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Decreased *de novo* synthesis of glomerular proteoglycans in diabetes: Biochemical and autoradiographic evidence

(sulfated proteoglycans/glomerular extracellular matrices/autoradiography/streptozotocin-induced diabetes)

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ABSTRACT **The experimental model of streptozotocin-induced diabetes in rats was utilized to determine the biosynthetic and biochemical alterations in the proteoglycans of the glomerular extracellular matrices (glomerular basement membrane and mesangial matrix) in diabetic nephropathy. Isolated kidneys from diabetic and control groups of animals were radiolabeled in an organ perfusion apparatus with [^{35}S]sulfate of high specific activity (>1,200 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) and processed for electron microscopic autoradiography, and the proteoglycans of the glomerular extracellular matrices were characterized. The results indicate that [^{35}S]sulfate incorporation into glomerular extracellular matrices of diabetic animals was 30–40% less than that of the control group; however, no differences in the biochemical properties of the *de novo* synthesized proteoglycans from either group were observed. The relevance of the decreased *de novo* synthesis of sulfated proteoglycans of glomerular extracellular matrices is discussed in terms of increased glomerular permeability to plasma proteins and reduction in the glomerular filtration rate.**

Severe ultrastructural alterations in the glomerular basement membrane (GBM) and the mesangial matrix (Mes) have been observed in diabetic nephropathy (1). These changes in the glomerular extracellular matrices (GEMs: GBM and Mes) are usually associated with enhanced permeability to large macromolecules, including plasma albumin (2). Recent studies have established that one of the determinants of glomerular permeability is the electrical charge of the GBM (3–5), contributed mostly by the heparan sulfate-proteoglycan (HS-PG) (6).

In this investigation, we have utilized the streptozotocin-induced diabetes experimental model in rats to determine whether the ultrastructural abnormalities in the GEMs can be attributed to alterations in the biosynthesis or content of HS-PG.

METHODS

Induction of Diabetes. Male Charles River CD rats that initially weighed 100 g were used. The animals were divided into two groups: the diabetic group was given an intravenous injection of streptozotocin (75 mg/kg) and the age-matched control group was given an injection of normal saline. The animals, after maintenance on rat chow and water ad lib for 7 months, were sacrificed and their kidneys were utilized for radiolabeling of the GEMs with [^{35}S]sulfate (Amersham).

Radiolabeling of GEMs. Radiolabeling of GEMs with [^{35}S]-sulfate was carried out under sterile conditions in an *ex situ* organ perfusion apparatus (7). An *in vitro* rather than *in vivo* approach was utilized in view of the inherent difficulties in labeling the GEMs for autoradiography. Satisfactory labeling was achieved by constantly recirculating the isolated kidneys via their renal arteries for 7–8 hr with a chemically defined medium (7) containing [^{35}S]sulfate (300 μCi/mmol; 1 Ci = 3.7 × 10^{10} Bq) of high specific activity (>1,200 Ci/mmol). The labeled kidneys from both groups of normal and diabetic rats were then processed for electron microscopic autoradiography or for extraction and characterization of glomerular proteoglycans (PGs).

Isolation and Characterization of Glomerular PGs. Incorporation of [^{35}S]sulfate into the PGs of GEMs was determined from kidneys perfused for 1, 2, 3, 5, and 7 hr. At the end of each time period, the glomeruli were isolated, the extracellular matrices were prepared by detergent treatment (8, 9), and the labeled PGs were extracted with 4 M guanidine·HCl containing protease inhibitors (7). The extracts were dialyzed against distilled water to remove free sulfate. Radioactivity in the extracts was determined and expressed as counts per microgram of GEM protein. Total protein of the GEMs was determined by the method of Lowry *et al.* (10). Five determinations were made for each time point.

The radiolabeled PGs and their chains [treated with nitrous acid or chondroitinase ABC (chondroitin ABC lyase)] were characterized by chromatographing them on a Sepharose CL-6B column and eluting them with a solution containing 4 M guanidine·HCl, 0.1 M Tris·HCl, 0.1 M Na_2SO_4, 0.5% (vol/vol) Triton X-100 at pH 7.0 (7).

The radiolabeled glycoaminoglycans (GAGs; chains) obtained from PG fractions by alkaline borohydride treatment (7) were also characterized by cellulose acetate electrophoresis (9). The GAG standards and radiolabeled GAGs, treated as well as untreated, were electrophoresed, and the cellulose acetate strips were stained with 0.1% alcian blue and fixed in 10% acetic acid. They were then dried at room temperature, impregnated with EN^{3}HANCE (New England Nuclear), and covered with Kodak X-Omat film and the films were subsequently exposed for 4–5 days at −70°C and developed.

***In Situ* Characterization of PGs by Quantitative Electron Microscopic Autoradiography.** Specific species of [^{35}S]sulfate-labeled PGs were digested *in situ* from the labeled GEMs of the perfused kidneys by an additional perfusion with specific GAG-degrading enzymes. The enzymes used were chondroitinase ABC (degrades chondroitin sulfate-PG, CS-PG) and purified heparinase (specifically degrades HS-PG and CS-PG) under conditions previously described (8, 9). After digestion, the kidneys were fixed by perfusion and pieces of cortical tissue (≈1 mm^3) were obtained and processed for quantitative electron microscopic autoradiography (11).

For each animal of each kidney group, 10 micrographs were obtained from at least three tissue samples. Only tissue areas

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Abbreviations: GBM, glomerular basement membrane; Mes, mesangial matrix; GEM, glomerular extracellular matrix (GBM and Mes); PG, proteoglycan; HS-PG, heparan sulfate-PG; GAG, glycoaminoglycan; CS-PG, chondroitin sulfate-PG.

containing elements of both peripheral GBM loop and the Mes were photographed (at a magnification of ×5,000; printed at a final magnification of ×15,000). Two grain compartments were defined for the tissue areas photographed—i.e., GBM and the Mes. The grain density (concentration of radiation) of each compartment was determined as follows. First, the center of each autoradiographic grain was determined by the best-fit circle method of Salpeter and Bachman (12). Subsequently, the grain centers directly over the defined compartments were counted and tabulated. Next, the relative area of each compartment was determined by the point counting method, as described by Weibel (13). The grain density was computed by dividing the total grains by total area points. Finally, the mean grain density (mean of grain densities of five rats) and the standard deviation about the mean were determined.

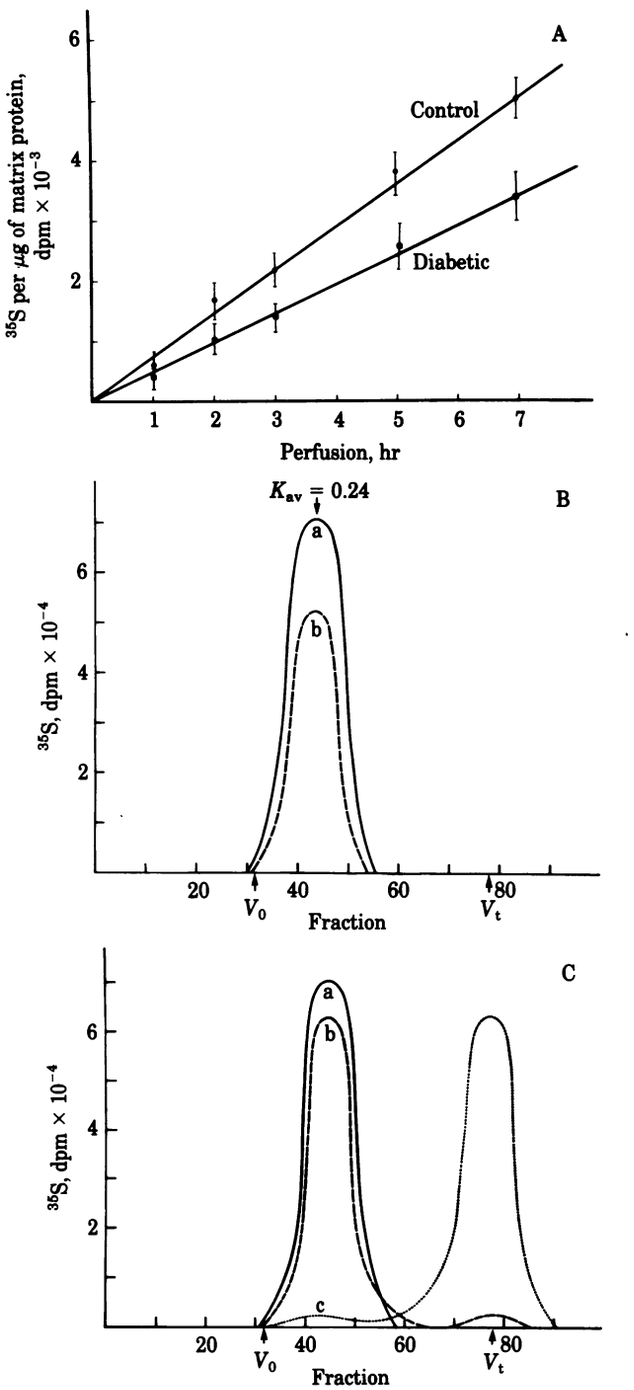

FIG. 1. (*A*) Linear incorporation of [^{35}S]sulfate into GAGs of *ex situ* perfused normal or control (●) and diabetic (■) kidneys. The [^{35}S]sulfate incorporation into the GEMs is lower in the diabetic group when compared with the control group. (*B*) Sepharose CL-6B chromatograms of sulfated PGs extracted from GEMs of normal (peak a; ——) and diabetic (peak b; ----) kidneys. No differences in K_{av} values of peaks a and b are observed, indicating the molecular weights of PGs do not differ. (*C*) Sepharose CL-6B chromatograms of sulfated PGs extracted from GEMs of diabetic kidneys. Peak a (——), intact PGs; peak b (----), treated with chondroitinase ABC; and peak c (·····), treated with nitrous acid. The majority of the *de novo* synthesized PGs are nitrous acid-sensitive (i.e., HS-PG) because almost all the counts are recovered in the V_t fraction.

RESULTS

Linear Incorporation of [^{35}S]Sulfate into PGs of the Matrices. Glomerular morphology remained normal and [^{35}S]sulfate was linearly incorporated into the PGs of the GEMs during 7 hr of *ex situ* kidney perfusion, indicating that the organ perfusion conditions were satisfactory for determining *de novo* synthesis of PGs (Fig. 1*A*). However, although kidneys from both normal and diabetic animals exhibited linear incorporation into the GEMs, it was 30–40% less in kidneys from diabetic animals (Fig. 1*A*).

Characterization of Glomerular PGs. Sepharose CL-6B chromatograms of glomerular extracts revealed similar K_{av} values for both diabetic and control groups, which were 0.24 (Fig. 1*B*) and 0.44 (not shown) for the intact PG and their chains, respectively. This indicated that there was no change in the molecular weights of the intact PGs ($M_r \approx 130{,}000$) and their chains ($M_r \approx 26{,}000$) in the diabetic state. The determination

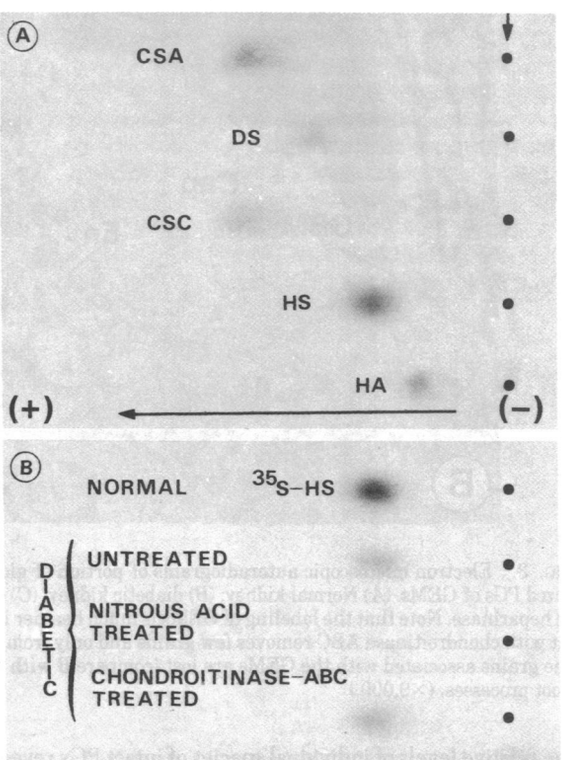

FIG. 2. Autoradiograms of the electrophoretograms of (*A*) known GAG standards, which include chondroitin sulfate A (CSA) and C (CSC), dermatan sulfate (DS), heparan sulfate (HS), and hyaluronic acid (HA), and (*B*) radiolabeled GAGs extracted from normal and diabetic kidneys. Equal volumes (1 μl) of GAG extracts were applied. With nitrous acid treatment the spot comigrating with HS disappears, whereas it persists after treatment with chondroitinase ABC.

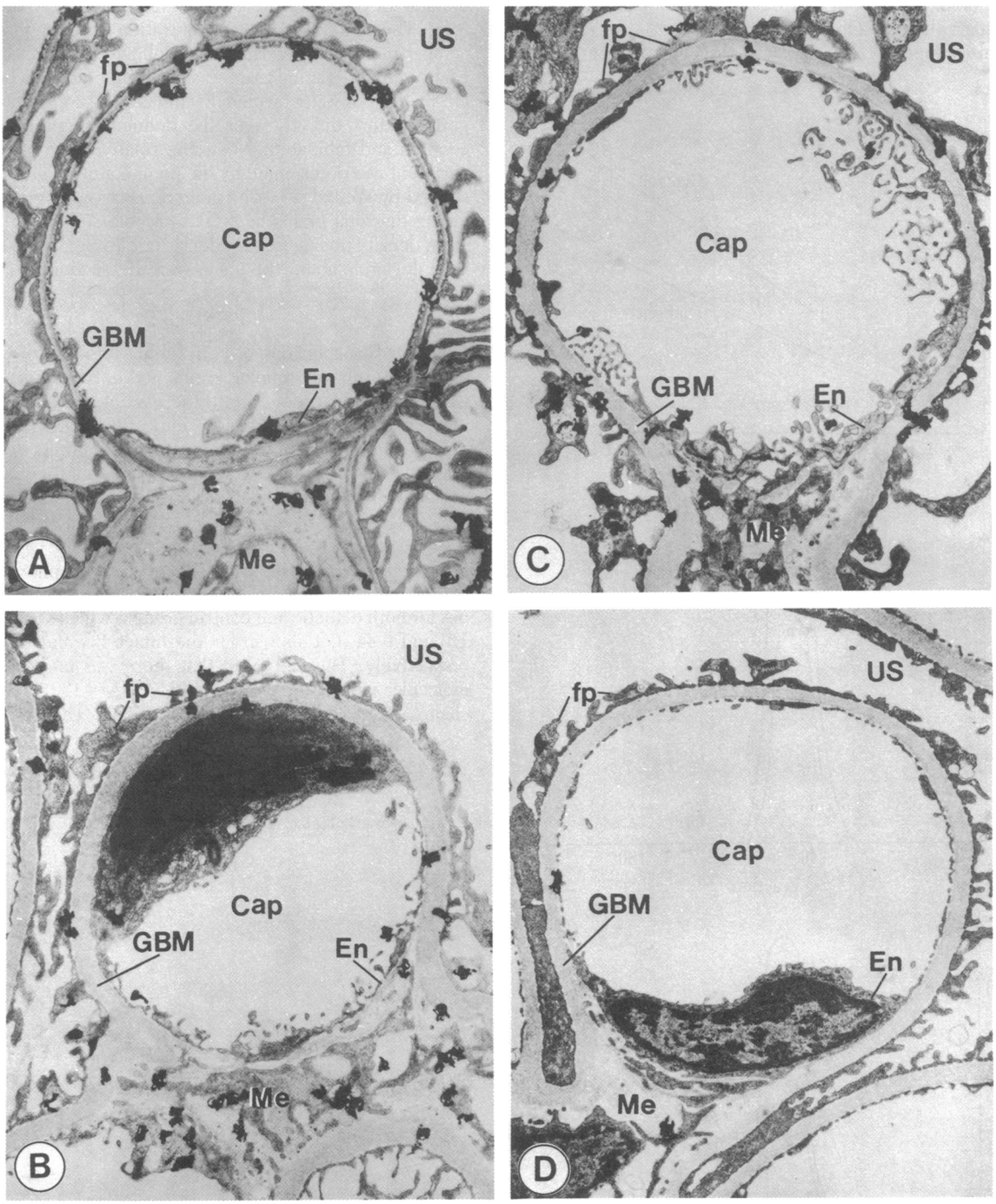

FIG. 3. Electron microscopic autoradiograms of portion of glomeruli from kidneys perfused with [^{35}S]sulfate in order to label the newly synthesized PGs of GEMs. (*A*) Normal kidney; (*B*) diabetic kidney; (*C*) diabetic kidney digested with chondroitinase ABC; and (*D*) diabetic kidney treated with heparinase. Note that the labeling of GEMs is much heavier in normal kidney when compared with diabetic kidney (compare *A* with *B*). Treatment with chondroitinase ABC removes few grains and only from the Mes (compare *B* with *C*), whereas after digestion with heparinase almost all of the grains associated with the GEMs are lost (compare *B* with *D*). Cap, capillary lumen; US, urinary space; Me, mesangium; En, endothelium; fp, foot processes. (×9,000.)

of the relative levels of individual species of intact PGs revealed that >90% of the newly synthesized PG was nitrous acid-sensitive (i.e., HS-PG) and <10% was chondroitinase ABC-degradable (i.e., CS-PG) (Fig. 1*C*).

The autoradiograms of the electrophoretograms of the radiolabeled GAG fractions indicated that in the diabetic state, the predominant GAG synthesized was heparan sulfate. This GAG did not exhibit any change in its migratory behavior; however, the amount synthesized was substantially decreased (Fig. 2). Chondroitin-GAG could not be detected by this method.

***In Situ* Characterization of Glomerular PGs.** All kidneys utilized for electron microscopic autoradiography were perfused with [^{35}S]sulfate for 7 hr. Analysis of electron microscopic autoradiograms revealed markedly thickened basement membranes with fewer grains associated with the GEMs in the diabetic group when compared with the control group (compare

Table 1. Sulfate incorporation into GEMs

Rat	Grain density	
	GBM	Mes
Normal untreated		
1	0.580	1.151
2	0.642	1.198
3	0.597	0.912
4	0.603	0.985
5	0.549	1.166
Mean ± SD	0.594 ± 0.033	1.082 ± 0.126
Diabetic		
Untreated		
1	0.392	0.620
2	0.402	0.525
3	0.354	0.510
4	0.357	0.565
5	0.394	0.541
Mean ± SD	0.379 ± 0.022	0.552 ± 0.043
Chondroitinase ABC-treated		
1	0.375	0.502
2	0.356	0.462
3	0.379	0.450
4	0.346	0.500
5	0.395	0.522
Mean ± SD	0.370 ± 0.019	0.487 ± 0.030
Heparinase-treated		
1	0.034	0.036
2	0.026	0.029
3	0.040	0.045
4	0.043	0.048
5	0.038	0.044
Mean ± SD	0.036 ± 0.006	0.040 ± 0.007

Fig. 3*B* with Fig. 3*A*). The mean grain densities of the GBM and the Mes were 0.594 and 1.082, respectively, in the normal group and decreased to 0.379 and 0.552, respectively, in the diabetic group (Table 1). Interestingly, the synthetic rate of the Mes remained proportionately higher than the GBM in both groups (Table 1). After chondroitinase ABC digestion, ≈10% of the grains associated with the Mes were lost (compare Fig. 3*C* with Fig. 3*B*) and the mean grain density dropped from 0.552 to 0.487 with no appreciable change in that of the GBM. This indicated that all of the *de novo* synthesized CS-PG was associated with the Mes. Heparinase treatment removed almost all of the grains that were associated with the GBM and the Mes (compare Fig. 3*D* with Fig. 3*B*). The mean grain densities decreased from 0.379 and 0.552 to 0.036 and 0.040 for the GBM and Mes, respectively (Table 1). This indicated that the majority of the *de novo* synthesized PGs was HS-PG.

DISCUSSION

The combined biochemical and autoradiographic data obtained in this investigation indicate that: (*i*) [^{35}S]sulfate is directly incorporated into the sulfated PGs of the GEMs; (*ii*) the incorporation of [^{35}S]sulfate is ≈30–40% less in the matrices of diabetic groups when compared with the control group; (*iii*) the rate of incorporation of [^{35}S]sulfate is higher for the Mes when compared with the GBM in either group; and (*iv*) in both groups, the molecular weights of *de novo* synthesized PGs and their chains as well as the electrophoretic mobilities of the GAGs are similar.

Prior to the establishment of HS-PG as a constituent of the GBM, intensive efforts were made by numerous workers to determine the biochemical or biosynthetic defects of diabetic nephroangiopathy (14–16). However, results were inconclusive and the mechanisms leading to the morphological alterations of GEMs and the consequential derangements in glomerular functions, proteinuria, and decreased glomerular filtration rate remained obscure. From the data obtained in the present investigation and from recent biochemical and functional studies on the glomerulus (3–5, 17), we may be able to answer certain questions regarding these mechanisms.

In the diabetic state, the decrease in *de novo* synthesis of sulfated PGs may lead to a reduction in the overall charge density of GEMs with eventual loss of the charge-selective properties of the glomerular capillary wall. This abolition of the negative electrical charge may, in turn, enhance transglomerular passage of plasma proteins, especially those which bear neutral or anionic charges. In addition, the loss of sulfated polyanionic macromolecules in the diabetic GEMs may allow formation of hydrogen bonds and ready adsorption of plasma proteins onto the GEMs (18). In fact, the nonspecific adsorption (noncomplement-mediated) of plasma proteins (IgG and albumin) is a well-known occurrence in diabetic nephropathy (19). Finally, the extensive adsorption of plasma proteins may serve to "clog" the basement membrane (20), thereby possibly contributing to the hypofiltration and reduction in glomerular filtration rate that is always associated with the late stages of diabetic nephropathy.

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