Lysosomal enzymes are decreased in the kidney of diabetic rats

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A B S T R A C T

The objective of the present study was to investigate the expression and activities of lysosomal enzymes that act upon proteins and sulfated polysaccharides in diabetic rat kidney. Cathepsins, glycosidases and sulfatases were studied on the 10th (DM-10) and on the 30th (DM-30) day of streptozotocin-induced diabetes mellitus (DM). The activity of cathepsin B, the main kidney cysteine protease, was decreased both in DM-10 and DM-30. Gel filtration chromatography of urinary proteins has shown the prevalence of low molecular weight peptides in normal and DM-10 urine, in contrast to the prevalence of high molecular weight peptides and intact proteins in DM-30. These results show that the decrease in lysosomal proteases could explain, at least in part, the increased albuminuria detected by radial immunodiffusion (RID), due to the excretion of less degraded or intact albumin. Concerning sulfated polysaccharides, the activities of β-glucuronidase, N-acetyl-β-D-glucosaminidase, and N-acetyl-β-D-galactosaminidase were also decreased in DM-30, while aryl sulfatasates did not vary. Increased toluidine blue metachromatic staining of the tissue suggests that the lower activities of glycosidases could lead to intracellular deposition of partially digested molecules, and this could explain the decreased urinary excretion and increased tissue buildup of these molecules. The main morphological changes observed in kidney were proximal convoluted tubules with thinner walls and thinner brush border. Immunohistochemistry revealed that most of cathepsin B was located in the brush border of proximal tubular cells, highlighting the involvement of proximal convoluted tubules in diabetic nephropathy.

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1. Introduction

Diabetic nephropathy is a serious complication of diabetes mellitus (DM) and is the most common cause of end-stage renal disease [1]. It affects approximately one third of patients with type 1 or type 2 DM [2].

The initial development of diabetic nephropathy in type 1 DM generally is indicated by microalbuminuria [3]. The changes in albumin excretion rate have traditionally been attributed to changes in the glomerular permselectivity barrier and intraglomerular pressure, with the assumption that albumin remains intact during filtration and renal passage [4]. However, studies with 3H-albumin in humans have shown that 3H-albumin was metabolized to small fragments during renal passage, and less than 4% of radioactivity was associated with intact albumin. These fragments were not detected by radioimmunoassay (RIA), one of the methods traditionally used to reveal microalbuminuria [5]. In diabetic patients, intact albumin represented up to 55% of total radioactivity, possibly due to an inhibition of the degradation rate, and this inhibition seems to be directly proportional to the level of albuminuria detected by RIA [6].

On the other hand, there are evidences suggesting that proximal convoluted tubules are important elements for albuminuria in streptozotocin (STZ)-type 1 diabetic rats. Using fluorescent-labeled albumin and intravital microscopy, it was shown that diabetic rats presented similar glomerular permeability in the 12th week, while demonstrating significantly less fluorescent albumin in proximal tubule cells in comparison to non-diabetic controls [7]. Also, there are evidences indicating that cubilin, a 460 kDa peripheral membrane protein, is essential for albumin reabsorption by proximal tubule cells, while megalin, a 600 kDa transmembrane protein, drives internalization of cubilin-albumin complexes [8]. The concentration of these two proteins was found to be increased in the urine of type 1 diabetes + microalbuminuria, suggesting that abnormal shedding of megalin and cubilin could contribute to albuminuria [9].

Other aberrant renal processing in DM concerns sulfated polysaccharides. We have previously shown a marked decrease in the excretion of urinary glycosaminoglycans in rats with STZ-DM (expressed as GAGs/24 h), from the second week on [10]. The excretion rate of exogenous dextran sulfates of different molecular weights was also decreased in diabetic rats (10 and 30 days) [11]. In contrast, higher amounts of dextran sulfates, especially those of high molecular weights, accumulated in diabetic kidney and liver, suggesting cellular
Table 1
Body weight, kidney weight, and glycemia of rats 10 days and 30 days after induction of diabetes mellitus (streptozotocin) and their matched controls.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Glycemia (mg/dl)</th>
<th>Kidney weight (g)</th>
<th>Kidney/body weight (× 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Initial</td>
<td>72 h</td>
</tr>
<tr>
<td>NL-10</td>
<td>308 ± 16</td>
<td>326 ± 19</td>
<td>105 ± 9</td>
<td>106 ± 17</td>
</tr>
<tr>
<td>DM-10</td>
<td>313 ± 33</td>
<td>282 ± 32</td>
<td>111 ± 13</td>
<td>424 ± 123</td>
</tr>
<tr>
<td>NL-30</td>
<td>300 ± 22</td>
<td>325 ± 19</td>
<td>108 ± 13</td>
<td>123 ± 25</td>
</tr>
<tr>
<td>DM-30</td>
<td>326 ± 28</td>
<td>247 ± 6</td>
<td>111 ± 10</td>
<td>416 ± 144</td>
</tr>
</tbody>
</table>

* The numbers are mean ± standard deviation. The groups are as follows: NL-10, controls, 10 days; DM-10, diabetic, 10 days; NL-30, controls, 30 days; DM-30, diabetic, 30 days.

2. Materials and methods

2.1. Animals, urine samples, and tissues

The present work was approved by the Ethical Committee of Escola Paulista de Medicina—UNIFESP (CEP 0170/09), and was carried out in accordance with UNIFESP guidelines, and also in accordance with Directive 86/609/EEC for animal experiments http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm.

Male Wistar rats (n = 52), 12 weeks of age (275–360 g body weight), were randomly assigned to one of four groups: control, 10 days (NL-10), diabetes, 10 days (DM-10), control, 30 days (NL-30), diabetes, 30 days (DM-30). Before the induction of DM, the animals were weighed, blood glucose was measured (Advantage Kit Roche, Basel, Swiss), and the animals were placed in metabolic cages for 24 h-urine collection. Diabetes was induced in the 26 rats of DM groups by a single i.p. injection of STZ (60 mg/kg body weight). The drug was dissolved in 300 μl of 10 mM sodium citrate buffer, pH 4.5. These animals were fed standard laboratory chow and a 5% glucose solution ad libitum, for 72 h. Afterwards, the glucose solution was replaced by water. The glycemia was measured 72 h after STZ administration, and also at the end of each experiment (either 10th or 30th day). Only animals that, at 72 h, presented blood glucose higher than 250 mg/dl were considered “diabetic” [16]. The 26 age-matched animals that served as controls (NL-10 and NL30) received only buffer (300 μl) and were fed standard laboratory chow and water ad libitum.

At the end of each experiment, the body weight was again determined, and the rats were placed in metabolic cage for 24 h-urine collection. The 24 h-urine volume was measured, the urine was centrifuged to remove debris, and used for determination of creatinine, total protein, and albumin. Creatinine was quantified by the picric acid reaction in alkaline conditions (CELM creatinine kit, São Paulo, SP, Brazil); total protein was measured by the pyrogallol red-molybdate complex method (Sensiprot, Labtest, Lagoa Santa, MG, Brazil) [17]; albumin was determined by two methods: (1) radial immunodiffusion (RID) based on precipitation with rabbit antibodies against rat albumin [10], and (2) ELISA with Bethyl E110-125 Rat Albumin Quantification Set (Montgomery, TX, USA).

After urine collection, the rats were euthanized, both kidneys were removed, weighed, and carefully cut in small fragments (~100 mg each). These fragments were used for RNA extraction (Section 2.3), measurement of enzyme activities (Section 2.4), Western blotting and

Table 2
24 h-urine volume, urinary creatinine, total protein, and albumin (measured by radial immunodiffusion, RID, and by ELISA) on days 0, 10 and 30 after diabetes mellitus induction (streptozotocin) and their matched controls.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (NL)</th>
<th>Diabetes mellitus (DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>10</td>
</tr>
<tr>
<td>24 h-urine volume (ml)</td>
<td>7.0 ± 0.9</td>
<td>9.1 ± 1.2</td>
</tr>
<tr>
<td>Urinary creatinine (mg/ml)</td>
<td>1.42 ± 0.21</td>
<td>1.31 ± 0.20</td>
</tr>
<tr>
<td>Urinary protein (mg/ml)</td>
<td>1.84 ± 0.27</td>
<td>1.86 ± 0.16</td>
</tr>
<tr>
<td>Urinary protein (mg/24 h)</td>
<td>12.9 ± 2.7</td>
<td>16.8 ± 2.0</td>
</tr>
<tr>
<td>Urinary albumin (RID, mg/24 h)</td>
<td>0.47 ± 0.18</td>
<td>0.66 ± 0.36</td>
</tr>
<tr>
<td>Urinary albumin (ELISA, mg/24 h)</td>
<td>1.12 ± 0.37</td>
<td>1.67 ± 0.94</td>
</tr>
</tbody>
</table>

* The numbers are mean ± standard deviation.

P = 0.05 vs. Day 0.
quantification of total protein (Section 2.2). The kidney fragments were put in sterile tubes, frozen in liquid nitrogen, and stored at $-70 \, ^\circ\text{C}$ until use.

2.2. Gel filtration chromatography and Western blotting

The molecular weight of urinary proteins was estimated by gel filtration chromatography. Urine samples (0.5–2 ml) were applied to a column (1×30 cm) of Sephadex G-100 Superfine, equilibrated and eluted with PBS (0.1 ml/min). The collected fractions (0.72 ml each) were analyzed for creatinine and protein.

Protein was quantified in the tissue extracts and also in the Sephadex G-100 eluted fractions by a modified Lowry procedure with bicinchoninic acid [18] (BCA Protein Kit Assay, Pierce, IL, USA), using bovine serum albumin as standard. Urinary protein was measured by the pyrogallol red-molybdate method because creatinine interferes with the BCA method. BCA was used to measure proteins in Sephadex G-100 fractions because it detects both intact proteins and peptides. Aliquots (10 μl) of the protein-containing fractions were submitted to SDS–PAGE (12%), stained with silver nitrate in alkaline medium [19], and also revealed by Western blotting for albumin (Bethyl A110-134P, 1:10,000). Western blotting was also used to test the specificity of anti-cathepsin B and anti-β-actin antibodies, as previously described [20]. In brief, after SDS–PAGE of tissue extracts, proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking (2% casein in Tris-buffered saline containing 0.05% Tween 20–TBST), membranes were probed with either rabbit polyclonal anti-cathepsin B antibody (1:250, Cat. No. 06-480, Millipore) or rabbit monoclonal anti-β-actin antibody (1:500) in 1% bovine serum albumin (BSA) in TBST. Then, the membranes were washed with TBST and incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:3,000, Millipore). The antibody binding was visualized first through chemiluminescent substrate (ECL Advance Western Blotting Detection Kit, GE Healthcare, Piscataway, NJ, USA), and then tetramethylbenzidine (KPL, Gaithersburg, MD, USA). Images were obtained in MF-ChemiBIS gel documentation system (DNR Bio Imaging Systems Ltd., Jerusalem, Israel) with the software GelCapture 7.0.6 for Windows™.

2.3. RNA extraction, analysis, and qPCR

Each tissue fragment (~100 mg) was disrupted in liquid nitrogen, homogenized in 1 ml of QiAzoL Lysis Reagent (QiAGEN Inc., CA, USA), and processed according to the manufacturer’s instructions (QiAzoL® Handbook, Sample and Assay Technologies, 2009). $A_{260/280}$ and $A_{260/230}$ were measured (ND-1000, NanoDrop, Delaware, USA), and samples with ratios lower than 1.8 and 1.7, respectively, were discarded. The integrity of RNA was also evaluated through 28S and 18S rRNA bands in agarose gel electrophoresis, Tris-borate-EDTA buffer, as previously described [21,22]. To avoid contamination with genomic DNA, RNA samples were treated with DNase I (Fermentas International, Inc., Ontario, Canada) followed by 25 mM EDTA (1 μl/ enzyme unit, 10 min, 65 °C), to inactivate DNase I.

One microgram of RNA was reverse-transcribed to complementary DNA (cDNA) with RevertAid M-MuLV (Fermentas International, Inc., Ontario, Canada). The resulting single-strand cDNA was amplified by qPCR in a reaction mixture containing 0.4 μM of each primer

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**Fig. 1.** Gel filtration chromatography of urinary proteins. Urine samples from normal (NL) and diabetic (DM-10 and DM-30) rats were applied to a Sephadex G-100 Superfine column (1×30 cm), equilibrated and eluted with PBS. Fractions (0.72 ml) were collected at a flow rate of 0.12 ml/min. Panel (A) shows the protein profiles (BCA, A562); panel (B) shows the SDS–PAGE (12%) of aliquots (10 μl) of selected fractions (2, 4, 6, 8, 9, 10, 11, 12, 14), stained by silver nitrate in alkaline medium; panel (C) shows Western blotting revealed for rat albumin of NL and DM-30 urine samples. Molecular weight markers are also shown.
and 7.5 μl of SYBR Green (Rotor-Gene SYBR Green PCR Kit) (15 μl final volume). The thermal cycling conditions were as follows: initial 5 min at 95 °C, followed by 40 – 45 cycles of denaturing at 95 °C for 5 seconds, annealing at 60 °C for 10 seconds, and extension at 95 °C for 10 min. Cycle number and cDNA concentration were adjusted so that amplified products remained within the linear range of the PCR reaction. PCR amplification was conducted on a Corbett Rotor-Gene 6000 (QIAGEN, Inc., CA, USA). Each PCR was done in duplicate.

Relative gene expression was calculated by the 2^−ΔΔC_T method developed by Livak and Schmittgen [23]. In this method, it is assumed that the expression of a reference gene (housekeeping gene) is independent of external factors and that its expression is quite constant. In the present paper, two genes were used as references: ribosomal protein 29S (RPS29) and β-actin (ACTB).

The PCR primers (Bioneer Corporation, Alameda, CA, USA) were as follows: cathepsin B, forward 5′ GCTATCCCTCTGGAGCATGGAAC 3′, and reverse, 5′ GACGGGAGCCATTGACATGGT 3′; cathepsin L, forward 5′ AGGCAATCCAGGCTTGTAATGGAG 3′, and reverse, 5′ CGTAGCCACA GCATACCTAGTC 3′; β-glucuronidase, forward 5′ CAGTGCTTCCAGCGAGGACAGGGA 3′, and reverse 5′ GTAGCTAGCCTCAAAGGGGAG 3′; β-actin forward 5′ GGATGACGATATCGCTGCGCT 3′, and reverse 5′ CTGACCCATACCCACCATCACAC 3′, and S29 ribosomal protein forward 5′ GTCACTAGCGAAGAGCATAGGG 3′, and reverse 5′ CAGGTTAGAC AGTTGGTCTCATGGG 3′.

### 2.4. Enzyme activities

Tissue samples (~100 mg) were disrupted in liquid nitrogen, and resuspended in 1 ml of 50 mM Tris–HCl buffer, pH 7.4, containing 200 mM NaCl and 250 mM sucrose [24] plus 1 ml of 0.2% Triton X-100. After standing for 10 min in an ice bath, debris was removed by centrifugation (12,000 × g, 10 min, 4 °C). Aliquots of the supernatant (100 μl) were stored in sterile tubes at −70 °C until use (tissue extracts).

Protease activities were quantified by fluorometric assays using either carbobenzyo-Phe-Arg-7-amide-4-methylcoumarin (Z-FR-MCA, [Fig. 2. Expression (mRNA) of cathepsin B, cathepsin L, and β-D-glucuronidase in diabetic (DM) and normal (NL) rat kidney. The expression of mRNA was normalized either by ribosomal protein S29 (RPS29) or β-actin (ACTB). The expression of RPS29 varied in diabetes relative to ACTB, indicating that it is not a good housekeeping gene in DM. Data represent mean±standard deviation. Statistically significant differences between NL and DM are shown as P<0.05.
Sigma-Aldrich Corp., St. Louis, MO, USA) or ε-NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA (synthesized by Prof. Dr. Maria A. Juliano) [25], as substrates. These substrates were used to quantify total cysteine-proteases and cathepsin B, respectively. The incubations were carried out in dark microplates (Corning, MA, USA), in 50 mM phosphate buffer, pH 6.3, containing 10 mM EDTA. The enzymes (10 μg of protein) were...
pre-activated by incubation of tissue extract aliquots with 2 mM dithiothreitol (DTT, 10 min, room temperature), and then the substrate was added (20 μM, 200 μl final volume). The fluorescence produced upon hydrolysis of the substrates was measured every 20 seconds in FlexStation 3 (Molecular Devices, CA, USA), using λ excitation = 380 nm and λ emission = 460 nm. The assays were also performed in presence of inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF, inhibitor of serine-proteases), 5 μM E64 (irreversible inhibitor of cysteine-proteases) and 1 μM CA074 (irreversible inhibitor of cathepsin B).

The activities of β-D-glucuronidase, N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-galactosaminidase, and sulfatas, were measured by spectrophotometric assays using the following substrates, respectively: 4-nitrophenyl β-D-glucuronide, 4-nitrophenyl N-acetyl-β-D-glucosaminide, 4-nitrophenyl N-acetyl-β-D-galactosaminide, and 4-nitrophenyl sulfate (Sigma-Aldrich Corp., St. Louis, MO, USA). All assays were performed in microplates, always as triplicates, and the incubation mixtures (150 μl final volume) contained 50 mM sodium acetate buffer, pH 5.0, 2 mM substrate and increasing amounts of tissue extracts (2–30 μl containing 20–120 μg of protein). After 1 h incubation at 37 °C, the 4-nitrophenol released was solubilized by addition of 1 M NaOH (150 μl), and the absorbance was measured immediately at λ405 in a microplate reader (Molecular Devices, CA, USA).

2.5. Histology and immunohistochemistry

For histology and immunohistochemistry, one rat of each group was anesthetized with 10% chloral hydrate (4 ml/kg body weight), and perfused with filtered saline (150 ml, 12 ml/min) followed by 4% formalin in 0.1 M phosphate buffer, pH 7.4 (500 ml, 12 ml/min). The kidneys were removed, cut, dehydrated, embedded in paraffin, and cut in 4 μm sections. These sections were transferred to silane-coated microscope slides and dewaxed as previously described [26]. Kidney sections were stained with either hematoxylin and eosin (H&E) or toluidine blue, and images were obtained with a Zeiss Axiolab microscope (Carl Zeiss Microlmaging GmbH, Göttingen, Germany), equipped with AxioCam MRc digital camera and the software AxioVision.

For immunohistochemistry, the dewaxed slides were transferred into 200 ml of pre-warmed 10 mM sodium citrate buffer, pH 6.0 (95 °C). Antigen retrieval was 30 min at 95 °C, 20 min on the bench, and 5 min in running water. Endogenous peroxidase was blocked by 3% hydrogen peroxide (10 min, 10 times), followed by running water (10 min) and phosphate buffered saline (PBS, 3 min, 3 times). Unspecific protein binding was blocked by 200 μl of 1% bovine serum albumin (BSA, Cat. No. A3059, Sigma-Aldrich, St. Louis, MO, USA) and 2% fetal calf serum in PBS in a moist chamber at room temperature. The primary antibody was rabbit anti-cathepsin B (Cat. No. 06-480, EMD Millipore, Billerica, MA, USA) diluted 1:100 in blocking solution. After overnight incubation at 4 °C and 3 × 5 min washes in PBS, secondary antibody goat anti-rabbit IgG, HRP-conjugate (Cat. No. 12-348, EMD Millipore, Billerica, MA, USA) diluted 1:200 in blocking solution was added. After 90 min and 3 × 5 min washes in PBS, nickel enhanced diaminobenzidine (Cat. No. 54-74-00, HistoMark Orange Peroxidase System, KPL, Inc., Gaithersburg, MD, USA) was used as HRP substrate. After running water, the tissues were counterstained with hematoxylin solution, Harris modified (Cat. No. HHS16, Sigma-Aldrich, St. Louis, MO, USA), 30 sec. The excess of hematoxylin was removed by 0.1% HCl in 70% ethanol, followed by 5 min in running water. Slides were dehydrated and mounted in Entellan (Cat. No.
2.6. Statistical analysis

Statistical analysis was performed using PASW Statistics (SPSS Statistics) for Windows™ (version 18.0.0). Data were evaluated for normality by the Shapiro–Wilk test, and were standardized (z-score) when the parametric distribution was not observed. Bootstrap, a resampling method, was also used to check for the stability of the experimental data. Results are presented as mean ± standard deviation, except for enzyme kinetics, when mean ± 95% confidence interval is shown. Differences between groups were analyzed by ANOVA. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Glycemia, body weight, kidney weight, 24-h urine volume, urinary creatinine, total protein, and albuminuria

Table 1 shows that 10 days and 30 days after STZ injection most of the experimental rats presented increased blood glucose concentrations. Two animals of the DM-10 group did not become diabetic and were excluded, and three animals of the DM-30 group died before the end of the experimental period. In contrast to the control animals (NL-10 and NL-30), which gained weight, all the diabetic rats have shown a progressive and significant decrease in body weight. In contrast, the kidney weight increased in diabetic rats, as well as the kidney/body weight ratios.

Table 2 shows the 24 h-urine volume, and the concentrations of creatinine (colorimetric, picric acid reaction), total protein (measured by the pyrogallol red-molybdate complex colorimetric method), and albumin (measured by RID and ELISA). As expected, the urine volume increased and the creatinine concentration decreased in diabetic animals. The protein concentration also decreased in the urine (expressed as mg/ml), but 24 h protein excretion increased. Urinary albumin also significantly and progressively increased. Although different values were obtained by RID and ELISA, the correlation coefficient of the two methods was very high ($R^2 = 0.9947$).

3.2. Urinary proteins

Urine samples were submitted to gel filtration chromatography in Sephadex G-100, and the eluted fractions were analyzed for creatinine and total protein (Fig. 1-A). Aliquots of the fractions were also submitted to SDS–PAGE (silver staining, Fig. 1-B) and Western blotting for albumin (Fig. 1-C). In normal urine, there was a prevalence of low molecular weight peptides, which were revealed by BCA reaction and SDS–PAGE-silver staining, although intact albumin was also present (Western blotting). Similar results were obtained for DM-10 urine samples. In contrast, in the urine of rats that remained diabetic for 30 days (DM-30), a prevalence of high molecular weight peptides and proteins occurred.

3.3. Quantitative PCR (qPCR) for cathepsin B, cathepsin L and β-glucuronidase

Two housekeeping genes—ribosomal protein S29 ($R_{PS29}$) and β-actin ($ACTB$) were included in the qPCR analysis, and the results obtained for $2^{-\Delta\Delta C_T}$ is shown in Fig. 2. The expression of $R_{PS29}$ decreased in diabetes-30 days relative to $ACTB$, indicating that at least one of them is not a good housekeeping gene. Relative to $R_{PS29}$, an apparent increase of lysosomal enzymes was observed on DM-30. In contrast, no changes occurred in the expression of cathepsin B when the expression was normalized by $ACTB$, while a significant decrease in the relative expression of cathepsin L (10 days) and β-glucuronidase (10 days and 30 days) occurred.

3.4. Activities of lysosomal enzymes

The kinetics of formation of fluorescent product from two different substrates – Z-FR-MCA and ε-NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA – are shown in Fig. 3, either with or without inhibitors (PMSF, CA074 and E64). PMSF inhibits serine-proteases, E64 inhibits cysteine-proteases, and CA074 inhibits only cathepsin B.
PMSF had no effect upon enzymatic activities on the substrates here used, and no enzyme activities were detected in presence of E64, indicating that only cysteine-proteases were acting upon the substrates. Z-FR-MCA was substrate for cathepsin B and also other cysteine-proteases, while ε-NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA was substrate for cathepsin B only.

Fig. 4 shows that cathepsin B was the main cysteine protease present in rat kidney, both normal and diabetic. A decrease in cathepsin B specific activities occurred in diabetic kidneys, both on the 10th and on the 30th days. No changes were observed for other cysteine-proteases (resistant to CA074, Z-FR-MCA as substrate).

Concerning lysosomal glycosidases, a decrease in the specific activities was observed for β-D-glucuronidase, N-acetyl-β-D-glucosaminidase, and N-acetyl-β-D-galactosaminidase only on the 30th day of diabetes. In contrast, the specific activities of aryl sulfatases did not vary (Fig. 5).

3.5. Histology of diabetic kidney

Fig. 6 shows that, in diabetic kidney, the tubular lumen was increased, both on the 10th and on the 30th days, with thinner walls and increased stroma. The brush border of proximal convoluted tubules was also thinner, in comparison to normal controls.

Toluidine blue staining (Fig. 7) shows nucleus (blue) and proteoglycans (metachromatic, purple). A higher intensity of metachromatic staining appeared in diabetic tubules, especially at the 30th day, in comparison to controls.

3.6. Western blotting and immunohistochemistry of cathepsin B

Fig. 8 shows that the expected bands of pro-cathepsin B (~40 kDa) and native cathepsin B (26 and 30 kDa) were detected by Western
Immunohistochemistry for cathepsin B is shown in Fig. 9. Strong labeling appeared in the brush border of proximal convoluted tubules, especially for diabetic kidney.

4. Discussion

The present paper reports decreased activities of lysosomal enzymes on the 10th and the 30th days of DM. It also shows that, in contrast to normal and early DM (10 days), on the 30th day of DM a prevalence of high molecular weight peptides and proteins appeared in the urine, indicating the excretion of less digested proteins.

Although microalbuminuria, which is considered the earliest manifestation of diabetic nephropathy, has been traditionally attributed to changes in the glomerular permselectivity barrier, Comper et al. have published a series of papers suggesting that the glomerular filtration barrier is more leaky to albumin than previously accepted [27]. It was shown that, in normal kidney, albumin is internalized by endocytosis in proximal tubule cells, digested in lysosomes to low molecular weight fragments, and returned to the tubular lumen [28]. Our results on urinary proteins and albumin are in agreement with this idea.

In 2001, using FITC-labeled albumin Tojo et al. have shown that, in the early stages of diabetes mellitus (2 weeks), tubular reabsorption of albumin was decreased in the proximal tubule of diabetic rats via a decrease in endocytosis of albumin associated with decreased expression of megalin [29]. Furthermore, it was shown that the degradation of reabsorbed proteins is inhibited in the proximal tubule of diabetes, via decreased cathepsin B and L activity in lysosomes [5,6]. In 2003, it was shown that angiotensin II blockage restored albumin

**Fig. 7.** Optical microscopy of diabetic (DM) and normal (NL) rat kidney stained by toluidine blue. The experiment was performed as described in Fig. 6, except that the kidney sections were stained by toluidine blue. Arrows indicate metachromatic staining. Bars = 20 μm.

**Fig. 8.** Representative Western blottings of cathepsin B and β-actin extracted from diabetic (DM) and normal (NL) rat kidney. Tissue extracts (3–5 μl containing 30 μg of protein) were submitted to SDS–PAGE (12% acrylamide with 0.32% bisacrylamide for cathepsin B; 7.5% acrylamide with 0.2% bisacrylamide for β-actin). Proteins were transferred to nitrocellulose membranes for Western blottings probed with either rabbit polyclonal anti-cathepsin B or rabbit monoclonal anti-β-actin antibody. Specific bands were detected by secondary antibody (anti-rabbit IgG) conjugated with horseradish peroxidase (HRP) and ECL-chemiluminescent substrate. Images were obtained with MF-ChemiBIS gel documentation system. Each lane represents a pool of four animals of each group.
reabsorption by increasing megalin expression in the early stages of diabetes [30]. In 2011, the colocalization of cathepsin B and Alexa-bovine serum albumin (BSA) in proximal convoluted tubules of normal mice was reported [31]. The same authors have also shown that the intrinsic lysosomal protein Limp-2 (the murine homolog of human SCARB2) plays a role in the fusion of endosomes containing reabsorbed proteins to lysosomes. The megalin/cubilin-dependent uptake of injected Alexa-BSA in Limp-2-deficient mice was similar to normal, but Alexa-BSA persisted in proximal convoluted tubule, and did not colocalize with cathepsin B [31]. Nevertheless, there is no evidence of impaired expression/function of SCARB2/Limp-2 in DM, suggesting that the lower than normal protein digestion here reported is due to decreased lysosomal enzyme activities (mainly cathepsin B) and/or decreased megalin/cubilin-dependent protein uptake, not to decreased endosome/lysosome fusion.

Additionally, decreased urinary excretion of GAGs occurred. Metachromatic staining of tubular cells by toluidine blue suggested build up of GAGs. The activities of all the lysosomal glycosidases here studied – β-D-glucuronidase, N-acetyl-β-D-glucosaminidase, and N-acetyl-β-D-galactosaminidase – were decreased on the 30th day of DM. Although no changes in sulfatase activities were observed in our experimental model, decreased sulfatases in DM isolated glomeruli have been previously reported [32]. Experimental evidences suggest that sulfated polysaccharides are filtered and internalized by kidney cells, although the receptor has not yet been characterized. In DM, due to a lower digestion rate, these compounds accumulate in the tissue (see references [10] for GAGs and [11] for dextran sulfate), possibly in proximal tubule cells as well.

We have previously reported that mesangial cells isolated from long term diabetes mellitus (rats) synthesize and secrete increased amounts of dermatan sulfate proteoglycans, proportional to the time of disease [33]. These proteoglycans are supposed to be deposited in the mesangial extracellular matrix. Now, we report an increase in tubular cell metachromatic staining. The decreased activity of lysosomal enzymes could lead to intracellular accumulation of partially digested macromolecules (including proteoglycans), and these engorged endosomes could impair tubular cell function in many ways, including endocytosis, shedding of receptors, thinning of brush border, etc.

Concerning the expression of cathepsin B and β-glucuronidase assessed by qPCR, different results were obtained relative to the two reference genes here used. Relative to ACTB, the expression of cathepsin B did not vary and β-glucuronidase decreased in DM, while relative to RPS29, an apparent increase occurred on the 30th day (DM-30). Nevertheless, the expression of RPS29 decreased relative to ACTB, indicating that at least one of them is not a good housekeeping gene. As Western blotting did not show any important increase (or decrease) in cathepsin B concentration (protein), and the enzyme activities decreased in diabetic kidney, we considered ACTB as a better...
reference gene. Other gene commonly used as reference is GAPDH (glyceraldehyde 3-phosphate dehydrogenase), but its expression also varies in diabetes [34,35], and could not be used.

The main morphological changes observed in kidney were proximal convoluted tubules with thinner walls and thinner brush border. Immunohistochemistry staining has shown that most of the rat kidney cathepsin B occurred in proximal convoluted tubules. The strong staining observed in diabetic tubules should be due to higher antigen concentration due to brush border thinning. Nevertheless, an increased expression of cathepsin B on the 30th day is also possible, potentially trying to counteract the decreased activity, although no increase has been observed in Western blotting (see Fig. 8).

Taken together, these results show that the decreased lysosomal protease activities could explain, at least in part, the increased albuminuria detected by RID, due to the excretion of less degraded albumin. If the decrease in lysosomal enzymes here reported reflects a general lysosomal failure, it is possible that the lysosome-mediated autophagy, which has been recently recognized to play a renoprotective role under several conditions, could be deficient. This autophagy deficiency in diabetic kidney could make tubular cells fragile under stress, possibly leading to progression of diabetic nephropathy [15].

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