (Ay-27,773, Ayerst, New York) on AA level and PRLase activity in diabetes.

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 180–200 g were used for this study. Each experiment was started with ~30 rats divided into approximately equal numbers for each of the four subgroups (normal and diabetes ± treatment with AA or normal and diabetes ± tolrestat). Diabetes was induced by the injection of streptozocin (65 mg/kg i.v.; Calbiochem, San Diego, CA), and only animals with tail blood glucose levels >20 mM were used. After 4 wk of treatment by the appropriate agent (AA or tolrestat), the animals were killed by an overdose of ketamine (Parke Davis, Sydney, Australia), and blood and tissue were obtained for study. All experiments were approved by the Animal Experiments Ethics Review Committee of The University of Sydney.

Ascorbic acid and tolrestat treatment. AA treatment of normal and diabetic animals was given for 4 wk from the onset of diabetes by adding AA (Sigma, St. Louis, MO) to drinking water at a concentration of 1 g/L (prepared fresh each day). All animals continued to receive their normal chow containing a negligible amount of AA. In this manner, the nondiabetic animals had an AA intake of ~10 mg/day, whereas diabetic animals consumed 20–40 mg/day, representing approximately the maximal synthetic rate of this substance in the rats (4). Tolrestat was prepared fresh each day and given by gavage at a dose of 5 mg/kg body wt.

Ascorbic acid measurement. AA was measured by HPLC with a uBondapak C-18 column (Waters, Milford, MA). Blood

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was obtained from rats by cardiac puncture after death by an overdose of ketamine (200 mg/kg). It was immediately centrifuged, the plasma was deproteinized by the addition of 20% trichloroacetic acid to a final concentration of 5% and centrifuged again, and the supernatant was stored at −80°C and used for AA assay within 2 wk. Control experiments showed <2% degradation of AA collected in this manner. AA was monitored by absorbance at 254 nm. Its concentration was measured by the area under the peak and read against a standard of AA prepared fresh each day from a stock solution.

Measurement of granulation tissue PRLase activity. Granulation tissue was obtained from normal and diabetic animals by a technique described by us previously (7). Three days after injection of streptozocin, animals were anesthetized (100 mg/kg i.p. ketamine) and two steel-mesh cylinders (4 × 1 cm) were implanted subcutaneously in the back of each rat. Infection was prevented by weekly intramuscular injections of penicillin, and granulation tissue was recovered from the cylinders at death 4 wk later. The activity of granulation tissue PRLase was measured according to the method of Peterkofsky and DiBlasio (8). Briefly, the collagen substrate was prepared by incubating frontal bones of 16-day-old chick embryos with L-[3,4-^{3}H]proline. PRLase was extracted from granulation tissue by homogenization in Tris-HCl buffer (0.05 M, pH 7.6). The assay was carried out by incubating 60 μl of the enzyme extract and 10–25 μl of collagen substrate (~5 × 10^7 dpm/ml). The activity of PRLase was quantitated by measuring the ^3H_2O released from the substrate and separated by Dowex AG-50XW ion-exchange chromatography (Bio-Rad, Richmond, CA). The validity of the assay was confirmed by showing proportionality of enzyme activity to protein concentration and appropriate stimulation and inhibition by ascorbate, α-ketoglutarate, catalase, Fe_2SO_4 and α,α′-dipyridyl. All samples from each experiment were studied in the same assay to eliminate interassay variation due to changes in the specific activity of the substrate.

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

RESULTS

Results of plasma AA concentration studies in normal and diabetic rats are shown in Fig. 1. The plasma level of AA was reduced by 29% in the diabetic animals and normalized by AA treatment. AA treatment had no effect on the severity of diabetes assessed by blood glucose levels or the degree of weight loss.

Results of the granulation tissue PRLase activity in normal and diabetic rats are shown in Fig. 2. There was a significant reduction (15%) in the activity of PRLase in diabetes that was normalized by AA treatment.

The results of tolrestat treatment on plasma AA concentration and granulation tissue PRLase activity are shown in Fig. 3. Treatment with this agent normalized both plasma AA level and PRLase activity.

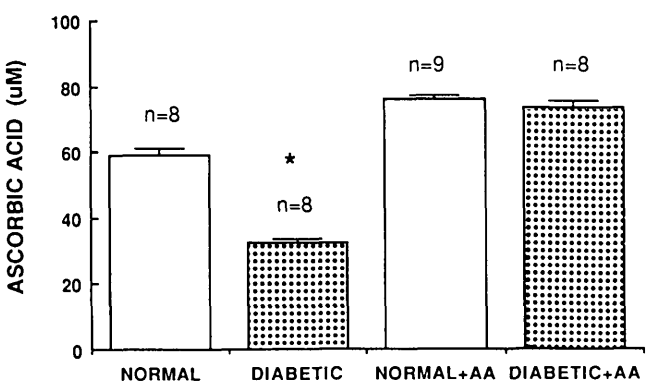


FIG. 1. Plasma ascorbic acid (AA) in normal and diabetic rats treated with AA. *P < .05, different from other groups.

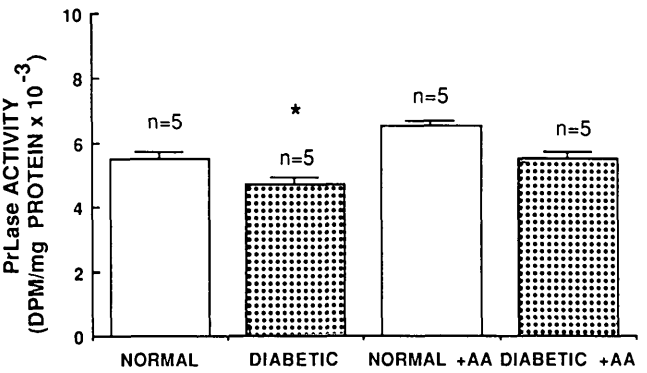
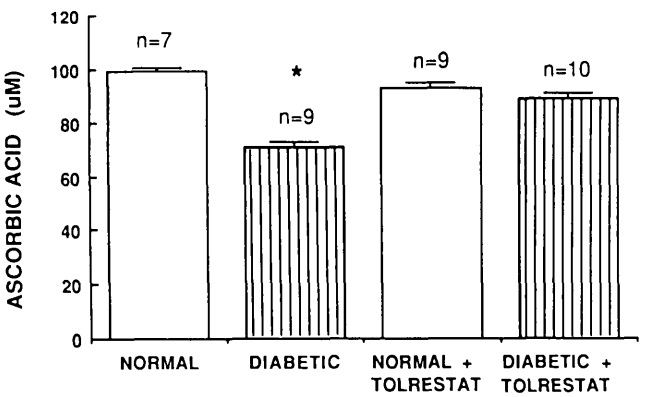


FIG. 2. Granulation tissue prolyl hydroxylase (PRLase) activity in normal and diabetic rats treated with ascorbic acid (AA). *P < .05, different from other groups.



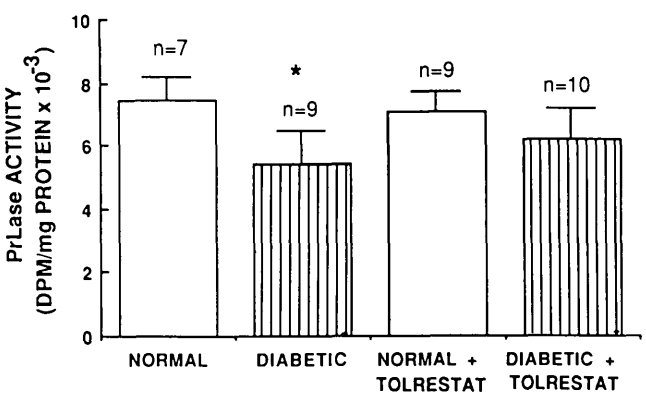


FIG. 3. Plasma ascorbic acid and prolyl hydroxylase (PRLase) activity in normal and diabetic rats treated with tolrestat. *P < .05, different from other groups.

DISCUSSION

The metabolism of AA is abnormal in diabetes, with AA concentration reported to be decreased in both plasma and tissues (1–3). Despite these observations, it has not been established whether the decreased AA in diabetes has any functional significance or whether diabetic patients should receive AA treatment. The best-known function of AA is the prevention of scurvy, an effect mediated by its action on the synthesis and posttranslational modifications of collagen (4). The interaction between AA and collagen biosynthesis is complex, but a major factor is the ability of AA to maintain in vivo the enzyme PRLase in its active state (5,6). This enzyme catalyzes the hydroxylation of proline to form hydroxyproline, an amino acid required for the stability of the collagen molecule (9).

We and others have previously observed a reduction of PRLase activity in the tissues of diabetic animals (10,11). Results herein showed that this phenomenon is a manifestation of AA depletion, which suggests a true deficiency of AA in diabetes and that its supplementation should be considered in the treatment of diabetic patients. This has the potential of preventing the abnormal synthesis and biomechanical properties of collagen in diabetes.

Because AA has many other important biochemical actions, including the scavenging of free radicals (4), its therapeutic usage in diabetes has even wider implications. Further studies are required to determine if these findings are also applicable to humans. The pattern of changes in AA metabolism is very similar in diabetic rats and humans, although rats can synthesize AA, whereas humans must rely on exogenous sources.

The aldose reductase inhibitor tolrestat, which normalized the plasma AA concentration in diabetes, also prevented the fall in PRLase activity, further supporting a causal relationship between these two phenomena. How aldose reductase inhibition leads to normalization of plasma AA is uncertain but may be related to the ability of this class of substance to raise the tissue concentration of reduced glutathione, which is required for the recycling of dehydroascorbate to AA by both enzymatic and nonenzymatic mechanisms (12,13). The urinary excretion of AA and *myo*-inositol is increased in experimental diabetes, and aldose reductase inhibition could also affect the metabolism of AA at this level (14).

This study confirms the low AA level in diabetes and reveals its relationship with the abnormalities of collagen and the polyol pathway. The depletion of AA in diabetes may not be a harmless phenomenon, and further studies are required to evaluate the need of treating diabetic patients with this vitamin.

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