Effect of Diltiazem on Glomerular Heparan Sulfate and Albuminuria in Diabetic Rats

Garikiparthy N. Jyothirmayi and Alluru S. Reddi

Calcium entry blockers, particularly diltiazem, have been shown to lower not only systemic blood pressure but also improve proteinuria in non–insulin-dependent diabetic patients. The presence of proteinuria is attributed to the loss of glomerular heparan sulfate, which confers a negative charge on the basement membrane. In the present study, we evaluated the efficacy of diltiazem in lowering blood pressure and proteinuria in diabetic rats and also examined the possibility that diltiazem prevents proteinuria through glomerular preservation of heparan sulfate. Diabetes was induced in male Wistar rats by streptozotocin (60 mg/kg). One group of diabetic rats was treated with diltiazem (25 mg/L) in drinking water for 20 weeks. Another group of diabetic rats and a group of nondiabetic rats were given tap water only. Systolic blood pressure was measured at 4, 8, 12, and 20 weeks. Urinary excretion of albumin was done at 4, 8, 12, 16, and 20 weeks. At the end of 20 weeks, all rats were killed, kidneys were removed, and glomeruli were isolated. Total glycosaminoglycan and heparan sulfate synthesis were determined by incubating glomeruli in the presence of [35S]sulfate. Diltiazem lowered blood pressure significantly in diabetic rats at 8, 12, and 20 weeks. Diabetic glomeruli synthesized less total glycosaminoglycan and heparan sulfate than glomeruli from normal rats. Characterization of heparan sulfate by ion-exchange chromatography showed that the fraction eluted with 1 M NaCl was significantly lower and the fraction eluted with 1.25 M NaCl significantly higher in diabetic than in normal rats. Diltiazem therapy returned not only glomerular synthesis but also various fractions of heparan sulfate to normal. Urinary albumin excretion was significantly higher in diabetic than in normal rats; diltiazem therapy significantly lowered albuminuria in diabetic rats. The data suggest that diltiazem therapy prevents albuminuria through preservation of glomerular heparan sulfate in diabetic rats. (Hypertension 1993;21:795–802)

KEY WORDS • diltiazem • calcium channel blockers • proteoglycans • diabetes mellitus, experimental • heparan sulfate • proteinuria

The pathology of the kidney in diabetes mellitus is characterized by thickening of the glomerular and tubular basement membranes and accumulation of basement membrane–like material in the mesangium.1 The onset of these changes is believed to be signaled by the appearance of microalbuminuria (urinary albumin excretion of 30–300 mg per day).2–4 If untreated, this microalbuminuria may progress to macroalbuminuria and then to nephrotic syndrome. It appears likely that albuminuria of either degree indicates the escape of albumin across the glomerular basement membrane (GBM). This escape appears to be due to the loss of negative electrostatic properties of the GBM. One constituent of the membrane, heparan sulfate–containing proteoglycan, can confer a negative charge on the lamina rara interna and externa of this structural element. It therefore seems probable that a decrease in heparan sulfate content would result in altered electrostatic properties and thus produce proteinuria.5 Indeed, it has been shown, for example, that removal of heparan sulfate from the GBM of rats by means of heparanase treatment resulted in increased permeability to [125I]albumin.6 A more recent study by Van Den Born et al7 showed that administration of a monoclonal antibody against GBM heparan sulfate induced an increase in urinary albumin excretion in rats. In human diabetic subjects, a decreased content of heparan sulfate proteoglycan has been found in the GBM.8 Other evidence, consistent with this hypothesis, is that decreased synthesis of renal proteoglycans has been reported in diabetic rats9–12 and in Engelbreth-Holm-Swarm tumor grown in genetically diabetic mice.13

In a previous report, we demonstrated a significant decrease in glomerular synthesis of heparan sulfate associated with an increase in albuminuria in long-term diabetic compared with normal rats.14 Further studies have shown that the changes observed in these diabetic rats were prevented by treatment with angiotensin converting enzyme (ACE) inhibitors, captopril,15 or enalapril.16 The beneficial effects of ACE inhibitors in preventing proteinuria in diabetic rats and human subjects have been well established.17–23 However, the role of calcium entry blockers in the prevention of proteinuria in diabetic rats or human subjects has received little attention compared with ACE inhibitors. Calcium entry blockers are being extensively used to treat systemic hypertension24,25 and to prevent renal damage.26,27 The objectives of this study, therefore, were 1) to evaluate the efficacy of diltiazem (DZM) (a benzothiazepine...
derivative of a calcium entry blocker) in lowering blood pressure in diabetic rats, 2) to study the effect of DZM on glomerular synthesis of heparan sulfate in diabetic rats, and 3) to study whether DZM prevents albuminuria by preserving glomerular synthesis of heparan sulfate.

**Methods**

**Animals**

A total of 26 male Wistar rats (Charles River) weighing 70–90 g were used in the study. After an overnight fast, diabetes was induced in 17 rats with a single intraperitoneal injection of streptozotocin in 0.1 M citrate buffer, pH 4.5, at a concentration of 60 mg/kg body weight. The remaining nine rats received an equivalent amount of buffer and served as normal control rats. One week after induction of diabetes, eight diabetic rats were given ad libitum as drinking fluid tap water that contained DZM (25 mg/L); the remaining nine diabetic and nine nondiabetic rats were given only tap water. Water was changed every day in all groups of rats. The daily consumption of DZM was 5.72±0.65 mg (mean±SEM). The drug treatment was continued for 20 weeks. All groups of rats were fed Purina rodent Chow (5001) ad libitum with the following composition by weight: protein (23.4%), fat (4.5%), fiber (5.8%), sodium (0.4%), calcium (1%), phosphorus (0.61%), potassium (1.1%) and with vitamin supplementation. The energy provided was 17.9 kJ (4.25 kcal/g).

Systolic blood pressures were measured in conscious rats at 4, 8, 12, and 20 weeks by the tail-cuff method. At 4, 8, 12, 16, and 20 weeks, each rat was placed in a metabolic cage for 24-hour urine collection, at which time the body weight and food and water intake were recorded. After the total volume was measured, the urine was centrifuged and used for determinations of albumin, heparan sulfate, Na⁺, and K⁺.

**Determination of Glomerular Gycosaminoglycan Synthesis**

At the time they were killed, each rat was anesthetized with a single intraperitoneal injection of pentobarbital (5 mg/100 g) and bled from the abdominal aorta. The kidneys were then removed, weighed, and the cortices separated from the medulla. Glomeruli were isolated from the cortex by differential sieving through 150-, 250-, and 63-μm meshes, using ice-cooled 0.02 M phosphate-buffered saline, pH 7.2. After centrifugation at 100g, the glomeruli were washed twice with Krebs-Ringer solution, and the washed pellet was uniformly suspended in the same solution. About 10,000 glomeruli were incubated in 2 mL Krebs-Ringer containing 0.15 mg/mL glutamine, 50 μg/mL ascorbate, and 50 μCi/mL [35S]sulfate (specific activity, 788 mCi/mmol) in an atmosphere of 95% O₂ and 5% CO₂. After incubation for 4 hours at 37°C, protein synthesis was terminated by the addition of 1 mM puromycin. The contents and further washings of the medium were

**Figure 1.** Line graph shows elution patterns of 35S-labeled heparan sulfate (standard) and testicular hyaluronidase-digested glomerular material from Sephadex G50 column before and after nitrous acid degradation.

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**Table 1. Data for Normal, Diabetic, or Diltiazem-Treated Diabetic Rats at End of Experimental Period**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt. (g)</th>
<th>Kidney wt. (g)</th>
<th>Glucose (mg/dL)</th>
<th>K⁺ (mEq/L)</th>
<th>Creatinine (mg/dL)</th>
<th>Urinary Na⁺ (mEq/day)</th>
<th>Urinary K⁺ (mEq/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N+H₂O (n=9)</td>
<td>&gt;610</td>
<td>3.90±0.09*</td>
<td>147±13†</td>
<td>3.38±0.24</td>
<td>0.82±0.04</td>
<td>3.90±0.40‡</td>
<td>6.39±0.48†</td>
</tr>
<tr>
<td>D+H₂O (n=9)</td>
<td>325±31</td>
<td>5.10±0.33</td>
<td>514±65</td>
<td>4.02±0.37</td>
<td>0.97±0.11</td>
<td>5.84±0.49§</td>
<td>11.09±0.78</td>
</tr>
<tr>
<td>D+DZM (n=8)</td>
<td>366±31</td>
<td>4.92±0.18</td>
<td>454±58</td>
<td>4.53±0.27</td>
<td>0.74±0.07</td>
<td>8.10±0.88</td>
<td>10.26±0.87</td>
</tr>
</tbody>
</table>

N+H₂O, normal rats given tap water to drink; D+H₂O, diabetic rats given tap water to drink; D+DZM, diabetic rats treated with diltiazem. Values are mean±SEM.

*P<0.01, N+H₂O vs. D+H₂O.
†P<0.001, N+H₂O vs. D+H₂O.
‡P<0.02, N+H₂O vs. D+H₂O.
§P<0.05, D+H₂O vs. D+DZM.
transferred to a preweighed tube and centrifuged at 1,000g for 15 minutes. To the glomerular pellet was added 2 mL acetone to remove lipids. This step was repeated twice, and the pellet was dried and weighed. The dried glomeruli were taken up in 2 mL of 0.1 M acetate buffer containing 5 mM EDTA, 5 mM cysteine, and 2.5 mL/mL crystalline papain (Sigma) and incubated at 60°C for 24 hours to release glycopeptides. Incubation was continued for another 24 hours with addition of papain (1.5 mL/mL). At this time, 1 mg carrier chondroitin sulfate (Sigma Chemical Co., St. Louis, Mo.) was added. The glomerular glycosaminoglycans (GAGs) were precipitated with 0.3 mL of 10% cetylpyridinium chloride. The precipitate was washed twice with NaCl-saturated 95% ethanol, then with 95% ethanol, and dried. The GAGs were further purified by removing proteins and nucleic acids by precipitation twice with 10% trichloroacetic acid and supernatants combined. The pH of the supernatant was adjusted to 5 with 0.5 M sodium acetate, and GAGs were precipitated with 3 vol 95% ethanol. The precipitate was dried and then dissolved in a known volume of distilled water. An aliquot of this sample was used for quantitation of radioactivity, which represented counts in total GAGs. Total GAG synthesis is expressed as disintegrations per minute per milligram glomerular weight or per glomerulus.

To identify individual GAGs, another aliquot was treated with 5 mg testicular hyaluronidase (Sigma) in 0.15 M NaCl and 0.1 M acetate buffer, pH 5.6, at 37°C for 24 hours to degrade chondroitin sulfate. The digested material was placed on a Sephadex G50 column (5×550 mm) and eluted with 0.15 M NaCl in 10% ethanol. The included volume contained over 95% of the uronic acid due to the degraded carrier, and the radioactivity associated with this fraction represented the counts in chondroitin sulfate. The voided volume contained mostly heparan sulfate, and the radioactivity associated with this fraction represented counts in heparan sulfate. The presence of heparan sulfate in the void volume was verified by its resistance to testicular hyaluronidase and its degradation by nitrous acid (Figure 1).

**Characterization of Heparan Sulfate**

Since heparan sulfate is heterogeneous, it was further fractionated on a Bio-Rad Agl-x2 (CT) ion-exchange column (4×1 cm) to determine the distribution of radioactivity within each fraction. The column was eluted in a stepwise manner with 8 mL each of 1, 1.25, and 2 M NaCl. Each fraction was fractionated, dried, and dissolved in distilled water; radioactivity was determined in each fraction. Since chondroitin sulfate was removed with the testicular hyaluronidase and by clu-

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerular wt. (mg)</th>
<th>Incorporation of [35S]sulfate into total GAG</th>
<th>Incorporation of [35S]sulfate into heparan sulfate (%)</th>
<th>Incorporation of [35S]sulfate into chondroitin sulfate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N+H2O (n=9)</td>
<td>55.8±4.0</td>
<td>251±37*</td>
<td>1.46±0.25†</td>
<td>83.59±2.61*</td>
</tr>
<tr>
<td>D+H2O (n=9)</td>
<td>55.0±5.5</td>
<td>152±20</td>
<td>0.77±0.16</td>
<td>66.91±6.96</td>
</tr>
<tr>
<td>D+DZM (n=8)</td>
<td>67.5±5.3</td>
<td>199±33</td>
<td>1.32±0.26</td>
<td>85.79±3.76</td>
</tr>
</tbody>
</table>

GAG, glycosaminoglycan; N+H2O, normal rats given tap water to drink; D+H2O, diabetic rats given tap water to drink; D+DZM, diabetic rats treated with diltiazem. Values are mean±SEM.

* p<0.05, N+H2O vs. D+H2O.
† p<0.01, N+H2O vs. D+H2O.
TABLE 3. Percent Radioactivity in Glomerular Heparan Sulfate Fractions From Normal, Diabetic, or Diltiazem-Treated Diabetic Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>NaCl</th>
<th>1 M</th>
<th>1.25 M</th>
<th>2 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>N+H₂O (n=9)</td>
<td>36.82±2.77*</td>
<td>29.12±2.19*</td>
<td>34.05±1.83</td>
<td></td>
</tr>
<tr>
<td>D+H₂O (n=9)</td>
<td>31.97±0.17</td>
<td>35.36±1.40</td>
<td>30.98±1.31</td>
<td></td>
</tr>
<tr>
<td>D+DZM (n=8)</td>
<td>35.26±1.34</td>
<td>32.05±1.38</td>
<td>33.91±1.71</td>
<td></td>
</tr>
</tbody>
</table>

N+H₂O, normal rats given tap water to drink; D+H₂O, diabetic rats given tap water to drink; D+DZM, diabetic rats treated with diltiazem. Values are mean±SEM.

*p<0.05, N+H₂O vs. D+H₂O.

Urine Protein Electrophoresis

Randomly selected urine samples (20 weeks) from one normal, one diabetic, and four diabetic rats treated with DZM were used for electrophoresis, using PhastSystem and PhastGel separation media (Pharmacia LKB Biotechnology, Uppsala, Sweden). Urine samples and low molecular weight standard protein samples were prepared in 1× sample buffer containing 10 mM Tris/HCl, 1 mM EDTA, 2.5% sodium dodecyl sulfate (SDS), and 5.0% B-mercaptoethanol at pH 8.0 and heated at 100°C for 5 minutes. Approximately 0.01% bromophenol blue was used as a tracking dye. Using an 8-track applicator, a 2 μL sample containing 2 μg protein per track was applied on a PhastGel homogenous medium (12.5% and 0.45 mm thick gel) with a buffer system of 0.112 M acetate and 0.112 M Tris, pH 6.5. The gels were run with PhastGel buffer strips containing 0.2 M tricine, 0.2 M Tris, and 0.55% SDS, pH 8.1. (The buffer strips are made of agarose isoelectric focusing.) Optimal resolution of SDS–polyacrylamide gel electrophoresis (PAGE) was obtained at a field strength of 250 V, 10.0 mA, 3.0 W, and 70 Vh at 15°C. The gels were stained with Coomassie blue. The total time required for separation and staining was 75 minutes.

Determination of Glucose, Creatinine, Na⁺, and K⁺

Plasma glucose was determined by the glucose oxidase method, using the reagents supplied by Sigma and plasma creatinine by alkaline picrate reagent (Sigma). Plasma K⁺ and urine Na⁺ and K⁺ were determined by flame photometry.

Statistical Analysis

Data were analyzed by both parametric (Tukey's test) using one-way analysis of variance and nonparametric method applying Kruskal-Wallis test and are expressed as mean±SEM. The significance between two unpaired means was evaluated by Student's t test and Fisher's least significant detection test. A value of p<0.05 was considered significant.

Results

Table 1 shows pertinent information at the time the rats were killed. Diabetic rats had significantly lower body weight but higher kidney weight, protein intake, plasma glucose levels, and urinary volume than normal rats. DZM had no effect on any of these parameters in diabetic rats. No differences in either plasma potassium or creatinine were found among the normal, diabetic, or DZM-treated diabetic rats. Urinary excretions of Na⁺ and K⁺ were higher in diabetic rats compared with normal rats. DZM treatment significantly increased Na⁺ but not K⁺ excretion in diabetic rats.

Figure 2 shows systolic blood pressure levels at 4, 8, 12, and 20 weeks in various groups of rats. Blood pressures were significantly higher in diabetic than in normal rats. DZM lowered blood pressure significantly in diabetic rats as shown at 8, 12, and 20 weeks of treatment.

Table 2 shows the incorporation of [³⁵S]sulfate into total GAG and heparan and chondroitin sulfates by isolated glomeruli in various groups of rats. A significant decrease in incorporation into total GAG, when expressed either per milligram dry glomerular weight or...
per glomerulus, was found in diabetic compared with normal rats. Similarly, a decrease in heparan sulfate synthesis was also observed in diabetic rats. However, the incorporation into chondroitin sulfate was significantly increased in diabetic rats. Treatment of diabetic rats with DZM returned these changes to normal. There were no significant differences in glomerular weight between the normal and the two groups of diabetic rats.

Percent radioactivity in various fractions of heparan sulfate is shown in Table 3. The fraction eluted with 1 M NaCl was significantly lower and the fraction eluted with 1.25 M NaCl significantly higher in diabetic than in normal rats. DZM corrected these fractions to normal. No difference in the fraction eluted with 2 M NaCl was found among various groups of rats.

Figures 3–5 demonstrate changes in body weight, protein intake, and urine volume at various time intervals. As evident, both groups of diabetic rats had significantly lower body weights than normal rats from 8 to 20 weeks of treatment (Figure 3). Protein intake (Figure 4) and urine volumes (Figure 5) were significantly higher in diabetic than in normal rats. Between the two groups of diabetic rats, no difference either in body weight, protein intake, or urine volume was observed.

Twenty-four–hour urinary albumin excretion at various time intervals in all groups of rats is presented in Figure 6. Diabetic rats had significantly higher urinary albumin excretion than normal rats that increased with duration of diabetes. DZM prevented this increase in diabetic rats.

Figure 7 shows urine protein electrophoresis pattern in normal, diabetic, and DZM-treated diabetic rats. DZM-treated diabetic rats had lower concentrations of albumin (molecular weight, 66 kd) when compared with diabetic rats. Also, proteins corresponding to molecular weights between 14 and 21 kd were prominent in diabetic compared with normal rats.

Discussion
The present study demonstrates the following important findings: 1) DZM lowers systolic blood pressure in
diabetic rats at a mean concentration of 5.72±0.65 mg per day; 2) glomerular heparan sulfate and total GAG synthesis are decreased in diabetic rats, and this decrease is prevented by DZM treatment; 3) urinary excretion of albumin is increased in diabetic rats, and this increase is prevented by DZM treatment; and 4) DZM therapy does not affect either fasting plasma glucose levels, body weight, kidney weight, plasma K⁺, or creatinine, but it promotes natriuresis in diabetic rats.

Calcium entry blockers have been shown to have variable effects on proteinuria in diabetic rats and human subjects. Jackson et al demonstrated lack of effect of verapamil (a phenylalkylamine derivative) on proteinuria in streptozotocin diabetic rats. Mimran et al reported an increase in urinary albumin excretion with nifedipine (a dihydropyridine derivative) treatment in insulin-dependent diabetic patients with incipient diabetic nephropathy (urinary albumin excretion >15 μg/min). This is in contrast to the results reported by the Melbourne Diabetic Nephropathy Study Group, in which nifedipine treatment significantly reduced albuminuria in hypertensive diabetic patients. A decrease in exercise-induced microalbuminuria was observed with nifedipine in patients with insulin and non-insulin-dependent diabetes. Holdaas et al reported no effect of nifedipine on proteinuria in insulin-dependent diabetic subjects. Nicardipine (a dihydropyridine derivative) has also been shown to reduce albuminuria in non-insulin-dependent diabetic subjects. Bakris reported a significant reduction in proteinuria in diabetic patients with nephrotic syndrome treated with DZM for 6 weeks. In a subsequent study, DeMarie and Bakris compared the effects of DZM and nifedipine on proteinuria in non-insulin-dependent diabetic patients and reported a significant decrease with the former and an increase with the latter drug. Our results of a decrease in albuminuria in DZM-treated diabetic rats are consistent with the reports of Bakris.

The present study demonstrates not only a decrease in the synthesis and concentration of heparan sulfate proteoglycan in diabetic glomeruli, but also changes in the type of heparan sulfate that is present. This is evident from the elution pattern on ion-exchange columns with NaCl. These results are consistent with our previous data in diabetic rats. It is known that the sulfate content of the eluted fractions increases with increasing salt concentrations, the heparan sulfate with highest sulfate content being eluted with 2 M NaCl. A significant decrease in the radioactivity of the 1 M fraction in diabetic glomeruli indicates substantial loss of low-sulfate-containing heparan sulfate compared with normal glomeruli. On the other hand, a shift of elution from 1 M fraction to the 1.25 M fraction may be due to an increase in sulfation, a change in iduronic-
glucuronic acid ratios, or an increase in molecular size of heparan sulfate. This change in the diabetic glomerular heparan sulfate probably represents a different and newly synthesized heparan sulfate. Since heparan sulfates are a heterogeneous group of anionic GAGs with variable charge density, it is possible that diabetic glomeruli synthesized heparan sulfate with different physicochemical and functional properties. DZM therapy normalized glomerular changes of heparan sulfate in diabetic rats. It is of interest to note that Nakamura and Kojima reported that cancerous human liver synthesizes a molecular species of heparan sulfate that is lower in sulfate content than the heparan sulfate synthesized by normal human liver tissue.

How DZM prevents glomerular and urinary loss of heparan sulfate is not clearly understood. It is possible that DZM may have lowered glomerular hypertension early in the course of diabetes and thus protected the glomerular capillary wall from thickening and leakage of heparan sulfate. Recently, Anderson reported lowering of glomerular hypertension by DZM in nondiabetic rats with reduced renal mass. Additional data are needed to evaluate pressure-induced biochemical changes in the glomerulus and the basement membrane.

Increase in albuminuria with duration of diabetes in untreated diabetic rats is not unexpected. However, it is of interest to note that DZM therapy blunted this increase without lowering plasma glucose. Reduction in albuminuria was associated with glomerular preservation of heparan sulfate, suggesting an inverse relation between charge density and albuminuria. The present study emphasizes that DZM normalizes albuminuria even in severely hyperglycemic rats. The improvement in proteinuria is not related to changes in either dietary protein content or urinary volume, since both untreated and DZM-treated diabetic rats had similar food consumption and urinary volume. Furthermore, the observed decrease in albumin excretion in DZM-treated diabetic rats was confirmed by demonstrating lower concentrations of albumin on SDS-PAGE.

We have consistently observed an increase in chondroitin sulfate synthesis in diabetic rats. The significance of this increase is not clearly understood. Additional work is clearly warranted.

Acknowledgments

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