

Streptozotocin-induced diabetes mellitus affects lysosomal enzymes in rat liver

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Abstract

It has been previously shown that dextran sulfate administered to diabetic rats accumulates in the liver and kidney, and this could be due to a malfunction of the lysosomal digestive pathway. The aim of the present study was to evaluate the expression and activities of lysosomal enzymes that act upon proteins and sulfated polysaccharides in the livers of diabetic rats. Diabetes mellitus was induced by streptozotocin in 26 male Wistar rats (12 weeks old), while 26 age-matched controls received only vehicle. The livers were removed on either the 10th or the 30th day of the disease, weighed, and used to evaluate the activity, expression, and localization of lysosomal enzymes. A 50-60% decrease in the specific activities of cysteine proteases, especially cathepsin B, was observed in streptozotocin-induced diabetes mellitus. Expression (mRNA) of cathepsins B and L was also decreased on the 10th, but not on the 30th day. Sulfatase decreased 30% on the 30th day, while glycosidases did not vary (or presented a transitory and slight decrease). There were no apparent changes in liver morphology, and immunohistochemistry revealed the presence of cathepsin B in hepatocyte granules. The decrease in sulfatase could be responsible for the dextran sulfate build-up in the diabetic liver, since the action of sulfatase precedes glycosidases in the digestive pathway of sulfated polysaccharides. Our findings suggest that the decreased activities of cathepsins resulted from decreased expression of their genes, and not from general lysosomal failure, because the levels of glycosidases were normal in the diabetic liver.

Key words: Liver; Diabetes mellitus; Lysosomal enzymes; Cathepsin; Glycosidase; Sulfatase

Introduction

Streptozotocin-induced diabetes mellitus (STZ-DM) in rats leads to a marked decrease in the urinary excretion of glycosaminoglycans (1). Decreased urinary excretion of exogenous dextran sulfate also occurs in STZ-DM, with accumulation of dextran sulfate in liver and kidney (2). Because dextran sulfate is very soluble in water, its presence in liver and kidney 48 h after administration suggests intracellular localization of these molecules.

The concentration of glycosaminoglycan also increased in diabetic kidney (2,3), and there is evidence that both anabolic and catabolic pathways are involved (4,5). Decreased expression of lysosomal enzymes in diabetic kidney was recently reported (5), and autophagy was recently proposed as a therapeutic target for diabetic nephropathy (6).

Lysosomal enzymes are the main agents of the digestive process that takes place in autophagic vacuoles (reviewed in Ref. 7). Even though much of the pioneering

research on autophagy comes from 40 years of studies on liver and isolated hepatocytes (8), and although lysosomes were discovered back in the 1950s by Christian de Duve (9), who also maintained a lifetime interest in the actions of insulin and glucagon on the liver (10), there are few studies on the expression and activities of lysosomal enzymes in the diabetic liver.

Most of the recent work on liver autophagy has focused on lipid digestion (lipophagy) (11,12) and glycogen digestion (13). There are few studies of liver lysosomal enzymes that act upon proteins and sulfated polysaccharides. The aim of the present study was to investigate the expression and activities of lysosomal hydrolases in the liver of rats with STZ-DM, in order to elucidate the mechanisms responsible for the dextran sulfate build-up. Two different stages of the disease were studied: 10 days (initial diabetic state) and 30 days (diabetic nephropathy).

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Material and Methods

Animals, urine, and tissue

The Ethics Committee of Escola Paulista de Medicina, UNIFESP approved the present research (CEP No. 0170/09), which was carried out in accordance with UNIFESP guidelines, and also in accordance with EC Directive 2010/63/EU for animal experiments.

Male Wistar rats ($n = 52$), 12 weeks of age (275-360 g body weight), were randomly assigned to one of four groups: control day 10, diabetes (DM) day 10, control day 30, and diabetes day 30. Before the induction of DM, the animals were weighed, blood glucose was measured (Advantage Kit Roche, Switzerland), 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 intraperitoneal injection of 60 mg/kg body weight STZ. The drug was dissolved in 300 μL 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. Afterward, the glucose solution was replaced by water. 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" (14). The 26 age-matched animals that served as controls received only 300 μL buffer and were fed standard laboratory chow and water *ad libitum*.

At the end of each experiment, body weight was again measured, and the rats were placed in metabolic cages for 24-h urine collection. The 24-h urine volume was measured and the urine was centrifuged at 1000 g for 10 min at room temperature to remove debris and used for determination of creatinine, total protein, and albumin. Creatinine was quantified by the picric acid reaction under alkaline conditions (CELM creatinine kit, Brazil), total protein was measured by the pyrogallol red-molybdate complex method (Sensiprot, Labtest, Brazil) (15), and albumin was determined by two methods: radial immunodiffusion based on precipitation with rabbit antibodies against rat albumin (1), and ELISA using a Bethyl E110-125 Rat Albumin Quantification Set (USA). The results obtained for total protein and creatinine were published in Peres et al. (5). After the urine was collected, the rats were killed, and the livers were removed, weighed, and carefully cut into small fragments (~100 mg each). These fragments were used for RNA extraction, measurement of enzyme activities, Western blotting, and quantification of total protein. The liver fragments were put into sterile tubes, frozen in liquid nitrogen, and stored at -70°C until use.

Liver enzyme activities

To measure the enzyme activities, liver samples (~100 mg) were disrupted in liquid nitrogen and resuspended in 1 mL 50 mM Tris-HCl buffer, pH 7.4, containing 200 mM NaCl and 250 mM sucrose (16) plus 1 mL

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), and 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 carbobenzoxy-Phe-Arg-7-amide-4-methylcoumarin (Z-FR-MCA; Sigma-Aldrich, USA) or ϵ -NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA (synthesized by Prof. Dr. Maria A. Juliano) (17) as substrates. These substrates were used to quantify total cysteine proteases and cathepsin B, respectively. Incubation was carried out in dark microplates (Corning, USA), in 50 mM phosphate buffer, pH 6.3, containing 10 mM EDTA. The enzymes (50 μg protein) were preactivated by incubation of tissue extract aliquots with 2 mM dithiothreitol (10 min, room temperature), and the substrate was then added (20 μM , 200 μL final volume). The fluorescence produced upon hydrolysis of the substrates was measured every 20 s in a FlexStation 3 microplate reader (Molecular Devices, USA), using $\lambda_{\text{excitation}} = 380$ nm and $\lambda_{\text{emission}} = 460$ nm. The assays were also performed in the presence of the following 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 sulfatases were measured by spectrophotometric assays using the following substrates: 4-nitrophenyl β -D-glucuronide, 4-nitrophenyl *N*-acetyl- β -D-glucosaminide, 4-nitrophenyl *N*-acetyl- β -D-galactosaminide, and 4-nitrophenyl sulfate, respectively (Sigma-Aldrich). All assays were performed on 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 protein). After 1 h of incubation at room temperature, the 4-nitrophenol released was solubilized by addition of 1 M NaOH (150 μL), and the absorbance was measured immediately at λ_{405} in a microplate reader (Molecular Devices).

Quantification of liver protein and Western blotting

Protein was quantified in tissue extracts by a modified Lowry procedure with bicinchoninic acid (18) (BCA Protein Assay Kit, Thermo Scientific Pierce, USA), using bovine serum albumin as standard.

The specificity of anti-cathepsin B and anti- β -actin antibodies was evaluated by Western blotting (19). Tissue extracts (1.5-2.5 μL containing 30 μg 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 and 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 enhanced chemiluminescence (ECL) substrate. Images were obtained with the MF-ChemiBIS gel documentation system, where each lane represents a pool of four animals for each group.

Color images (tetramethylbenzidine; KPL, USA) and chemiluminescence images (ECL Advance Western Blotting Detection Kit; GE Healthcare, USA) were obtained with the MF-ChemiBIS gel documentation system (DNR Bio Imaging Systems Ltd., Israel) with the GelCapture 7.0.6 software for Windows™.

RNA extraction and real-time reverse transcriptase-polymerase chain reaction (PCR)

Each liver fragment (~100 mg) was disrupted in liquid nitrogen, homogenized in 1 mL QIAzol Lysis Reagent (QIAGEN, USA), and processed according to the manufacturer's instructions. Absorbances ($A_{260/280}$ and $A_{260/230}$) were measured (ND-1000, NanoDrop, USA), and samples with ratios lower than 1.8 and 1.7, respectively, were discarded. RNA integrity was also evaluated from 28S and 18S rRNA bands after agarose gel electrophoresis using Tris-borate-EDTA buffer, as previously described (20,21). To avoid contamination with genomic DNA, RNA samples were treated with DNase I (1 U/ μ g RNA, 30 min, 37°C; Fermentas International, Canada) followed by 25 mM EDTA (1 μ L/enzyme unit, 10 min, 65°C) to inactivate the DNase I.

One microgram of RNA was reverse-transcribed to complementary DNA (cDNA) with RevertAid M-MuLV (Fermentas International), and the resulting single-strand cDNA was amplified by quantitative PCR (qPCR) in a reaction mixture containing 0.4 μ M of each primer and 7.5 μ L SYBR Green (15 μ L final volume; Rotor-Gene SYBR Green PCR Kit, QIAGEN). 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 s, annealing at 60°C for 10 s, 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. PCR amplification was conducted on a Corbett Rotor-Gene 6000 (QIAGEN). Each PCR was done in duplicate.

Relative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ method developed by Livak and Schmittgen (22). 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*). Other genes were tested, but they varied in the diabetic liver, compared with controls, and therefore were not used. The PCR primers (Bioneer Corp., USA) are shown in Table 1.

Histology and immunohistochemistry

For histology and immunohistochemistry, one rat in 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 liver was removed, cut, dehydrated, embedded in paraffin, and cut in 4- μ m sections. These sections were transferred to silane-coated microscope slides and dewaxed as previously described (23).

Liver sections were stained with either hematoxylin and eosin or toluidine blue, and images were obtained with a Zeiss Axiolab microscope (Carl Zeiss MicroImaging GmbH, Germany), equipped with an AxioCam MRc digital camera and AxioVision software.

For immunohistochemistry, the dewaxed slides were transferred to 200 mL prewarmed 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 under 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). Nonspecific protein binding was blocked by 200 μ L 1% bovine serum albumin (Cat. #A3059, Sigma-Aldrich) and 2% fetal calf serum in a moist chamber at room temperature. The primary antibody was rabbit anti-cathepsin B (Cat. #06-480; EMD Millipore, USA) diluted 1:100 in blocking solution. After an overnight incubation at 4°C and three 5-min washes in PBS, HRP-conjugated goat anti-rabbit IgG, secondary antibody (Cat. #12-348, EMD Millipore) diluted 1:200 in blocking solution was added. After a 90-min incubation and three 5-min washes in PBS, nickel-enhanced diaminobenzidine (Cat. #54-74-00, HistoMark Orange Peroxidase System; KPL) was used as HRP substrate. Then, the tissue sections were rinsed under running water and counterstained by Harris modified hematoxylin solution (Cat. #HHS16, Sigma-Aldrich) for

Table 1. PCR primers.

Enzyme	Forward	Reverse
Cathepsin B	5'-GCTATCCCTCTGGAGCATGGAAC-3'	5'-GACGGGAGCCATTGACATGGT-3'
Cathepsin L	5'-AGGCAATCAGGGCTGTAATGGAG-3'	5'-CGTTAGCCACAGCATACTCAGCTC-3'
β -D-glucuronidase	5'-CAGTGCTTCCACAGCGATGGA-3'	5'-GTGATGTCAGCCTCAAAGGGGAG-3'
β -actin	5'-GGATGACGATATCGCTGCGCT-3'	5'-CTGACCCATACCCACCATCACAC-3'
Ribosomal protein S29	5'-GTCAGTACGCGAAGGACATAGGC-3'	5'-CAGGGTAGACAGTTGGTTTCATTGGG-3'

Table 2. Body and liver weights, and glycemia of rats 10 and 30 days after streptozotocin-induced diabetes mellitus (DM) and their matched controls.

	10 Days		30 days	
	Control (n = 13)	DM (n = 11)	Control (n = 13)	DM (n = 10)
Body weight (g)				
Initial	307 ± 15	313 ± 33	300 ± 22	326 ± 28
Final	326 ± 19	282 ± 32*	325 ± 19	247 ± 6*
Glycemia (mg/dL)				
Initial	105 ± 9	111 ± 13	108 ± 13	111 ± 10
Final	116 ± 28	461 ± 108*	108 ± 21	456 ± 81*
24-h urine volume (mL)	9.1 ± 1.2	97.0 ± 8.4*	8.0 ± 2.1	84.6 ± 11.9*
Urinary albumin (mg/24 h)				
RID	0.66 ± 0.36	0.89 ± 0.19	0.48 ± 0.23	2.70 ± 1.85*
ELISA	1.67 ± 0.94	2.03 ± 0.60	1.31 ± 0.70	7.78 ± 2.95**
Liver weight (g)	8.9 ± 0.7	10.3 ± 1.5	9.9 ± 0.7	9.3 ± 1.3
Liver/body weight ($\times 10^{-2}$)	2.81 ± 0.33	3.35 ± 0.24	3.33 ± 0.20	3.79 ± 0.10*

Data are reported as means \pm SD. RID: radial immunodiffusion assay. *P<0.05 DM vs matched control (*t*-test). **P<0.01 vs matched control (*t*-test).

30 s. Excess hematoxylin was removed by 0.1% HCl in 70% ethanol, followed by a 5-min rinse under running water. Slides were dehydrated and mounted in Entellan (Cat. #14800, Merck, Germany) and sealed with clear nail polish. Negative controls were incubated with blocking solution at each step.

Statistical analysis

Statistical analysis was performed with the PASW Statistics software (SPSS Statistics) for Windows™ (version 18.0.0). The Shapiro-Wilk test was used to evaluate data for normality, and data 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 reported as means \pm SD, except for enzyme kinetics, when means \pm 95% confidence interval are reported. Differences between groups were analyzed by ANOVA. Values of P<0.05 were considered to be significant, except where otherwise stated.

Results

Glycemia, albuminuria, and liver weight

The body weight of the animals at the beginning (day zero) and at the end (either 10th or 30th day) of the experiment, glycemia, urine volume, albuminuria, and liver weight are reported in Table 2. Two animals of the DM day 10 group did not become diabetic (glycemia <250 mg/dL) and were excluded, and three animals of the DM day 30 group died before the end of the experimental period. In contrast to normal controls, which gained weight during the experimental period, all the diabetic rats showed a progressive and significant loss of body weight. In the

controls, glycemia was maintained in the normal range during the entire experimental period, but in the diabetic rats glycemia, as well as urine volume, were very high on days 10 and 30. Significant albuminuria (evaluated by two different methods) appeared only on day 30. Liver weight did not vary in the diabetic rats, compared with the controls, but liver/body weight increased on day 30.

Activity of liver proteases

The kinetics of formation of fluorescent product (MCA) from two different substrates [Z-FR-MCA and ϵ -NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA] is shown in Figure 1. The kinetics was measured in the presence and absence of the inhibitors PMSF, E64, and CA074, which inhibit serine proteases, cysteine proteases, and cathepsin B, respectively. PMSF had no effect on enzymatic activities of the substrates used here. In contrast, enzyme activities were very low in the presence of E64, indicating that cysteine proteases were the main enzymes acting on the substrates. Z-FR-MCA was the substrate for cathepsin B and also for other cysteine proteases, while ϵ -NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA was the substrate mainly for cathepsin B (inhibited by CA074).

Cathepsin B corresponds to about one-half of total cysteine proteases in rat liver, both normal and diabetic (Figure 2), and all cysteine protease activities were decreased in the diabetic liver compared with normal activity, on both the 10th and 30th days.

Activities of glycosidases and sulfatase

In contrast to the proteases, only the specific activity of β -D-glucuronidase was decreased, and only on the 10th day of STZ-DM (Figure 3). The specific activities of *N*-acetyl- β -D-glucosaminidase and *N*-acetyl- β -D-galactosaminidase

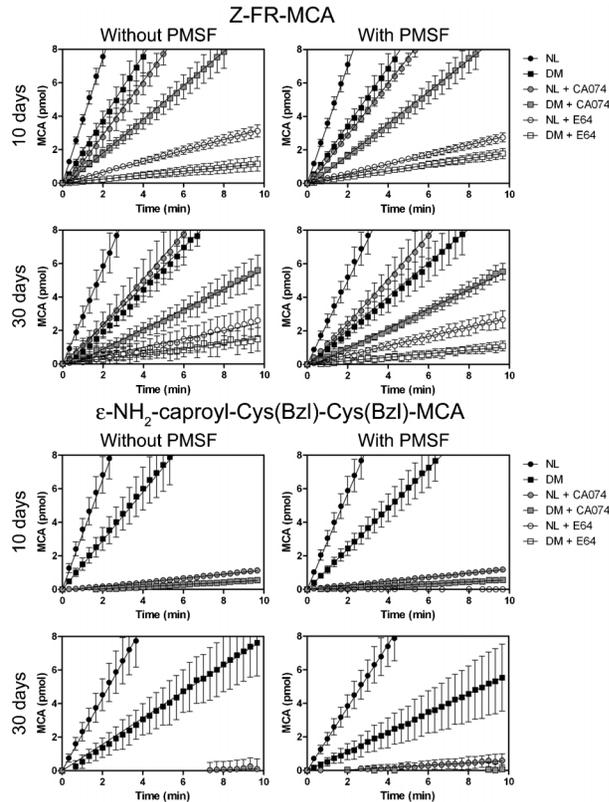


Figure 1. Kinetics of cysteine protease activities in diabetic (DM) and normal (NL) rat livers. Tissue extracts (2-6 μ L containing 50 μ g protein) were preactivated by incubation of tissue extract aliquots with 2 mM dithiothreitol (10 min, room temperature). The following substrates were then added (20 μ M, 200 μ L final volume): Z-FR-MCA (substrate for cysteine proteases), or ϵ -NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA (substrate for cathepsin B). The fluorescence produced upon hydrolysis of the substrates was measured every 20 s in FlexStation 3 (Molecular Devices, USA), using $\lambda_{exc} = 380$ nm and $\lambda_{emi} = 460$ nm. The assays were also performed in the presence of inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF, inhibitor of serine-proteases), 5 μ M E64 (irreversible inhibitor of cysteine-proteases), and 1 μ M CA074 (irreversible inhibitor of cathepsin B). A maximum of 10% substrate consumption was considered, and each point represents the mean \pm 95% confidence interval of four replicates for all animals in each group.

did not vary in the diabetic liver, compared with normal activity. Decreased specific activity of sulfatases was observed only on the 30th day of DM.

Liver expression (mRNA) of cathepsin B, cathepsin L, and β -D-glucuronidase (qPCR)

The expression of three lysosomal enzymes (cathepsin B, cathepsin L, and β -D-glucuronidase) was analyzed by qPCR in normal and diabetic rat liver. The results are reported as $2^{-\Delta\Delta C_T}$ relative to the two housekeeping genes, *RPS29* and *ACTB* (Figure 4).

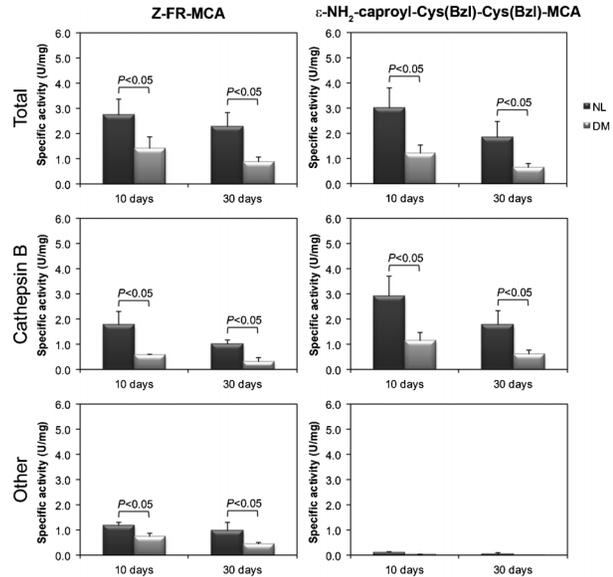


Figure 2. Specific activities of cysteine proteases in diabetic (DM) and normal (NL) rat livers. The assays were performed as described in Figure 1, except that specific activities are shown (enzyme units/mg protein, U/mg). The following substrates were used: Z-FR-MCA (substrate for cysteine proteases), or ϵ -NH₂-caproyl-Cys(Bzl)-Cys(Bzl)-MCA (substrate for cathepsin B). One enzyme unit was defined as the amount of enzyme that produces 1 nmol product/min. Data are reported as means \pm SD. Statistically significant differences between NL and DM are reported as $P < 0.05$ (ANOVA).

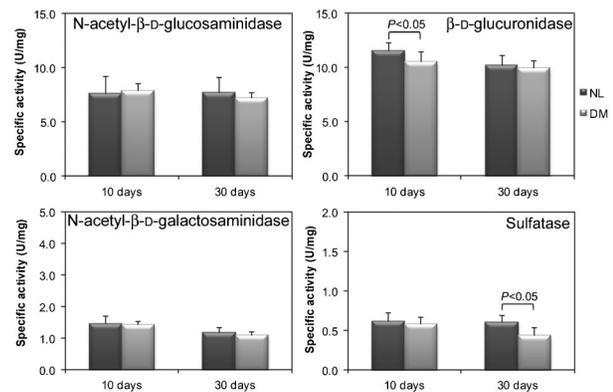


Figure 3. Specific activities of glycosidases and sulfatases in diabetic (DM) and normal (NL) rat livers. Tissue extracts (1-14 μ L containing 20-120 μ g protein) were incubated with p-nitrophenyl monosaccharides or p-nitrophenyl sulfate. One enzyme unit (U) was defined as the amount of enzyme that produces 1 nmol product/min. Data are reported as means \pm SD. Statistically significant differences between NL and DM are reported as $P < 0.05$ (ANOVA).

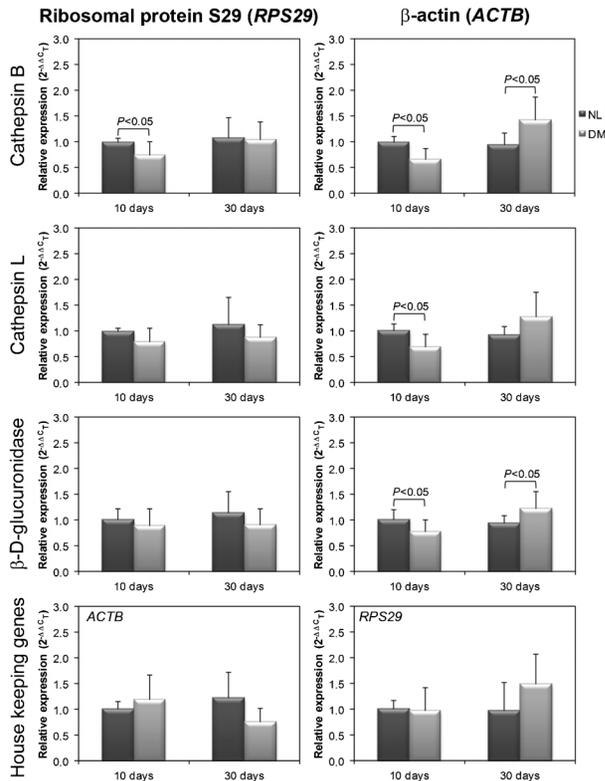


Figure 4. Expression (mRNA) of cathepsin B, cathepsin L, and β -D-glucuronidase in diabetic (DM) and normal (NL) rat livers. The expression of mRNA was normalized either by ribosomal protein S29 (*RPS29*) or β -actin (*ACTB*). Data are reported as means \pm SD. Statistically significant differences between NL and DM are reported as $P < 0.05$ (ANOVA).

The relative expression of the two housekeeping genes used here did not vary on the 10th day of DM compared with controls, indicating that both can be used as reference. Nevertheless, on the 30th day, the expression of *ACTB* decreased (relative to *RPS29*) or *RPS29* increased (relative to *ACTB*) in DM (Figure 4), indicating that at least one of them is not a good reference gene.

On the 10th day of DM, relative to *ACTB*, expression of the three enzymes decreased, whereas, relative to *RPS29*, only the expression of cathepsin B decreased. In contrast, on the 30th day, relative to *ACTB*, the expression of cathepsin B and β -D-glucuronidase increased, whereas, relative to *RPS29*, they did not vary. It is possible that the observed increase in expression of cathepsin B and β -D-glucuronidase relative to *ACTB* is only apparent, since *ACTB* decreased relative to *RPS29*. Furthermore, this apparent increase does not correlate to the specific activities of the enzymes (see Figures 2 and 3), suggesting that, in the diabetic liver, *RPS29* is a better housekeeping gene than *ACTB*.

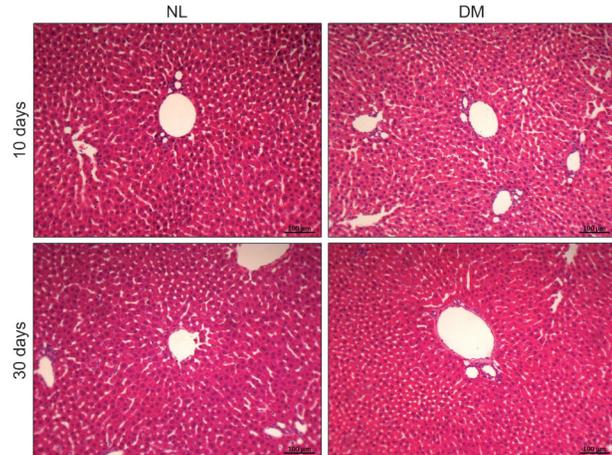


Figure 5. Optical microscopy of diabetic (DM) and normal (NL) rat livers. Tissue samples from normal and diabetic livers (10 and 30 days of diabetes) were included in paraffin, cut into 4- μ m sections, and stained with hematoxylin and eosin. No significant changes in liver morphology were observed. Magnification bar: 100 μ m.

Histology, immunohistochemistry, and Western blotting

No changes were observed in the general histological organization of the tissue (Figure 5). Figure 6 shows that, upon toluidine blue staining, metachromatic cells appeared both in normal and diabetic liver (perivascular), and cytoplasmatic granules appeared in all hepatocytes, both normal and diabetic. Tiny granules were also stained by immunohistochemistry for cathepsin B (Figure 7). The specificity of the antibodies was analyzed by Western blotting, which revealed the expected bands of pro-cathepsin B (~40 kDa) and native cathepsin B (26 and 30 kDa).

Discussion

Although much of the pioneering research on autophagy comes from studies in liver, and lysosomes were first described in liver (9), there are few studies on the expression and activities of lysosomal enzymes that act upon protein and sulfated polysaccharides in the diabetic liver. In 1974, Amherdt et al. (24) reported an increase in hepatic lysosome volume in severe diabetes induced by 100 mg/kg STZ, primarily due to autophagosomes. In 1978, Dice et al. (25) showed that the degradation of proteins is accelerated in the liver of insulin-deficient rats, and, in 1988, Jordá et al. (26) reported a decrease in the half-life of liver mitochondrial ATPase in the severe diabetic state, possibly associated with hepatic autophagy. These data indicate that the liver autophagy pathway seems to be implicated in diabetic complications. Nevertheless, the activities and expression of lysosomal enzymes were not measured.

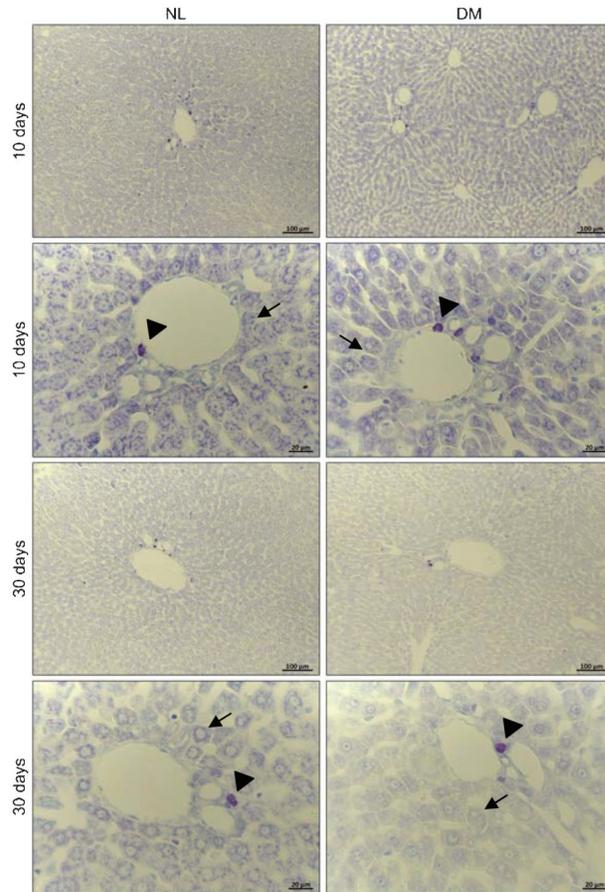


Figure 6. Optical microscopy of diabetic (DM) and normal (NL) rat livers. The experiment was performed as described in Figure 5, except that the liver sections were stained by toluidine blue. Note the metachromatic cells around vessels (arrowheads), and the cytoplasmic granules (arrows) in hepatocytes.

In this article, we report a significant decrease in the specific activities of cysteine proteases, especially cathepsin B, in the liver of rats with STZ-DM, on the 10th and 30th days of the disease. The decrease in liver cysteine protease activities was much greater than that of kidney (liver: 50-60% decrease; kidney: 11-15% decrease, see Ref. 5 for comparison), although in liver the specific activities of these enzymes were much lower (liver: 2.3-2.7 U/mg, Figure 2; kidney: 12.5-14.1 U/mg) (5). Decreases in the expressions (mRNA) of cathepsin B and cathepsin L were also observed on the 10th day after DM induction, suggesting that the lower gene expression may be one of the mechanisms responsible for the lower enzyme activities.

Conversely, the picture concerning sulfated polysaccharides was very different. The specific activities of most glycosidases were higher in liver than in kidney (5) as follows: β -D-glucuronidase, liver: 10.2-11.5, kidney: 2.6-3.1 U/mg; *N*-acetyl- β -D-glucosaminidase, liver: 7.7, kidney: 3.5-3.9 U/mg; *N*-acetyl- β -D-galactosaminidase, liver: 1.1-1.5, kidney:

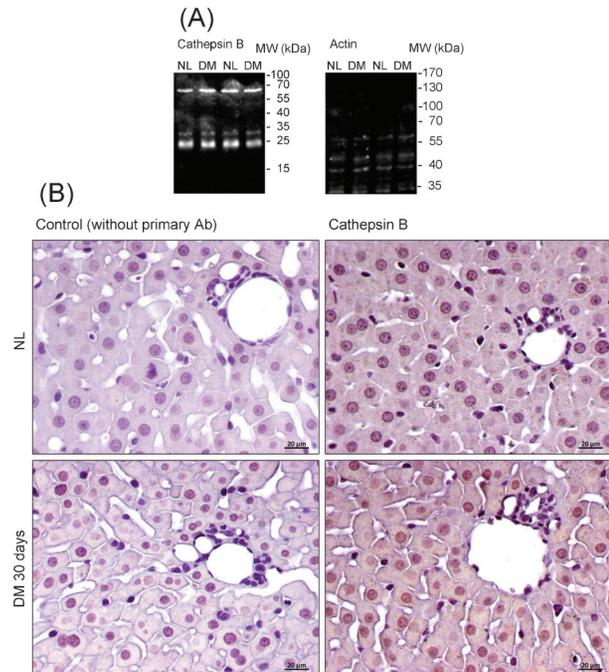


Figure 7. Western blotting and immunohistochemistry of cathepsin B in diabetic (DM) and normal (NL) rat livers. *A*, Tissue extracts (1.5-2.5 μ L containing 30 μ g protein) were submitted to SDS-PAGE (12% acrylamide with 0.32% bisacrylamide for cathepsin B; 7.5% acrylamide with 0.2% bisacrylamide for β -actin). Proteins were transferred to nitrocellulose membranes for Western blotting, and 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 substrate. Images were obtained with the MF-ChemiBIS gel documentation system. Each lane represents a pool of 4 animals of each group. *B*, Tissue sections were incubated with primary rabbit polyclonal anti-cathepsin B antibody. Controls were incubated with blocking solution, without any primary antibody. Anti-rabbit IgG conjugated with HRP was used as secondary antibody, and nickel enhanced diaminobenzidine was used as HRP substrate. Tissues were counterstained with hematoxylin. Cathepsin B-containing structures are brownish, while hematoxylin-stained nuclei are purple. Magnification bars: 20 μ m.

2.2-2.5 U/mg. Nevertheless, in contrast to kidney, most activities did not vary in STZ-DM relative to control. A transitory and slight decrease in the expression and activity of β -D-glucuronidase (8%) was observed only on the 10th day of diabetes, while the activities of *N*-acetyl- β -D-glucosaminidase and *N*-acetyl- β -D-galactosaminidase did not vary.

It is noteworthy that the specific activity of liver sulfatase was found to have decreased 30% on the 30th day after DM induction. This decrease could be responsible for the previously reported accumulation of dextran sulfate in the diabetic liver (2), since the action of sulfatases precedes the action of glycosidases in the digestive pathway of sulfated polysaccharides (27).

There were no apparent changes in liver morphology, suggesting that the observed effects on lysosomal proteases and sulfatases were not a consequence of STZ toxicity. Immunohistochemistry for cathepsin B showed cytoplasmatic granules in hepatocytes, corroborating the lysosomal location for these enzymes. The specificity of the antibodies was confirmed by Western blotting, which revealed the presence of the expected bands. Toluidine blue staining also showed cytoplasmatic granules in hepatocytes, both in normal and diabetic liver.

Taken together, our results suggest that STZ-DM leads to decreased cathepsin and sulfatase activities in liver. It seems that this decrease is not a consequence of general lysosome failure, since the activities of glycosidases did not concurrently decrease in the diabetic liver. Also, it is not a consequence of general STZ liver toxicity, since no histological changes were visible.

Degradation of sulfated polysaccharides is usually initiated by endoglycosidases (matrix or lysosomal), which produce sulfated poly- and oligosaccharides as products, and these are the substrates for sulfatases. Only desulfated products are susceptible to the action of

exoglycosidases that remove specific sugars, one by one, from the nonreducing end of the molecule. If one of these lysosomal enzymes is reduced (or absent), partially degraded molecules accumulate in lysosomes, and may impair cell function. Stuffed lysosomes may appear as cytoplasmatic granules in histological analysis, especially with toluidine blue staining.

The decrease in sulfatase activity observed in the present study explains the previously reported (2) dextran sulfate build-up in the diabetic liver.

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