Kidney International, Vol. 37 (1990), pp. 918–926

Effect of low molecular weight proteins and dextran on renal cathepsin B and L activity

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Effect of low molecular weight proteins and dextran on renal cathepsin B and L activity. Renal extraction of low molecular weight proteins (LMWP) accounts for 30% to 80% of their total metabolic clearance. Extraction includes glomerular filtration, proximal tubular uptake, and intralysosomal proteolysis. To characterize the anatomic sites and enzymes involved in digestion of reabsorbed LMWP, the lysosomal proteases, cathepsin B and L, were measured by ultramicroassay in isolated S1, S2 and S3 segments of the proximal tubule of proteinuric rats. Increased glomerular filtration and tubular uptake of LMWP were induced by i.v. and i.p. injections of myoglobin and cationic and anionic lysozyme. Both cationic lysozyme and myoglobin increased cathepsin B and L activities in the proximal tubule, while anionic lysozyme had no effect. Morphologic examination of kidney tissue suggested that proximal tubular uptake of anionic lysozyme was negligible in comparison with the cationic form. Hence, only LMWP absorbed by the proximal tubule cells stimulated cathepsin B and L activities. Proximal tubular uptake of cationic lysozyme was determined by measurement of lysozyme activities in S1, S2, and S3. S1 segments contained the highest lysozyme activity, while S2 and S3 had much lower activities, and cathepsin B and L activity following cationic lysozyme injection was stimulated only in S1 segments. These results suggest that cathepsin B and L participate in lysosomal digestion of certain LMWP. Furthermore, the activities of cathepsin B and L adapt to increased uptake of LMWP. To gain additional insight into the mechanism of cathepsin adaptation, the cathepsin B and L activities were measured following injection of dextran with a similar low molecular weight. Dextran uptake in proximal tubules was confirmed by morphologic examination of kidney tissue. Dextran increased cathepsin B and L activities in the proximal tubule. Hence, increased endocytic activity of proximal tubule cells or increased lysosomal load of macromolecules or both rather than direct protein-enzyme interaction seem to be involved in cathepsin stimulation.

The kidney is a major determinant of plasma concentrations and turnover rates of circulating proteins with molecular weights less than that of albumin. The renal extraction of these low molecular weight proteins (LMWP) accounts for 30% to 80% of their total metabolic clearance rate. The route of extraction includes glomerular filtration, uptake in proximal tubules by means of adsorptive endocytosis, and proteolytic digestion within the lysosomal system. The tubular uptake is characterized by a low affinity and a high capacity. The transport maximum for LMWP in the kidney is in the range of 50 to 200 times larger than normal filtered loads [1]. In order to maintain a steady state between tubular uptake of LMWP and proteolytic digestion and to avoid tubular accumulation, the proteolytic activity in the proximal tubule should increase as protein uptake increases.

In a recent study [2] we raised the likely possibility that the lysosomal proteases, cathepsin B and L, are involved in intralysosomal degradation of proteins reabsorbed by the proximal tubule. Hence, to evaluate this hypothesis further, the activities of cathepsin B and L were measured in microdissected S1, S2, and S3 segments of rat proximal tubule following injection of the animals with either cationic lysozyme or myoglobin. Lysozyme and myoglobin are LMWP with molecular weights of 14,600 daltons and 16,900 daltons, respectively. Either intravenous or intraperitoneal injection increases both the filtered load and the proximal uptake of cationic lysozyme and myoglobin [3, 4]. It is well known that the tubular absorption of anionic lysozyme is negligible in comparison with the cationic form [1]. Hence, animals injected with anionic lysozyme were studied also. The difference in tubular uptake of cationic versus anionic lysozyme was evaluated by morphologic examination of kidney tissue. To evaluate the relation between lysosomal protein load and cathepsin stimulation along the proximal tubule, the activity of cationic lysozyme in different segments of the proximal tubule was determined. Finally, to gain additional insight into the nature of the stimulus for cathepsin activity, rats were injected with neutral dextran (molecular wt 20,000 daltons). Dextran, which served as a non-protein macromolecular control, is filtered by the glomerulus, is taken up into proximal tubule cells by endocytosis, and is transferred to the lysosomes [5].

Methods

Female Sprague-Dawley rats weighing 120 to 230 g were studied. The animals had free access to standard rat chow (Purina) and tap water.

Experimental protocols

Lysozyme injection. Seven rats received injections of cationic lysozyme (Sigma Chemical Co., St. Louis, Missouri, USA;

Received for publication January 13, 1988
and in revised form September 27, 1989
Accepted for publication October 5, 1989

Grade I) via the i.p. route at a dose of 1 mg/g body weight. Another seven rats received i.p. injections of anionic lysozyme in a dose of 1 mg/g body weight. Thirteen control rats received i.p. injections of 0.9% saline. Cathepsin activities were measured 18 hours following injection. The dose of lysozyme was dissolved in 1 ml of 0.9% saline. Additional rats received either cationic ($N = 6$) or anionic ($N = 6$) lysozyme (1 mg/g body wt) or vehicle only ($N = 6$), after which the animals were placed in metabolic cages for measurement of their endogenous creatinine clearance. Cathepsin activities were not measured in these animals. An additional 13 rats were injected with either cationic lysozyme (1 mg/kg body wt; $N = 7$) or vehicle only ($N = 6$) and the lysozyme activity was determined 18 hours later in proximal tubule segments.

Myoglobin injection

Fourteen rats were injected i.v. with myoglobin (Sigma, Type III) at a dose of 0.75 mg/g body weight. In these studies, 0.2 g of myoglobin were dissolved in 1 ml of 0.9% saline. Six rats received one injection, and eight rats received three injections of myoglobin at 12 hour intervals. Fourteen control rats received injections of 0.9% saline via the tail vein.

Dextran injection. One gram of low molecular weight dextran (Dextran 20, mean molecular wt 20,000 daltons, Pharmacia, Uppsala, Sweden) was dissolved in 4 ml of isotonic saline. Six rats received three i.p. injections of 0.5 g of dextran at 12 hour intervals. Another six rats received three i.p. injections of 1 g dextran. Control animals were injected three times with vehicle only.

In all animals in the myoglobin and dextran groups, the enzyme activities were measured 24 hours following the last injection. Twenty-four hours prior to sacrifice all rats were kept in metabolic cages. Urine was collected and creatinine clearance, urinary protein and dextran contents were measured. The rats were sacrificed by stunning and dislocation of the neck.

Determination of creatinine, protein, and dextran. Endogenous creatinine in urine and plasma was determined using a modified Jaffes picric acid method (Beckman Creatinine Analyzer 2, Beckman Instruments, Fullerton, California, USA). Protein in the urine was determined by the Lowry method [6]. Urine proteins were precipitated with trichloroacetic acid and redissolved in sodium hydroxide. The concentration of dextran was determined by the anthrone method [7].

Determination of cathepsin B and L activity in microdissected segments of rat nephron. The activities of cathepsins B and L in microdissected nephron segments were measured by an ultramicroassay developed recently in this laboratory [2]. We used Z-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NMec) as the substrate for cathepsin B and L together [8]. The enzymes cleave the carboxy end of arginine and release the free 7-amino-4-methylcoumarin group, which is highly fluorescent and can be measured at very low concentrations in small fluid volumes.

Solutions. All solutions were prepared from glass distilled water to minimize the background fluorescence. All chemicals were reagent grade. The dissection solution contained (in mmol/liter): NaCl, 136; KCl, 3; K_2HPO_4, 1; $MgSO_4$, 1.2; $CaCl_2$, 2; sodium lactate, 4; sodium citrate, 1; L-alanine, 6; and glucose, 5.5. The collagenase solution which was prepared fresh daily was identical to the dissection solution except for the addition of 0.1% collagenase (Sigma, Type I). The rinsing solution contained (in mmol/liters): Na_2HPO_4, 11.5; KH_2PO_4, 55.2; and EDTA, 4. The pH was 6.0 and the osmolality was 141 mOsmol/kg H_2O. The preincubation solution was the same as the rinsing solution but, in addition, contained 0.2% Triton X 100 (Sigma) and 0.05% bovine serum albumin (Sigma). The substrate, Z-Phe-Arg-NMec, was obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. A 10 mmolar stock solution of the substrate was prepared in DMSO (dimethyl sulphoxide). This stock solution was diluted daily to a 1 mmolar solution with 0.1% Brij 35 solution (Sigma). To this final substrate solution, 8 mmol/liter cysteine were added immediately before use. The stop solution contained 100 mmol/liter iodoacetate in a buffer containing 30 mmol/liter sodium acetate and 70 mmol/liter acetic acid. The pH was 4.75. For preparation of standard curves the product 7-amino-4-methylcoumarin (NMec) was obtained from Bachem Feinchemikalien, Bubendorf, Switzerland.

Collagenase treatment. Immediately after sacrifice the left kidney was removed rapidly and immersed in ice-cold dissection solution. Twenty ml of the same solution were perfused through the renal artery to remove all blood remaining in the kidney. This was followed by perfusion of 10 ml of ice-cold collagenase solution. Pieces of tissue were then sliced along the corticomedullary axis and incubated for 30 minutes in the same collagenase solution gassed with 100% oxygen in a shaking water bath at 37°C. The tissue was then rinsed with the dissection solution and maintained on ice until dissection. Collagenase was used to facilitate dissection of the proximal tubule segments. In preliminary experiments performed on rabbit proximal tubule segments, the collagenase treatment did not alter cathepsin activities significantly [2].

Microdissection

Individual tubules were dissected as described previously by Burg and Orloff [9]. Nephrons with at least one segment of proximal tubule on the surface were identified as superficial and only superficial nephrons were dissected. Based on morphology and function three segments of the proximal tubule, S1, S2, and S3, can be identified and dissected separately [10, 11]. In the present study S1 was identified as the first 1.5 mm of the proximal tubule attached to the glomerulus, while S2 included the last 1.5 mm of the pars convoluta, and S3 was identified as the last 1.5 mm of the pars recta immediately before the transition into the thin descending limb of Henle. The dissected segments were straightened and the length was measured using an eyepiece micrometer at 30× magnification. The length varied from 200 to 1500 μm. Each segment was then transferred into a second dissection dish with the rinsing solution. After 10 seconds the segments were transferred with a 2 μl volumetric pipette into a reaction vial containing 25 μl of preincubation solution. The vials were then sealed and frozen in dry ice for 10 min before preincubation was initiated. In each rat between 3 and 10 samples of each segment were dissected and analyzed.

Incubation

The samples were preincubated for 10 minutes in a shaking water bath at 37°C. Next, the vials were kept on ice while 25 μl of substrate solution were added. At this point the samples were incubated for 60 minutes in a shaking water bath at 37°C. The

enzyme reaction was stopped by placing the vials on ice and adding 500 μl of stop solution. To evaluate the background fluorescence due to either substrate contamination with reaction product or spontaneous hydrolysis of the substrate during incubation, vials containing 25 μl of preincubation solution and 25 μl of substrate solution without nephron segments were treated identically. A standard curve was measured with each experiment.

Measurement of fluorescence

The fluorescence was measured with a Turner Model III fluorometer (Turner Assoc., Palo Alto, California, USA) using the reaction vial as a cuvette. The sample was excited at 365 nm (Corning filter CS-7-83) and the emission was recorded at 450 nm (Corning filter CS-3-72). The fluorometer reading was adjusted to zero with glass distilled water. Enzyme activity was calculated as pmol of NMec generated per mm tubule length per min incubation time from the standard curve using NMec.

Determination of cationic lysozyme activity in isolated segments of proximal tubule. Lysozyme in S1, S2, and S3 segments was assayed by a modification of the turbimetric procedure of Litwack [12]. In addition to the first mm of the proximal tubule attached to the glomerulus, the second and the third mm were also dissected and analyzed. Six to 12 samples of one proximal tubule segment were transferred into a vial containing 50 μl of potassium phosphate buffer (0.05 mmol/liter; pH 7) with 0.1% BSA. The total tubule length in the vial ranged from 7.2 to 11.2 mm. The vials were frozen in dry ice for three minutes and thawed. This procedure was repeated three times. Following dissolution of the tubule cells, the vials were vortexed thoroughly. Then 40 μl of this solution were added with immediate mixing of 300 μl of a freshly prepared suspension containing 30 mg/100 ml of lyophilized cells of *Micrococcus luteus* (ATCC strain 4698, Boehringer, Mannheim, FRG) and 90 mg/100 ml NaCl in 0.05 mmol/liter potassium-phosphate buffer (pH 7). The increase in transmittance was determined at 450 nm at a temperature of 25°C in microcuvettes (1 cm light path) using a spectrophotometer (LKB Ultraspec II; Pharmacia LKB Biotechnology, Uppsala, Sweden). The increase in transmittance was linear between 30 seconds and two minutes following incubation. The increase in transmittance of the sample between 30 and 60 seconds was related to that of lysozyme standards at the same time interval. Under the conditions used, the increase in transmittance was linear between 30 ng and 200 ng of lysozyme per vial. The transmittance of the *Micrococcus luteus* suspension without lysozyme or tubule segments did not increase significantly between 30 seconds and two minutes following incubation. The lysozyme activity was expressed as ng of lysozyme per mm tubule length. Anionic lysozyme in concentrations of 100 and 200 ng per vial did not decrease the transmittance of the *Micrococcus luteus* solution, indicating that enzymatic activity of lysozyme was not retained following anionization of the natural cationic form.

Histologic studies. Additional rats were injected with either cationic ($N = 6$) or anionic ($N = 6$) lysozyme at a dose of 1 mg/g body weight via the i.p. route. Two rats received dextran (3 × 1.0 g) at 12 hour intervals via the i.p. route. Twenty-four hours later the animals were anesthetized with pentobarbital (35 mg/kg body wt) in preparation for intravascular perfusion fixation of the kidneys as described by Maunsbach [10]. A midline laparotomy was performed and the abdominal aorta was cannulated and perfused with a Tyrode buffer solution containing 3% PVP (osmolality, 300 mOsm/kg H_2O) to remove the blood. The kidneys were then fixed by intravascular perfusion with 1% glutaraldehyde in a modified Tyrode solution containing 3% PVP (osmolality, 350 mOsm/kg H_2O). The kidneys were excised and fixed additionally by immersion in the same fixative for at least three hours. Tissue blocks were sampled from both the outer and inner half of the cortex, and from the outer stripe of the outer medulla, postfixed in 2% osmium tetroxide for one hour, dehydrated in a graded series of alcohols and propylene oxide, and embedded in Epon 812 or Medcast. One μm thick sections of randomly oriented tissue were cut on a Sorvall MT5000 ultramicrotome and stained with toluidine blue for light microscopy. The sections were examined and photographed with a Zeiss photomicroscope II. A minimum of four sections was examined from each animal that was studied.

Anionization of lysozyme. Cationic lysozyme (EC 3.2.1.17) (Egg white-grade 1), with an isoelectric point of 11.0 was obtained from Sigma. The polyanionic form was obtained by acylation with succinic anhydride [13, 14]. In this procedure 400 mg of succinic anhydride (Eastman Kodak Company, Rochester, New York, USA) was added slowly over a 15 to 60 minute period with continuous stirring to a solution containing 1 g of the lysozyme in 50 ml of distilled water. The pH was maintained at 9.0 by the addition of 0.2 M NaOH. The solution was then allowed to stand for 30 minutes after the addition of succinic anhydride was completed before dialysis overnight against distilled water. After dialysis the protein was filtered through a 0.45 μm pore size Millex filter (Millipore Corporation, Bedford, Massachusetts, USA). The isoelectric point of the modified lysozyme was determined by isoelectric focusing and ranged between 5.6 and 5.75.

Statistics. The significance of differences in values obtained from the lysozyme injected groups of animals was tested using the one-way analysis of variance (ANOVA). Where the ANOVA was significant, Duncan's multiple-range test was used to identify group differences. A two-sample *t*-test for unpaired data was used for comparison of values obtained from all other experiments. P values <0.05 were considered significant.

Results

Physiologic data

The physiologic data from the lysozyme injected rats are depicted in Table 1. The body weight did not differ in the three groups. The protein excretion in the control animals was 1.8 ± 0.8 mg per 24 hours. The injection of cationic and anionic lysozyme increased the urine protein excretion by as much as fiftyfold. The protein excretion was not significantly different between rats injected with cationic and with anionic lysozyme. In a separate group of 12 animals of the same size injected with lysozyme, the creatinine clearance (ml/min/100 g body wt) was 0.60 ± 0.08 after cationic lysozyme ($N = 6$) versus 0.63 ± 0.10 after anionic lysozyme ($N = 6$, P = NS). The creatinine clearance in a third group ($N = 6$) receiving the vehicle only was 0.72 ± 0.18, a value that was not different from values obtained in the lysozyme injected animals.

Table 1. Body weight and urine protein excretion of lysozyme-injected rats

Group	*N*	Body weight *g*	Proteinuria *mg/24 hr*
Controls	13	155 ± 10	1.8 ± 0.8
Cationic lysozyme 18 hr after injection	7	153 ± 14	66 ± 36[a]
Anionic lysozyme 18 hr after injection	7	147 ± 13	91 ± 23[a]

Values are mean ± SD.
[a] $P < 0.005$ vs. control

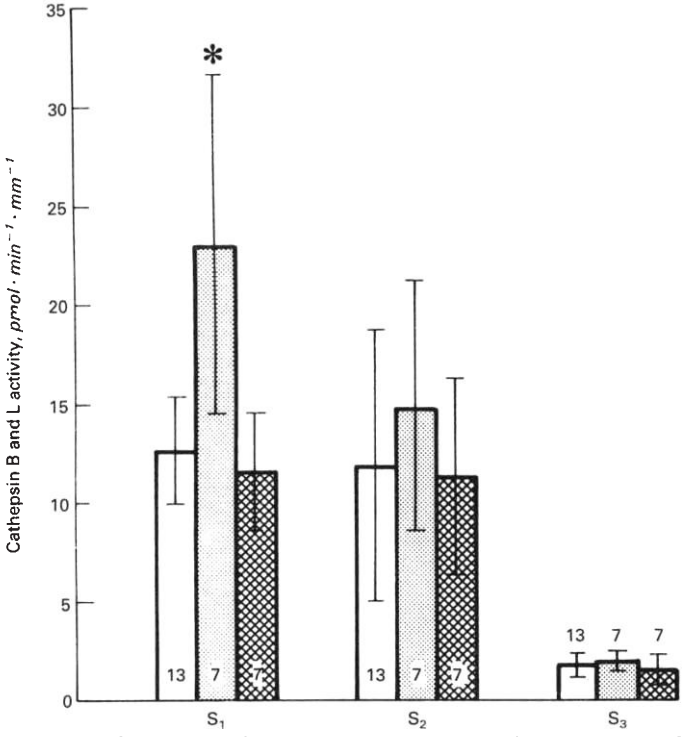

Fig. 1. *Cathepsin B and L activities in S1, S2, and S3 segments of proximal tubule 18 hours following lysozyme injection.* Open bars represent control rats. Stippled bars represent activities following cationic lysozyme administration and cross-hatched bars, activities following anionic lysozyme injection. Values represent the mean ± SD. Numbers in the columns indicate the number of rats. In each animal between 3 and 5 samples of each segment were analyzed. The asterisk denotes a significant difference in S1 between the cationic and anionic group ($P < 0.001$) and between controls and the cationic group ($P < 0.001$).

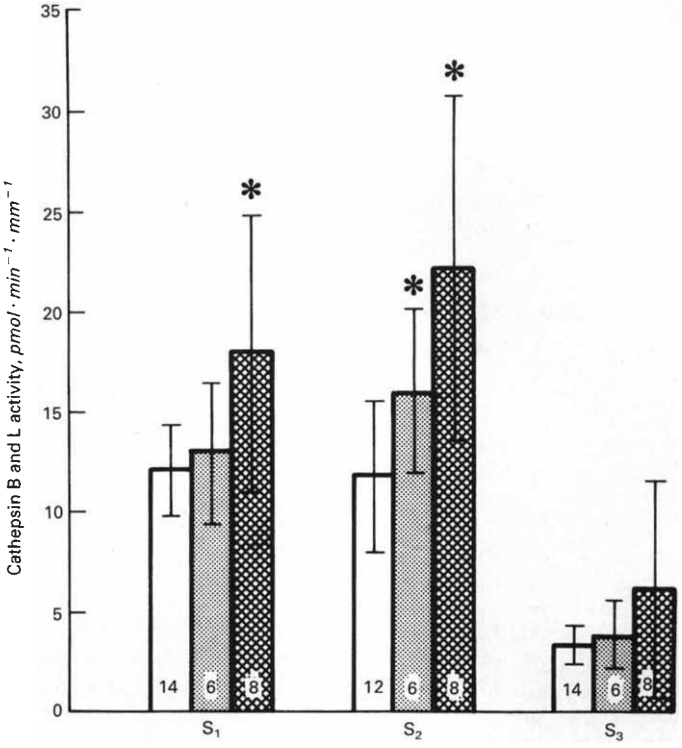

Fig. 2. *Cathepsin B and L activities in S1, S2, and S3 segments of proximal tubule following myoglobin injection.* Open bars represent control rats. Stippled bars represent activities following one injection of myoglobin of 0.75 mg/kg body weight. Cross-hatched bars depict activities following three injections of myoglobin. Values represent the mean ± SD. Numbers in the columns indicate the number of rats. In each animal between 3 and 10 samples of each segment were analyzed. Asterisks denote a significant difference ($P < 0.05$) in the same segments between myoglobin injected rats and controls.

Table 2 displays the physiologic data from the myoglobin injected animals. The body weights of the three groups were not different. The myoglobin injection induced a significant increase in urine output in both groups. However, the creatinine clearance remained unchanged. About 30 minutes following the injection, the rats started to excrete myoglobin which gave the urine a reddish-brown color. The total protein excretion was dramatically increased in both groups injected with myoglobin.

Table 3 displays the physiologic data from the dextran injected rats. Urine volume and protein excretion remained unchanged. In comparison with control animals, the creatinine clearance decreased in both groups injected with dextran ($P < 0.005$, respectively).

Cathepsin B and L activities

The cathepsin B and L activities in the S1, S2, and S3 segments of the proximal tubule 18 hours following lysozyme injection are depicted in Figure 1. In control rats (open bars), the enzyme activities were high in S1 (12.7 ± 2.6 pmol/min/mm) and S2 (11.8 ± 6.8), but low in S3 (1.8 ± 0.8). This confirms previous results [2]. Injection of cationic lysozyme was accompanied by a significant increase of cathepsin B and L activities (stippled bar) in the S1 segment (23.0 ± 8.6; $P < 0.001$). Enzyme activities in S2 (14.9 ± 6.2) and S3 (2.0 ± 0.5) remained unchanged. Anionic lysozyme (hatched bar) had no effect on cathepsin activities. In S1 the difference between enzyme activities following anionic and cationic lysozyme injection was significant ($P < 0.01$).

The cathepsin B and L activities in myoglobin-injected rats are depicted in Figure 2. A single injection significantly increased the activity in S2 segments (stippled bar; $P < 0.05$), but not in S1 and S3. Following three consecutive injections (cross-hatched bar), the enzyme activities were increased in S1 ($P < 0.005$) and in S2 ($P < 0.005$). Cathepsin in S3 was also increased but due to the large scatter of data, the level of significance was not achieved.

Table 2. Body weight, urine volume, creatinine clearance, and urine protein excretion of myoglobin-injected rats

Group	N	Body weight g	Urine volume ml/24 hr	Creatinine clearance ml/min/100 g	Proteinuria mg/24 hr
Controls	14	209 ± 42	11 ± 5	0.81 ± 0.45	3.4 ± 1.2
Myoglobin, 1 injection	6	195 ± 13	25 ± 9[a]	0.78 ± 0.25	33.1 ± 21.6[a]
Myoglobin, 3 injections	8	183 ± 9	18 ± 8[a]	1.06 ± 0.33	108.3 ± 24.5[b]

Values are mean ± SD.
[a] $P < 0.005$ vs. control
[b] $P < 0.05$ vs. one injection

Table 3. Body weight, urine volume, creatinine clearance, urine protein excretion, and urine dextran excretion in dextran-injected rats

Group	N	Body weight g	Urine volume ml/24 hr	Creatinine clearance ml/min/100 g	Protein excretion mg/24 hr	Dextran excretion mg/24 hr
Controls	12	196 ± 25	17.9 ± 6.5	0.86 ± 0.11	4.8 ± 2.1	0
Dextran-loaded						
3 × 0.5 g	6	220 ± 27	20.1 ± 10	0.64 ± 0.10[a]	5.3 ± 3.0	588 ± 216[a]
3 × 1.0 g	6	199 ± 13	19.6 ± 4.3	0.65 ± 0.09[a]	4.0 ± 0.9	1400 ± 610[a]

Values are mean ± SD.
[a] $P < 0.025$ vs. controls

Figure 3 shows the cathepsin activities following dextran injections. After three injections of 0.5 g dextran (stippled bars), the enzyme activity was increased in S2 segments only ($P < 0.01$). Following three injections of 1 g of dextran (cross-hatched bar) the cathepsin B and L activities were enhanced in all three segments of the proximal tubule (S1, $P < 0.01$; S2, $P < 0.005$; S3, $P < 0.005$).

Lysozyme activities

In control rats a low activity of lysozyme was present in the first two mm of the proximal tubule. The S2 and S3 segments did not contain any lysozyme activity (Fig. 4, open bars). Eighteen hours following injection of cationic lysozyme, lysozyme activity was measured in the first three mm of the proximal tubule (Fig. 4, hatched bars). In the first mm the activity was 23 ± 9 ng/mm tubule length; in the second and third mm of the S1 segment the activities amounted to 24 ± 6 and 22 ± 5 ng/mm, respectively. In S2 the lysozyme activity was 11 ± 4 ng/mm. No lysozyme activity could be detected in S3.

Histopathology

Sections of cortex from animals injected with either anionic or cationic lysozyme revealed excellent overall preservation of the glomeruli and tubules. Comparisons between tissue sections obtained from those animals injected with anionic lysozyme (Fig. 5) and control animals revealed no major differences in the appearance of the proximal tubule. The number and size of lysosomes was similar in the two groups of animals. However, those animals receiving cationic lysozyme exhibited large numbers of dense lysosomes throughout the cytoplasm of the S1 and S2 segments of the proximal tubule (Fig. 6). Many lysosomes were large and irregular in appearance. Far fewer dense lysosomes were observed in the pars recta of the proximal tubule. Glomeruli appeared to be unaffected by the administration of lysozyme when viewed by light microscopy. Following dextran injection the proximal tubule cells contained large vacuoles consistent with the presence of dextran-loaded lysosomes (Fig. 7).

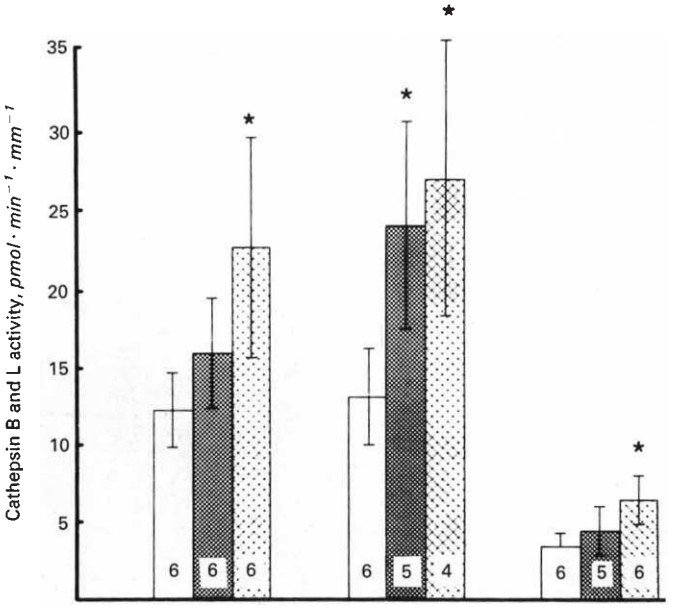

Fig. 3. *Cathepsin B and L activities in S1, S2, and S3 segments of proximal tubule following dextran (molecular wt = 20,000 daltons) injection.* Open bars represent control rats. Stippled bars represent activities following three injections of 0.5 g of dextran, and cross-hatched bars, activities following three injections of 1.0 g of dextran. Values represent the mean ± SD. Numbers in the columns indicate the number of rats. In each animal between 3 and 9 samples of each segment were analyzed. Asterisks denote a significant difference ($P < 0.05$) in the same segments between dextran injected animals and controls.

Discussion

In all groups of rats injected with either myoglobin or lysozyme, a huge increase in urine protein excretion occurred. This is consistent with the fact that LMWP, especially those with a molecular weight below 20,000 daltons, are filtered

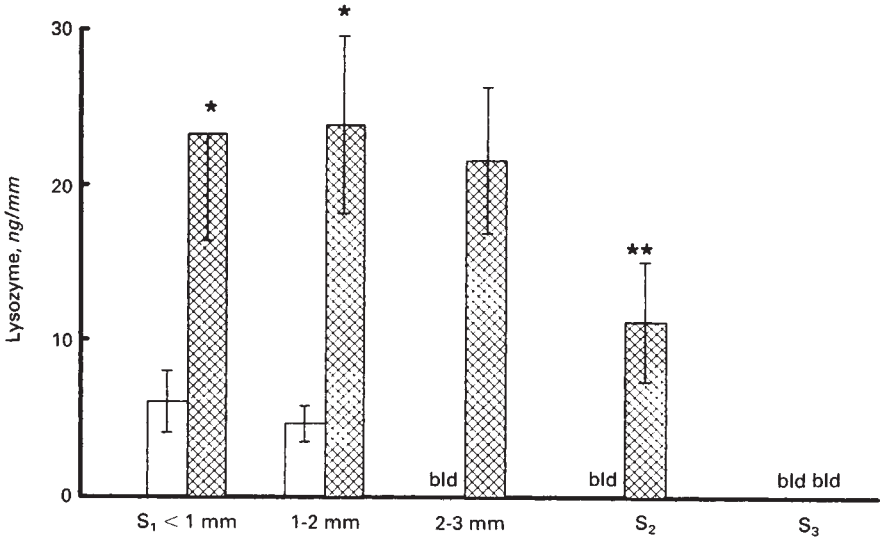

Fig. 4. *Lysozyme activities in the first three mm of the S1 segment and in S2 and S3 segments.* Open bars represent control animals ($N = 6$). Cross-hatched bars represent rats 18 hours following injection of cationic lysozyme, 1 mg/g body weight ($N = 7$). Values are given as mean ± SD. One asterisk indicates $P < 0.001$ versus control activity. Two asterisks indicate $P < 0.005$ versus S1 segments. bld = below level of detection.

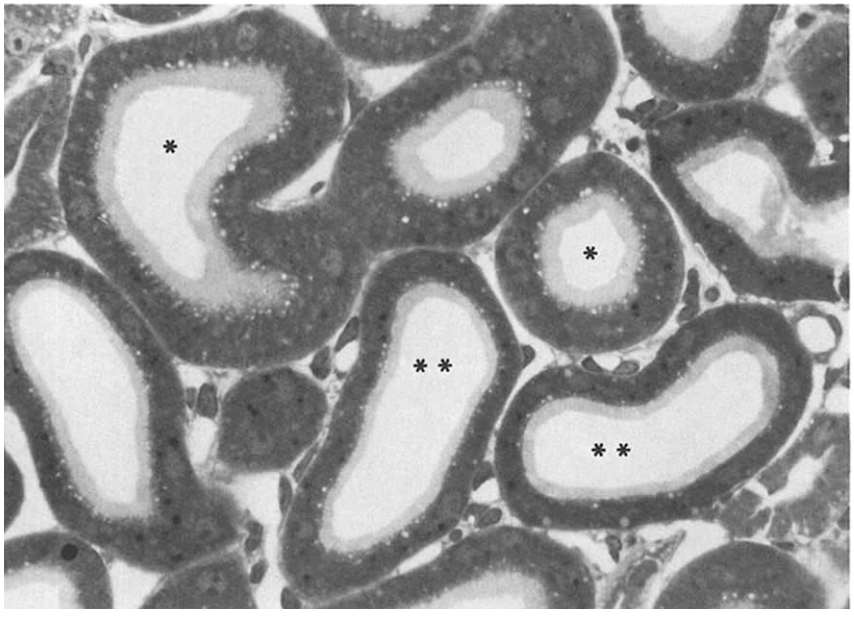

Fig. 5. *Photomicrograph illustrating the typical appearance of the kidney cortex of a rat sacrificed 24 hours after receiving anionic lysozyme.* The appearance of the proximal tubule was no different from control animals not receiving anionic lysozyme. S1 segments are indicated by a simple asterisk, and S2 segments by a double asterisk. One μm thick section stained with toluidine blue, magnification, × 700.

extensively by the kidney. The absorption of proteins in the proximal tubule is characterized by a high capacity and a low affinity. An increase in the tubular load increases both the reabsorption and the excretion of the respective protein [1]. Although we determined only the total urine protein, it seems likely that the bulk of the urine protein consisted of myoglobin or lysozyme. Myoglobin in the urine of myoglobin injected rats was visible by the dark brown color of the urine. In addition, others have found that the urine of mice injected intraperitoneally with lysozyme contains large amounts of lysozyme [15]. Hence, it seems safe to conclude that in our experiments all groups of rats injected with LMWP had an increased filtered load and an increased urinary excretion of the respective protein.

Despite the similarity in protein excretion and presumably protein filtration between the different groups of animals, the changes in cathepsin activities were less consistent. The results demonstrated that injection of cationic lysozyme and myoglobin were accompanied by an increase in cathepsin B and L activity in specific segments of rat proximal tubule. A similar increase of proteolytic activity in homogenates of kidney cortex following injection of cationic lysozyme has been described previously [15]. We found that the injection of anionic lysozyme, however, was not associated with changes in cathepsin activity in the proximal tubule. It is well established that the proximal reabsorption of myoglobin and cationic lysozyme increases with increased proximal loads of protein by a factor of 100 [3, 4]. In contrast, the proximal tubule uptake of anionic lysozyme is negligible despite increased proximal loads [16, 17]. Our morphologic data confirm this difference in uptake. In the kidney of rats injected with cationic lysozyme, the proximal tubules contained large numbers of dark staining lysosomes

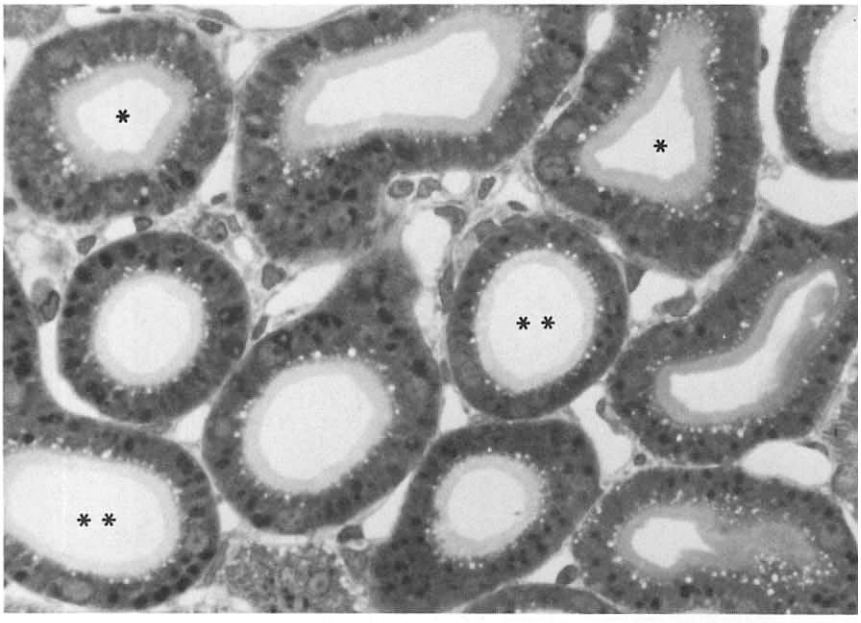

Fig. 6. *Photomicrograph depicting the characteristic appearance of the kidney cortex of a rat sacrificed 24 hours after receiving cationic lysozyme.* The proximal tubules contain large numbers of dense lysosomes, many of which are large and irregular in appearance. S1 segments are indicated by a single asterisk, and S2 segments by a double asterisk. One μm thick section stained with toluidine blue, magnification, × 700.

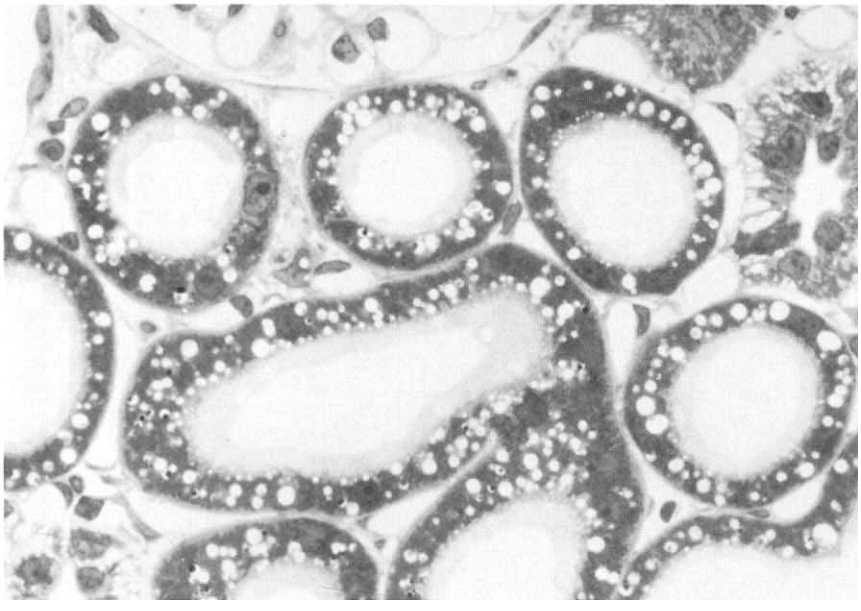

Fig. 7. *Photomicrograph depicting the characteristic appearance of the kidney cortex of a rat sacrificed 24 hours after receiving three injections of 1.0 g of dextran (molecular wt = 20,000 daltons).* The proximal tubules contain vacuoles consistent with the presence of dextran-loaded lysosomes, many of which are large and irregular in appearance. One μm thick section stained with toluidine blue, magnification, × 850.

secondary to increased endocytosis of protein. A comparable increase in lysosomes was not observed in those animals injected with anionic lysozyme. These findings are compatible with our hypothesis that increased protein uptake by the proximal tubule stimulates the lysosomal proteolytic enzymes, cathepsin B and L [2]. Presumably the increase in activity is the direct result of enhanced endocytosis of the LMWP. With respect to the segments where stimulation occurred, considerable differences existed between the myoglobin and the lysozyme injected groups. Following one injection of myoglobin the urine protein excretion was increased only moderately and the cathepsin B and L activity was enhanced in S2 segments only (Fig. 2). This finding is consistent with previous results demonstrating an increase in cathepsin activity in S2 and S3 segments, but not in S1 segments in the presence of an increased albumin load delivered to the proximal tubule [2]. Following three consecutive injections of myoglobin which were accompanied by a significantly higher urine protein excretion, cathepsin activities were stimulated in S1 segments in addition to S2 segments; however, due to very low values in S3 in some rats, the level of cathepsin activity did not quite reach the limits of significance. The results suggest that following a moderate increase in myoglobin loads to the proximal tubule, proteolytic capacity and presumably protein uptake increase in S2 seg-

ments only. With a further increase in the protein load, both protein uptake and proteolytic capacity also increase in the S1 and in the S3 segments.

In those animals injected with cationic lysozyme, the results revealed the ability of S1 to increase both the protein uptake and the proteolytic capacity following increased proximal loads. In the S1 segments there were large numbers of dark staining lysosomes secondary to increased endocytosis of cationic lysozyme, the activity of lysozyme was high, and the activity of cathepsin B and L was markedly increased.

The increased lysozyme content in S1 may be the result of either increased tubular uptake or decreased intralysosomal degradation of lysozyme in the presence of unchanged lysozyme uptake or both. In normal rats the plasma concentration of lysozyme is about 5 mg/ml [16], and the glomerular sieving coefficient for lysozyme is 0.8. Thus, under normal circumstances, lysozyme in the glomerular ultrafiltrate amounts to about 4 μg/ml. At normal plasma concentrations 99.5% of the filtered lysozyme is reabsorbed because only trace amounts appear in the final urine [16]. The lysozyme activities measured in S1 segments of control rats in the present study, although low, are in agreement with these published observations. However, a decrease in intralysosomal lysozyme degradation in the presence of unchanged uptake appears unlikely to explain our findings since microperfusion studies have clearly established an increase in uptake of lysozyme in the proximal tubule when the proximal load is increased [16]. The higher lysozyme activities in S1 segments compared to S2 and S3 following lysozyme injection and the elevated cathepsin activities in S1 but not in S2 and S3 segments suggest that proximal tubule uptake of lysozyme occurs preferentially in the S1 segment.

Repeated injections of neutral low molecular weight dextran served as a non-protein control to explore the mechanism responsible for the increase in proteolytic activity. Glomerular filtration and urine excretion is the major route of dextran removal from the body [5, 18], and dextran clearance increases with decreasing molecular weight. Following dextran injection extensive vacuolization of proximal tubule cells was observed in the present study and has been reported by others [5, 9–21]. Within these vacuoles dextran has been demonstrated by both light and electron microscopic techniques [5, 22–24]. These data support the concept that dextran is taken up into proximal tubule cells by endocytosis and is transferred to the lysosomes [19]. However, the amount of dextran absorbed in the kidney may be low in comparison to the proximal load. For dextran with a molecular weight of 80,000 daltons, an uptake of 2% of the filtered load has been measured [5]. In the present study extensive vacuolization of proximal tubule cells occurred, and it is our interpretation that these vacuoles represent dextran loaded lysosomes. The dextran uptake induced a significant increase in cathepsin B and L activities in a pattern similar to those observed in proximal tubule segments from rats following myoglobin injection. After three injections with 0.5 g of dextran, the enzyme activity was raised only in S2 segments of the proximal tubule. Increasing the dose to three injections of 1 g resulted in stimulation of cathepsin activities in all proximal tubule segments. These findings suggest that endocytic uptake or lysosomal storage of macromolecules or both may be the trigger for cathepsin stimulation rather than a direct protein-enzyme interaction.

The data contained in the present study and from other investigations [2, 15] support the view that increased proximal protein uptake increases intralysosomal proteolytic activity in the proximal tubule. This reaction may be important in order to maintain a steady state between tubular uptake of LMWP and proteolytic digestion and to avoid tubular accumulation of LMWP. The increased cathepsin activities following dextran injection indicate that endocytic uptake and the lysosomal load of a macromolecule may be the relevant stimuli for cathepsin activation. The precise molecular mechanism of stimulation remains to be elucidated.

Acknowledgments

This work was supported in part by U.S. Public Health Service Grant DK 28330 (Dr. Tisher) and a grant from the German Research Fund (DFG-1789) to Dr. Olbricht. Santiago Casanova provided technical support and Linda Michael and Judi Smith provided secretarial assistance. The authors thank Dr. R. Michael Roberts for determination of the isoelectric point of the anionic lysozyme, and Dr. Kirsten M. Madsen for helpful criticism during the preparation of this manuscript.

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