Reduced urinary excretion of sulfated polysaccharides in diabetic rats

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Abstract

The aim of the present study was to further understand the changes in renal filtration that occur in the early stages of diabetes mellitus. Diabetes was induced in male Wistar rats by a single injection of streptozotocin. Glycemia, body weight, 24-h urine volume and urinary excretion of creatinine, protein and glycosaminoglycans were measured 10 and 30 days after diabetes induction. All the diabetic animals used in the present study were hyperglycemic, did not gain weight, and presented proteinuria and creatinine hyperfiltration. In contrast, the glycosaminoglycan excretion decreased. Dextran sulfates of different molecular weights (6.0 to 11.5 kDa) were administered to the diabetic rats, and to age-matched, sham-treated controls. Most of the dextran sulfate was excreted during the first 24 h, and the amounts excreted in the urine were inversely proportional to the dextran sulfate molecular weight for all groups. Nevertheless, diabetic rats excreted less and accumulated more dextran sulfate in kidney and liver, as compared to controls. These differences, which were observed only for the dextran sulfates of higher molecular weights (>7 kDa), increased with the duration of diabetes. Our findings suggest differential renal processing mechanisms for proteins and sulfated polysaccharides, with the possible involvement of kidney cells.

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1. Introduction

In long-term diabetes mellitus, among the most clinically significant complications are the microangiopathies, which are disruption of the normal function of vascular capillary beds. A hallmark of these pathological processes is a significant thickening of microvascular basement membrane, with chronic progression with time. Paradoxically, this thickening of basement membranes is accompanied by a loss of function, allowing serum molecules that are normally retained within the circulation to pass across the matrix. This produces systemic effects, but most studies focused the kidney because the glomerulus provides concentrate and abundant amounts of capillary basement membrane. Furthermore, one of the most marked clinical consequences of diabetes is observed in the renal glomerulus, resulting in diabetic nephropathy [1]. Proteoglycans may play an important role in this process, since they are prominent components of extracellular matrices.

A close examination of the kidney reveals the potential complexity that proteoglycans may have in a very small region. The glomerular capillary wall consists of the epithelial and endothelial coats and glomerular extracellular matrices, which are composed of basement membrane and mesangial matrix. Cell surface proteoglycans as well as extracellular matrix proteoglycans are present, both in glomerular basement membrane and in mesangial matrix [2,3]. The mesangial matrix and the basement membrane appear to differ with respect to their proteoglycan composition [4,5].

Both the glomerular basement membrane and the mesangial matrix are increased in diabetic nephropathy [6] and this expansion is, possibly, a contributor to diabetic renal failure. Increased synthesis of extracellular matrix
components [7] and reduction in the charge density of proteoglycans [8] have been reported, suggesting that changes in extracellular matrix organization and metabolism could be involved in the pathogenesis of diabetic nephropathy, with alteration in filtration features.

Using an experimental model of type I diabetes mellitus, which was induced by chemical destruction of pancreatic islet cells, we have previously shown a marked decrease in the urinary excretion of glycosaminoglycans, from the second week after streptozotocin injection [9]. In contrast, the glycosaminoglycan concentration increased in diabetic kidney, due to dermatan sulfate buildup [9,10].

The aim of the present study was to further understand the parameters governing the changes in renal filtration that occur in diabetic nephropathy, especially concerning the early events of the process.

In the present study, we used a commercial preparation of dextran sulfate (modal MW 8 kDa) as a probe. This compound is highly negatively charged and is not reabsorbed by postglomerular tubules, so its clearance reflects interactions occurring at the glomerular level. Furthermore, it was recently shown that dextran sulfate does not undergo depolymerization during renal passage, and the effect of dextran sulfate binding to albumin upon fractional clearance is negligible [11].

2. Materials and methods

2.1. Animals, urine specimens, and diabetes induction

The experiments were performed in two phases. In the first phase, we used 12 male Wistar rats (12–16 weeks old, 250–350 g body weight), and in the second phase, 48 animals. At the beginning of each experiment, 24-h urine samples were collected in metabolic cages, and the animals were randomly sorted in four groups: “Control—10 days”, “Diabetes—10 days”, “Control—30 days” and “Diabetes—30 days”. Diabetes mellitus was induced in a half of the animals by a single injection of streptozotocin (60 mg/kg of body weight). The drug was dissolved in 300 μl of 10 mM sodium citrate buffer (pH 4.5) and injected by i.p. route. These animals were assigned to the “Diabetes” groups, and fed standard laboratory chow and a 5% glucose solution ad libitum, for 48 h. Afterwards, the glucose solution was replaced by water. The glycemia was measured (Glycofilm™, Bayer Diagnostics MFG, Bridgen, Mild Glamorgan, England) and all the diabetic animals of the present study have shown blood glucose concentrations higher than 200 mg/dl. Age-matched “Controls” received only citrate buffer and were fed standard laboratory chow and water ad libitum. After either 10 or 30 days, the glycemia of the corresponding groups was measured, and 24-h urine samples were collected. No animals of the “Diabetes” group died during the experimental period.

2.2. Dextran sulfate

A commercial preparation of dextran sulfate with 8-kDa modal molecular weight (Sigma Chemical, St Louis, MO, USA) was used as a probe to investigate the renal filtration of polyanionic polysaccharides by normal and diabetic rats. This commercial preparation, which was used in the first phase of the experiment, is heterogeneous and gives a polydisperse band in polyacrylamide gel electrophoresis.

In order to obtain more homogeneous dextran sulfate preparations and with different modal molecular weights, a dextran sulfate sample (2 g) was fractionated by gel filtration chromatography on a Sephadex G-50 column (2 × 120 cm) (Amersham Biosciences do Brazil, São Paulo, SP, Brazil). The column was equilibrated and eluted with 0.05 M acetic acid, and 3-ml fractions were collected at a flow rate of 2 ml/min, and analyzed by the 1,9-dimethylmethylene blue assay [12] adapted to microscale as previously described [13]. Aliquots of the fractions containing metachromatic compounds were submitted to polyacrylamide gel electrophoresis either in a BRL vertical mini-system or in a horizontal system [14]. Fractions that presented similar electrophoretic migrations were pooled, and the modal molecular weight of each pool was determined by polyacrylamide gel electrophoresis [15]. These preparations were used in the second phase of the experiments.

2.3. Urinary glycosaminoglycan extraction and analysis

Urinary glycosaminoglycans were extracted from 24-h urine samples (4–8 ml for non-diabetic and 25–40 ml for diabetic rats) by ion-exchange chromatography on Q-Sepharose (2 ml bed volume, 1 × 3 cm). The samples were diluted with 2 volumes of water, and then applied to the column, previously equilibrated with water. The column was eluted in a stepwise fashion by 0.3 M NaCl (5 ml) and 2 M NaCl (5 ml). Three volumes of methanol were added to each fraction, slowly and under agitation. After standing for 24 h at −20 °C, the precipitate formed was collected by centrifugation, vacuum-dried and resuspended in 50 μl of water. Aliquots (5 μl) were submitted to agarose gel electrophoresis in two buffer systems: 0.05 M 1,3-diaminopropane-acetate buffer, pH 9 (PDA) [16] and 0.05 M Tris-acetate buffer, pH 8 (Tris). After Toluidine Blue staining, the urinary GAGs were quantified by densitometry of the gel slabs in a CS-9000, Shimadzu densitometer. All glycosaminoglycans were found in the 2 M-fraction. These compounds were further characterized by enzymatic degradation with specific mucopolysaccharidases, as previously described [17].

2.4. Urinary dextran sulfate

Samples of dextran sulfate, either unfractionated (5 mg) or fractionated as described under Dextran sulfate (3.5 mg),
Fig. 1. Body weight, kidney wet weight, blood glucose, 24-h urine volume, and urinary excretion of creatinine and protein (expressed both as concentration in g/l and amounts excreted in 24 h, in mg) of rats that remained diabetic (DM) for either 10 or 30 days, and their respective controls (NL) (mean ± standard deviation). *P<0.0001; **P<0.01.
were dissolved in isotonic saline, and i.p.-injected in control and diabetic rats (three animals of each group for each dextran sulfate preparation). Again, 24-h urine samples were collected for 2 days (0–24 and 24–48 h).

Aliquots of the urine samples (10 μl for controls and 50 μl for diabetes) from dextran sulfate-injected rats were analyzed for the presence of dextran sulfate by agarose gel electrophoresis, as described above for glycosaminoglycans.

2.5. Tissue glycosaminoglycans and dextran sulfate

At the end of the experiments (48 h after dextran sulfate administration), control and diabetic rats were killed and glycosaminoglycans (and eventually dextran sulfate) were extracted from plasma, brain, heart, liver, muscle, lung and kidney as previously described [14]. Plasma was obtained from blood samples (2 ml) by centrifugation and GAGs and dextran were isolated as described [14]. The samples were then analyzed for the presence of dextran sulfate and glycosaminoglycans by a combination of agarose gel electrophoresis and enzymatic degradation with mucopolysaccharidases, as described above.

2.6. Urinary creatinine and protein measurement

Urinary creatinine was measured by the picric acid reaction in alkaline conditions (Sigma creatinine kit) and protein was quantified by the Coomasie blue method, both adapted to microscale, as previously described [13].

2.7. Statistical analysis

Parametric and nonparametric statistical tests were used. Student’s two-sided t test for paired samples and the Wilcoxon nonparametric sample rank test for independent groups were used to compare the mean difference in body weight, blood glucose concentration, 24-h urine volume, urinary creatinine, protein, and glycosaminoglycans of the experimental and control groups. The significance level is indicated in each experiment.

3. Results

3.1. Diabetic status of streptozotocin-treated rats

Diabetes was induced in a group of animals by streptozotocin injection. Fig. 1 shows that 10 and 30 days after streptozotocin injection, the experimental rats presented increased blood glucose concentration, increased 24-h urine volume and increased excretion of creatinine and protein in the urine, as compared to age-matched controls. In contrast to the control animals, which gained weight, the diabetic rats demonstrated a progressive decrease in body weight. All these differences remained significant, at \( P < 0.01 \) or better, from the 10th to the 30th day for the streptozotocin-injected animals (Fig. 1). The blood glucose levels were also increased to \( >200 \text{ mg/dl} \) beginning on the first week after injection and maintained until the end of the experiment, as controlled by glycemic levels monitoring every week. These data indicate that all the streptozotocin-injected rats used in the present study had developed diabetes.

3.2. Urinary glycosaminoglycans

The urinary glycosaminoglycan excretion (expressed both as mg/l, μg/24 h) was greatly decreased in the diabetic animals, both in the 10th day and in the 30th day (Fig. 2). The glycosaminoglycan concentration in the urine was decreased to less than 1/10 of the control in the diabetes groups (expressed as mg/l of urine), and the total amounts excreted in 24 h (μg/24 h) decreased to about a half (for 10 days) and 1/3 (for 30 days).

Agarose gel electrophoresis of rat urinary glycosaminoglycans is shown in Fig. 3A. Three bands were identified, migrating as chondroitin sulfate (CS), dermatan sulfate
(DS), and heparan sulfate (HS). The band migrating as chondroitin sulfate was totally degraded by chondroitinase AC, and the band migrating as heparan sulfate was susceptible to heparitinase II from *Flavobacterium heparinum*. Dermatan sulfate was degraded by chondroitinase B and was partially degraded by chondroitinase AC, indicating that this dermatan sulfate is composed of glucuronic acid- and iduronic acid-containing disaccharide units [18]. In some instances it was difficult to define the boundary between dermatan sulfate and chondroitin sulfate in the agarose gel. Because of their low molecular weights, there was some degree of diffusion and overlap during the electrophoresis (see Fig. 3A); so, they were quantified together, and Fig. 3B shows that both heparan sulfate and chondroitin sulfate/dermatan sulfate were found to be decreased in diabetic animals both in the 10th day and in the 30th day after streptozotocin injection.

### 3.3. Urinary excretion of dextran sulfate

When a commercial preparation of dextran sulfate (8 kDa modal molecular weight) was administered to normal and
diabetic rats, most of the dextran sulfate was excreted in the urine during the first 24 h in all groups (only a few animals excreted trace amounts of dextran sulfate during the subsequent 24 h). Fig. 4A shows that diabetic rats excreted significantly less dextran sulfate in the urine than their normal controls. At the end of the experiments (10 or 30 days), the rats were killed, and blood, brain, heart, liver, muscle, lung and kidney were analyzed for dextran sulfate and glycosaminoglycans. Dextran sulfate was found only in liver and kidney (Fig. 4B). No dextran sulfate was detected in blood (data not shown), and the diabetic animals accumulated more dextran sulfate in liver than normal controls. Moreover, the molecules that accumulated in the kidney were of higher molecular weights than those present in the urine (Fig. 5), suggesting a molecular size dependence for urinary excretion of dextran sulfate.

In order to further investigate this point, the commercial 8-kDa dextran sulfate, which is polydisperse (5 to 15 kDa), was fractionated by gel filtration chromatography in Sephadex G-50 (Fig. 6). As described in Material and methods, the fractions containing molecules of similar molecular weights were pooled. Four preparations were obtained, with different modal (and range of) molecular weights: 11.5 kDa (9.8 to 15.0 kDa), 9.8 kDa (6.0 to 13.5 kDa), 7.2 kDa (5.5 to 11.0 kDa), and 6.0 kDa (5.0 to 8.0 kDa).

These preparations (3.5 mg) were administered to normal and diabetic rats and their urinary excretion is shown in Fig. 7. A clear correlation between dextran sulfate molecular weight and amounts recovered in the urine is observed for all animals, again suggesting that the renal filtration of this anionic polymer is molecular weight-dependent. Nevertheless, diabetic animals excreted lower proportions of the larger molecules, as compared to their respective controls (Fig. 7). These data suggest that the molecular mass upper limit for renal permselectivity of dextran sulfate was decreased in diabetes mellitus. This difference increased with the duration of diabetes mellitus, but could be observed even in the animals that remained diabetic for only 10 days, again indicating that changes in renal filtration of charged polysaccharides are very early events in the development of diabetic nephropathy.

Conversely, a higher proportion of the high molecular weight dextran sulfates accumulated in the kidney and liver of diabetic animals (Fig. 8).

4. Discussion

We have previously shown that the chronic diabetic state induced by streptozotocin in rats results in marked decrease of urinary glycosaminoglycan excretion, on a daily basis

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Fig. 4. (A) Agarose gel electrophoresis of dextran sulfate excreted in the urine of normal (NL) and diabetic (DM), and total amounts excreted in the urine during the first 24 h by rats that remained diabetic for either 10 or 30 days, and their respective controls. (B) Agarose gel electrophoresis of glycosaminoglycans and dextran sulfate isolated from brain, heart, liver muscle, lung and kidney normal rats, and total amounts found in liver and kidney 48 h after dextran sulfate administration to rats that remained diabetic for either 10 or 30 days and their respective controls. S: mixture of standard glycosaminoglycans: chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS); DexS: dextran sulfate (mean±standard deviation). *P<0.02.
Nephropathy and other glomerular diseases [19]. Also been observed by others in patients with diabetic induction. Decreased excretion of glycosaminoglycans has development of diabetes, observed 2 weeks after diabetes [9]. The onset of this feature was an early event in the development of diabetes, observed 2 weeks after diabetes [9,10]. Heparan sulfate do not come from the kidney [9,10]. Heparan sulfate is present in almost all mammalian tissues but, since it is the main kidney glycosaminoglycan, minor amounts of the urinary heparan sulfate could be originated from the kidney. This is also true for cats [14] and possibly other mammalian species. We have recently shown that chondroitin sulfate is not present in cat kidney and urinary tract, but is found in the cat plasma. Circulating glycosaminoglycans are thought to represent products of the metabolism of connective tissue, en route to catabolism in liver or excretion in the urine. Kittens excrete high amounts of dermatan sulfate and heparan sulfate in the urine (on a daily basis), as reported by Cechowska-Pasko et al. [20], but it could also be due to altered renal permselectivity to negatively charged molecules.

Rats excrete a mixture of chondroitin sulfate, dermatan sulfate and heparan sulfate in the urine. Only trace amounts of dermatan sulfate were found in normal rat kidney, while chondroitin sulfate was not detected, indicating that the urinary chondroitin sulfate and dermatan sulfate do not come from the kidney [9,10]. Heparan sulfate is present in almost all mammalian tissues but, since it is the main kidney glycosaminoglycan, minor amounts of the urinary heparan sulfate could be originated from the kidney. This is also true for cats [14] and possibly other mammalian species. We have recently shown that chondroitin sulfate is not present in cat kidney and urinary tract, but is found in the cat plasma. Circulating glycosaminoglycans are thought to represent products of the metabolism of connective tissue, en route to catabolism in liver or excretion in the urine. Kittens excrete high chondroitin sulfate concentrations, possibly due to high metabolic rates and tissue remodeling in these animals. Moreover, it has been previously shown that part of the chondroitin sulfate administered by either i.p. or i.v. routes to rats appears in the urine, along with its degradation products [21,22]. These data indicate that glycosaminoglycans can be filtered in the kidney, and the changes observed in the urinary excretion of these compounds in diabetes could be due to changes in the renal permselectivity to these molecules.

Although the renal permselectivity for proteins and neutral polysaccharides has been thoroughly investigated [23,24] (reviewed in Ref. [25]), only a few reports on the renal filtration of charged polysaccharides have appeared [11,22,26]. Guimarães and Mourão [22] have shown that, in normal rats, the urinary excretion of sulfated polysaccharides is based on molecular size, but the upper limit for excretion in the urine depends on the structure of the molecules. For dextran sulfates the limit reported by these authors is ~8 kDa, while branched polysaccharides were cleared in the urine only in trace amounts. Nevertheless, diabetic animals were not studied.

Our objective was to further investigate the renal filtration of sulfated polysaccharides in animals that remained diabetic for short periods. In the present paper, we described the urinary excretion of glycosaminoglycans and of dextran sulfates of different molecular weights by rats that remained diabetic for either 10 or 30 days.

All the diabetic animals here studied were hyperglycemic, and did not gain weight during all the experimental period. Proteinuria and creatinine hyperfiltration also appeared. In contrast, all these animals excreted less glycosaminoglycans in the urine (on a daily basis), as compared to their age-matched controls.

When dextran sulfates of different molecular weights were administered by i.p. route to normal and diabetic rats, most of it was excreted in the urine during the first 24 h. This result is in agreement with those obtained by Guimarães and Mourão [22] for normal rats. The proportions recovered in the urine were inversely proportional to the dextran sulfate molecular weight, indicating that the renal filtration of these polyanionic molecules depends on the molecular size. This recovery was never 100%, suggesting accumulation in tissues, excretion in the feces, or partial degradation [27]. Diabetic rats excreted less and accumulated more dextran sulfate in kidney and liver than their respective age-matched controls. These differences, which were statistically significant only for the dextran sulfates of higher molecular weights (>7 kDa), increased with the duration of diabetes.

The apparent paradox between reduced dextran sulfate (and endogenous glycosaminoglycan) urinary excretion and increased protein excretion here reported has also been noticed by other authors, under different experimental

Fig. 5. Polyacrylamide gel electrophoresis of dextran sulfate (DexS) and glycosaminoglycans (GAGs) isolated from rat liver, kidney and urine. A commercial preparation of dextran sulfate (8 kDa) was i.p. injected to rats, and 24-h urine samples were collected for 2 days. Afterwards, some animal tissues and organs were analyzed for glycosaminoglycans and the presence of dextran sulfate, as described in Materials and methods. Dextran sulfate was found only in liver and kidney. Aliquots of the glycosaminoglycans and dextran sulfate extracted from these tissues and also excreted in the urine were submitted to polyacrylamide gel electrophoresis. The figure shows both the gel slab and the densitometry profiles thus obtained.
conditions. In 1982, Michels et al. [26] reported an increase in albumin excretion and a significant decrease in dextran sulfate clearance (50–60%) in rats that remained diabetic for 3 months, treated or not with insulin. Similar results were obtained by Burne et al. [11] with the isolated perfused kidney technique, for rats that remained diabetic for four weeks. The fractional clearance of neutral dextran was not different in diabetic and control kidneys, but dextran sulfate clearance was significantly lower than control.

This suggests the involvement of multiple mechanisms for the renal handling of macromolecules, which could include transglomerular filtration and tubular reabsorption or secretion, in addition to the involvement of renal cells in
the differential processing of dextran sulfate, glycosaminoglycans and proteins.

Recent studies on glomerular permselectivity strongly suggest that charge selectivity does not exist [28,29]. Attempts to measure the glomerular sieving coefficient of albumin in a low temperature perfusion system designed to inhibit cellular processes supported the charge selectivity concept [30], but the system used has the serious drawbacks pointed out by Osicka et al. [31]. There are evidences suggesting that protein exclusion from polyanion membranes is only size-based [32], and size and shape seem to influence the transglomerular passage of both proteins [33] and polysaccharides [22].

On the other hand, there are increasing indications of the involvement of renal cells in the processing of proteins and polysaccharides. Comper et al. [27] have shown partial desulfation of dextran sulfate during kidney filtration. Burne et al. [11] reported a decreased activity of glomerular sulfatases in diabetes, with corresponding lower degrees of dextran sulfate desulfation upon renal passage. This effect was reverted by lysosome-target drugs [34], suggesting that diabetes may induce changes in the lysosomal renal processing. The presence of albumin peptides in the urine of streptozotocin-diabetic rats agrees with this concept [35], signifying that albumin-specific mechanisms may be responsible for the increase in albumin peptides in the urine. Furthermore, changes in the renal proximal tubular cell cytoskeleton have been correlated to albuminuria in diabetes [36].

Our results support the notion of differential renal processing for proteins and sulfated polysaccharides, with the possible involvement of kidney cells. The accumulation of dextran sulfate molecules of larger molecular sizes in diabetic kidney and liver also agrees with the increased glomerular residence time previously observed in perfused kidney [11].

Basement membrane thickening and mesangial expansion characterize the morphological changes in diabetic nephropathy [37–39]. Some authors suggested that thickening is due to accumulation of basement membrane material deposited by successive cell layers [40], while others proposed either a decreased susceptibility of diabetic basement membrane to degradation [41] or overexpression of basement membrane components, such as laminin and type IV collagen [42]. Although we did not find any changes in the kidney heparan sulfate concentration [9], a decrease in glomerular heparan sulfate has been reported by other authors in diabetic nephropathy [1,43]. It is possible that the structural organization of the basement membrane and...
Mesangial matrix leads to a looser arrangement of proteoglycans, affecting the properties of the renal cells. Many authors reported the overexpression of glomerular extracellular matrix components in diabetic nephropathy [44], but the synthesis of these components may be unbalanced. For instance, the expression of type IV collagen $\alpha_3^{(IV)}$, $\alpha_4^{(IV)}$ and $\alpha_5^{(IV)}$ chains is dissociated from the $\alpha_1^{(IV)}$ and $\alpha_2^{(IV)}$ expression in the kidney of diabetic mice [45]. This unbalanced synthesis may affect the type IV collagen assembly and contribute to the basement membrane thickening.

Concerning the mesangium, we have previously shown that cultured mesangial cells synthesize a mixture of dermatan sulfate and heparan sulfate and most of the dermatan sulfate is released to the culture medium [46]. Mesangial cells from diabetic rats incorporate much more $^{35}$S-sulfate in glycosaminoglycans, especially dermatan sulfate, than mesangial cells from normal rats. This increase is proportional to the duration of diabetes, and could be related to altered Golgi or lysosomal function. The dermatan sulfate was secreted to the medium as two proteoglycans, identified as members of the small matrix proteoglycan decorin/biglycan family [47]. Decorin and other extracellular matrix proteoglycans may functionally facilitate the assembly of extracellular matrix. The stimulation of proteoglycan production in mesangial cells obtained from diabetic rats may be related to the accumulation of dermatan sulfate and chondroitin sulfate previously reported [9] and possibly to the mesangial matrix expansion observed in diabetes mellitus. Taken together, these data suggest morphological and biochemical alterations in diabetes that may affect the kidney physiology, leading to diabetic nephropathy. The measurement of the urinary glycosaminoglycans, in concert with protein and creatinine, could be of value in the early diagnostics and follow-up of diabetic nephropathy.

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