Alterations in lysosomal enzymes of the proximal tubule in gentamicin nephrotoxicity

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Alterations in lysosomal enzymes of the proximal tubule in gentamicin nephrotoxicity. Gentamicin accumulates in proximal tubule lysosomes, increases their number, and changes their structure. An important lysosomal function is degradation of intracellular proteins. To evaluate the effect of gentamicin on this lysosomal function, we measured the activity of the key lysosomal proteinases, cathepsin B and L, in microdissected S1, S2, and S3 segments of rat proximal tubules by means of a fluorometric microassay. The cathepsin activities were decreased in S1 and S2 following one and four gentamicin injections of 100 mg/kg body weight. The lysosomal enzyme, acid phosphatase, was also measured and was not decreased by gentamicin. The urine excretion of cathepsins B and L was decreased after gentamicin. This excludes an increase in urinary loss of cathepsins as the cause of decreased tubule activity. Structural changes of the lysosomes per se were excluded as the factor responsible for the reduced cathepsin activity by demonstrating increased cathepsin B and L activity in proximal tubule segments from rats injected with dextran, since dextran induces an increase in number and size of proximal tubule lysosomes. In vitro incubation of urine and tubule segments with gentamicin demonstrated a concentration-dependent reversible inhibition of cathepsin B and L. We conclude that gentamicin per se decreased cathepsin B and L activities in proximal tubule segments as early as 24 hours following one injection due to either enzyme inhibition or reduced generation of active intralysosomal cathepsin B and L. Gentamicin may, therefore, reduce renal protein catabolism by decreasing the activity of the key proteolytic enzymes, cathepsin B and L. Since cathepsin B and L are proteolytic activators of other lysosomal enzymes, their reduced activity may also decrease the activities of other lysosomal enzymes.

The aminoglycoside antibiotic, gentamicin, is nephrotoxic in man and experimental animals [1, 2]. Glomerular filtration is the major route of excretion [3]. Uptake occurs in the proximal convoluted tubule predominantly via the luminal cell surface [4, 5]. Gentamicin is incorporated into proximal tubule lysosomes after attachment to luminal surface receptors and endocytosis [6–9]. It accumulates in the lysosomes, and the renal cortical tissue half-life is approximately 100 hours [10, 11]. Morphologic alterations in gentamicin nephrotoxicity are present predominantly in the proximal tubule [2]. Due to the accumulation in the proximal tubule lysosomes impaired function of these organelles may be an important mechanism leading to proximal tubular toxicity [2].

The lysosomes are generally believed to play a major role in the breakdown of intracellular proteins [12–15]. Protein degradation by the lysosomal system involves sequestration of intracellular proteins in autophagic vacuoles, fusion of these vacuoles with primary lysosomes, and degradation of proteins within the lysosomes by proteases. Cathepsins B and L are highly active lysosomal proteases. It has been shown that inhibition of these proteases also inhibits the intracellular protein degradation by up to 70% [12–15]. In order to evaluate the effect of gentamicin on this important aspect of lysosomal function, we measured cathepsin B and L activities in microdissected segments of the proximal tubule from gentamicin injected rats. Measurements of the lysosomal marker enzyme, acid phosphatase, served as a control for the selectivity of the induced changes. Segmental analysis of enzyme activities was deemed essential since gentamicin uptake may be different between the consecutive segments of the proximal tubule [6, 7]. To gain further insight into the mechanism of cathepsin changes, the urinary excretion of these enzymes and the in vitro effect of gentamicin on cathepsins were both studied. To account for effects that were probably induced by enlargement of lysosomes observed in gentamicin nephrotoxicity, cathepsin activity was also measured in tubules from rats injected with dextran, that also induces similiar enlargement of lysosomes.

Methods

Female Sprague-Dawley rats weighing 165 to 240 g were studied. The animals had free access to standard rat chow (Altromin, Spezialfutter GmbH, 4937 Lage, Germany) and tap water.

Experimental protocols

Gentamicin injection. Seven rats received one subcutaneous injection of gentamicin (Byk-Essex Pharma, München, Germany) at a dose of 100 mg/kg body weight, and 13 rats received four s.c. injections of gentamicin at 24 hour intervals. Eleven control rats received four s.c. injections of vehicle only. An additional 16 rats received four s.c. injections of the same dose of gentamicin. These rats were kept continuously in metabolic cages, the urine was collected, and urinary cathepsin activities and creatinine clearance were measured daily. Blood was collected by retrobulbar puncture.

Dextran injection. One gram of dextran (Dextran 20, mean molecular wt of 20,000 daltons, Pharmacia, Uppsala, Sweden)

was dissolved in 4 ml of isotonic saline. Six rats received eight i.p. injections of 1 g of dextran at 12 hour intervals.

In all animals in the gentamicin 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 and urinary protein content were measured. The rats were sacrificed by stunning and dislocation of the neck. Blood was collected by cardiac puncture.

Determination of creatinine and protein. 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 urine was determined by Lowry's method [16].

Determination of gentamicin in kidney cortex. The concentration of gentamicin in the kidney cortex was determined in six rats following one injection and in six rats following four injections of gentamicin at a dose of 100 mg/kg of body weight. Tissue samples were prepared using an adaptation of the method of Kornguth and Kunin [17]. Previously weighed samples of kidney cortex (213 to 306 mg) were repeatedly frozen in dry ice and thawed. The samples were homogenized in 6 ml of a 10 mmolar phosphate buffer, pH 7.4, using a cooled Sorvall Omni-Mixer four times for four minutes at 50,000 RPM. During the third and the fourth steps, the homogenate was acidified with 4 ml trichloroacetic acid to obtain protein denaturation. After centrifugation ($\times$ 1200 g for 20 min), the supernatant was titrated to pH 6.5 to 7.5 with 10% sodium hydroxide. Gentamicin was measured by fluorescence polarization immunoassay using a commercially available system (TDx Unit Dose System Gentamicin, Abbott Health Care Products, Inc, Abbott Park, Illinois, USA).

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 [18]. We used Z-phenyl-alanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NMec) as the substrate for cathepsin B and L together. The enzymes cleave the substrate and release the free 7-amino-4-methylcoumarin group (NMec) which is highly fluorescent and can be measured at very low concentrations in small fluid volumes. The substrate is specific for cathepsin B and L [18].

Solutions. All solutions were prepared from glass distilled water to minimize the backround 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 was identical to the dissection solution except for the addition of 0.1% collagenase (Sigma, Type I). The rinsing solution contained (in mmol/liter): 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-Phen-Arg-NMec, was obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. A 10 mmolar stock solution of the substrate was prepared in DMSO (dimethyl sulphoxide). The 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 reaction product 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. The collagenase treatment did not alter cathepsin activities significantly, as demonstrated recently [18].

Microdissection. Individual tubules were dissected as described previously [18]. Based on morphology and function three segments of the proximal tubule, S1, S2, and S3 can be identified and dissected separately. In the present study S1 was identified as the first three mm of the proximal tubule attached to the glomerulus, while S2 included the last mm of the pars convoluta (S2c) and the first mm of the pars recta (S2r), and S3 was identified as the last 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. The length varied from 200 to 1000 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 24 μl of preincubation solution. The vials were sealed and frozen in dry ice for ten min before preincubation was initiated.

In each rat between three and ten samples of each segment were dissected and analyzed.

Incubation. The samples were preincubated for ten minutes in a shaking water bath at 37°C. The preincubation in the hypoosmolar preincubation solution lysed the tubules completely. 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. The enzyme assay was linear

with respect to incubation time and tubule length as described previously [18].

Determination of acid phosphatase activity in microdissected tubule segments. The activity of acid phosphatase in proximal tubule segments was measured utilizing 4-methylumbelliferyl phosphate (4-MUP, Sigma) as a substrate, according to the method previously published [19]. Acid phosphatase cleaves 4-MUP and releases free 4-methyl-umbelliferone (4-MU) which is highly fluorescent. The method is identical to the cathepsin determination, including tissue preparation, microdissection, preincubation, incubation, adding of stop solution and measurement of fluorescence. However, all solutions were different, except the dissection and collagenase solutions. The rinsing solution contained 0.2 M sodium acetate-acetic acid buffer and 2 mmol/liter EDTA, and had a pH of 4.8 and an osmolality of 158 mOsm/kg H_2O. The substrate solution was prepared from the rinsing solution by adding 1 mmol/liter 4-MUP. The preincubation solution was prepared from rinsing solution by adding 0.2% Triton X-100 and 0.05% bovine serum albumin. The stop solution contained 0.5 mol/liter of glycine adjusted to pH 10.4 with 5 N NaOH. A standard curve was measured with each experiment. The enzyme activity was expressed in pmol reaction product, 4-MU, liberated per min incubation time per mm tubule length. The assay was specific for acid phosphatase, and was linear with respect to incubation time and tubule length [20].

Determination of cathepsin B and L in plasma and urine. The enzyme activities were measured by a fluorometric assay utilizing Z-Phe-Arg-NMec as a substrate. The urine was centrifuged for 10 minutes at 5,000 RPM. Five ml of the supernatant were dialyzed for 24 hours in the cold against two changes of a solution composed of potassium citrate, 30 mmol/liter; NaCl, 100 mmol/liter and 0.1% Triton X, pH 6.0. Dialysis tubing made of regenerated cellulose with an exclusion limit of 8000 to 15000 daltons was employed (Visking Dialysis Tubing, Type 27/32, Serva Feinbiochemica, Heidelberg, Germany). The volume of the bath solution was 200 ml. In urine samples from animals obtained two, three, and four days following gentamicin injection, cathepsin activity was measured prior to and following dialysis. In preliminary experiments 1 ml of plasma was dialzed for 24 hours, and the cathepsin activities prior to and following dialysis were identical. Hence, determination of cathepsin B and L in plasma during the experiments was performed without dialysis. Plasma and urine were appropriately diluted. Two µl of the sample were added to 25 µl of a preincubation solution containing (in mmol/liter) Na_2HPO_4, 11.5 (A component); KH_2PO_4 (B component), 55.2; EDTA, 4; and 0.2% Triton X. The pH was 6.0. The vials containing the samples were kept on ice and 25 µl of substrate solution were added. A 10 mM stock solution of Z-Phe-Arg-NMec was prepared in dimethyl sulphoxide (DMSO, Sigma). This stock solution was diluted daily to a 1 mM solution with 0.1% Brij 35 solution (Sigma). To this final substrate solution, 8 mmol/liter cysteine was added immediately before use. The samples were incubated for 15 minutes in a shaking water bath at 37°C. The enzyme reaction was stopped by placing the vials on ice and adding 500 µl stop solution. The stop solution contained 100 mmol/liter iodoacetate in a buffer consisting of 30 mmol/liter sodium acetate and 70 mmol/liter acetic acid. The pH was 4.75. The fluorescence was determined as described above. The enzyme activity in plasma was ex-

pressed in nmol of the reaction product, NMec, released per hour and per ml of plasma. The activity in urine was expressed in nmol NMec released per hr per µmol of creatinine in urine.

The linearity of the assay with incubation time was evaluated by incubating two µl samples of the same urine or of the same plasma for different times between 5 and 30 minutes. The linearity with enzyme activity was evaluated by incubating two µl aliquots of the same urine or of the same plasma for 15 minutes that were differently diluted.

In vitro experiments. The in vitro effects of gentamicin on cathepsin B and L in urine and proximal tubules were studied by measuring cathepsin activities following preincubation with gentamicin. Urine samples from eight control rats were preincubated with 0.6, 1.0, and 5.0 mg/ml of gentamicin for five minutes. To verify the reversibility of the effect, urine samples preincubated with 5 mg/ml of gentamicin were dialyzed for 24 h. The effect of gentamicin on proximal tubule cathepsin activity was evaluated by incubating S1 segments of four control rats with 0.3, 0.6, 1.0, and 5.0 mg/ml of gentamicin. In preliminary experiments it was demonstrated that 5 mg/ml of gentamicin decreased the pH of the incubation solution from 6 to pH 5.1. The pH of 6.0 was maintained by changing slightly the concentration of the A and B component of the phosphate buffer in the preincubation solution. Standard curves were measured with each gentamicin concentration. The values were not different from normal standard curves without gentamicin. Hence, gentamicin did not interfere with the assay.

Statistics

Data are given as mean ± SD. Differences were evaluated by Student's *t*-test. Pre- and post-dialysis urine cathepsin activities were compared by Student's *t*-test for paired values. Multiple comparisons between urine and plasma cathepsin on different days were calculated by analysis of variance and by *t*-test with Bonferroni correction, and *P* values < 0.05 were regarded as significant. The significance of correlations was evaluated by linear regression analysis.

Results

Physiological data

The data describing the animal population under study are presented in Table 1. No changes were observed after one gentamicin injection. Four gentamicin injections were accompanied by an increase in urine volume ($P < 0.05$). The creatinine clearance was decreased to 79% of the control value ($P < 0.05$). Following eight injections with dextran the urine volume was increased ($P < 0.05$), while the creatinine clearance remained unchanged.

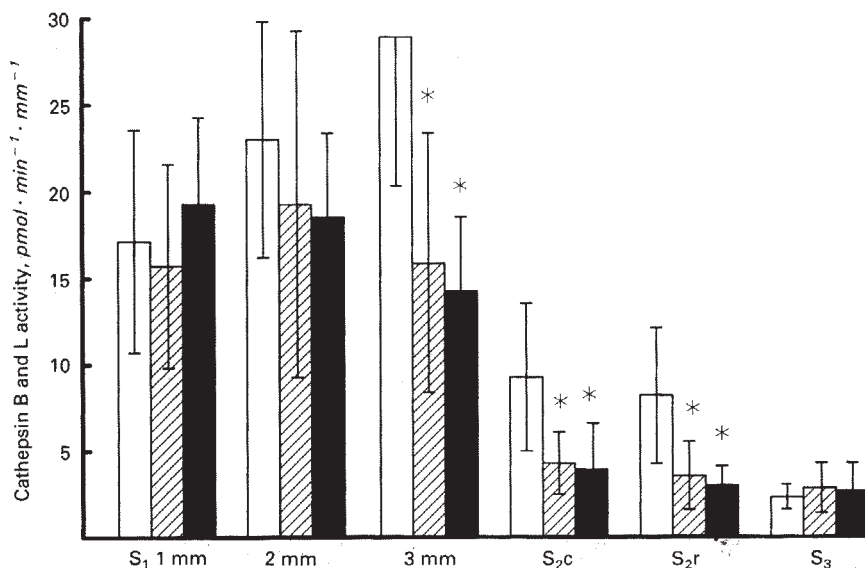### Cathepsin B and L activities in proximal tubule segments

In control rats the highest activities were found in the S1 segments (Fig. 1). The S3 segments had very low activities amounting to only 10% of the activities in S1 segments. Following one gentamicin injection the cathepsin B and L activity was decreased in the third mm of S1 ($P < 0.05$), in S2c ($P < 0.02$) and in S2r ($P < 0.01$). After four injections the enzyme activities were reduced in the third mm of S1 ($P < 0.01$), in S2c ($P < 0.02$), and in S2r ($P < 0.005$). The cathepsin activities in S3 segments remained unaltered by gentamicin. Dextran injections

Table 1. Functional parameters of rats injected with gentamicin, 100 mg/kg body weight, or with dextran, 8 × 1 g

Group	N	Body wt g	Urine volume ml/24 hr	Creatinine clearance ml/min/100 g	Urine protein mg/24 hr
Control	11	185 ± 9	11 ± 3	0.71 ± 0.09	4.3 ± 1.5
Gentamicin, 1 ×	7	186 ± 13	10 ± 3	0.68 ± 0.12	4.6 ± 1.8
Gentamicin, 4 ×	13	182 ± 15	26 ± 12[a]	0.55 ± 0.11[a]	5.5 ± 1.6
Dextran	6	217 ± 20	17 ± 6[a]	0.68 ± 0.12	4.1 ± 0.6

Values are mean ± SD. Creatinine clearance is calculated per 100 g body weight.
[a] $P < 0.05$ vs. control

Fig. 1. *Cathepsin B and L activity in microdissected segments of proximal tubules from gentamicin-injected rats.* Abbreviations are: S1, the first three mm attached to the glomerulus; S2c, the last mm of the pars convoluta. S2r: the first mm of the pars recta; S3, the last mm of the pars recta prior to the thin descending limb of Henle. Open bars represent values from control rats (N = 11). Hatched bars show data from rats following one injection of gentamicin (N = 7). Solid bars represent activities from rats after four injections of gentamicin (N = 13). The values are mean ± SD. Asterisks indicate $P < 0.05$ vs. control values.

were accompanied by significant increases in cathepsin B and L activities in all dissected proximal tubule segments (Fig. 2). The P values versus control were: S1, 1 mm, < 0.05; S1, 2 mm, < 0.005; S2c, < 0.001; S3, < 0.001.
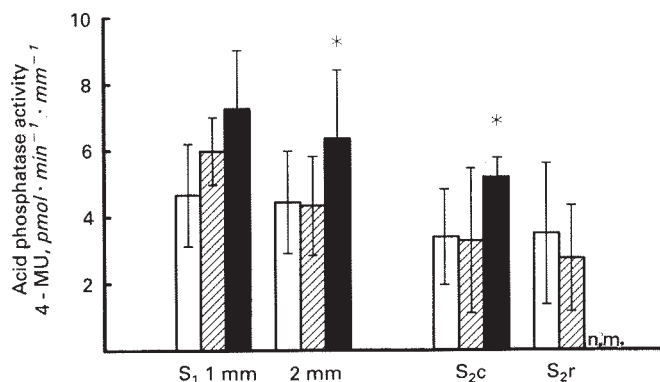
Acid phosphatase activities

The values of acid phosphatase activity are depicted in Figure 3. Gentamicin did not change the enzyme activities in the proximal tubule segments. Dextran injections were accompanied by a significant increase in acid phosphatase activity in the first mm of S1 and in S2c ($P < 0.05$).

Cathepsin B and L in plasma and urine

The enzyme assay developed for determination of cathepsin B and L in urine and plasma showed a linear correlation with respect to incubation time and urine and plasma volume, respectively (Figs. 4 and 5 for urine, plasma data not depicted). The enzyme activity in plasma was 404 ± 149 nmol/hr/ml during the control period 24 hour prior to gentamicin injection (Fig. 6). No significant changes were observed following gentamicin injection. The urine excretion of cathepsin B and L in the control period was 623 ± 274 nmol/hr/μmol creatinine (Fig. 6). This value remained unchanged during the first day following gentamicin injection. The enzyme activity dropped to 201 ± 91 nmol/hr/μmol creatinine on the second day and remained low on the third and fourth day ($P < 0.05$, respectively, vs. the
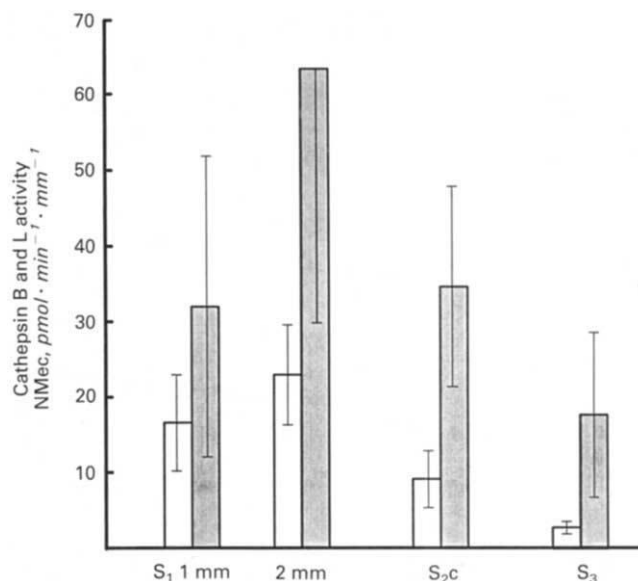
Fig. 2. *Cathepsin B and L activity in microdissected segments of proximal tubules from dextran-injected rats.* Abbreviations are: S1, the first two mm attached to the glomerulus; S2c, the last mm of the pars convoluta; S3, the last mm of the pars recta immediately prior to the thin descending limb of Henle. Open bars represent values from control rats (N = 11). Shaded bars represent activities from rats after eight injections of dextran (N = 6). The values are mean ± SD. Asterisks indicate $P < 0.05$ vs. control values.

Fig. 3. *Acid phosphatase activity in microdissected proximal tubule segments from rats after injection of gentamicin or dextran.* Abbreviations are: S1, the first two mm attached to the glomerulus; S2c, the last mm of the pars convoluta; S2r, the first mm of the pars recta. Open bars represent values from control rats ($N = 10$). Hatched bars show data from rats following four injections of gentamicin ($N = 8$). Solid bars represent activities from rats after eight injections of dextran ($N = 6$). The values are mean $\pm$ SD. Asterisks indicate $P < 0.05$ vs. control values. n.m., not measured.
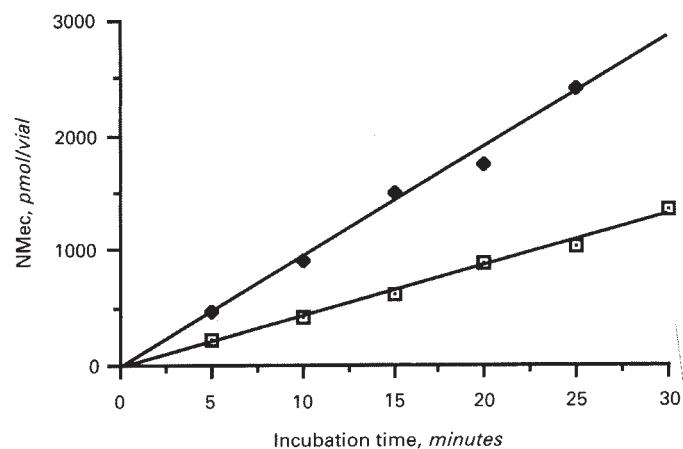

Fig. 4. *Assay of cathepsin B and L in urine.* Linearity of liberated reaction product (NMec) with time of incubation. Two μl of identical urine were incubated for different time intervals. Each symbol represents values from one rat. The correlation coefficient for both urines is 0.99.

control period). The creatinine clearance in these animals remained constant during the experimental period (Fig. 6).

Urine cathepsin activities from rats three and four days following gentamicin injection were measured before and after 24 hours of dialysis (Fig. 7). No changes in enzyme activities were observed.

In vitro experiments

In vitro incubation of proximal tubule S1 segments and of urine with gentamicin showed a concentration dependent inhibition of cathepsin B and L activity (Fig. 8). For instance, 5 mg/ml of gentamicin reduced the tubular enzyme activity to 15 $\pm$ 6% and the urine enzyme activity to 33 $\pm$ 8% of the control value ($P < 0.001$, respectively). The inhibition of cathepsin B
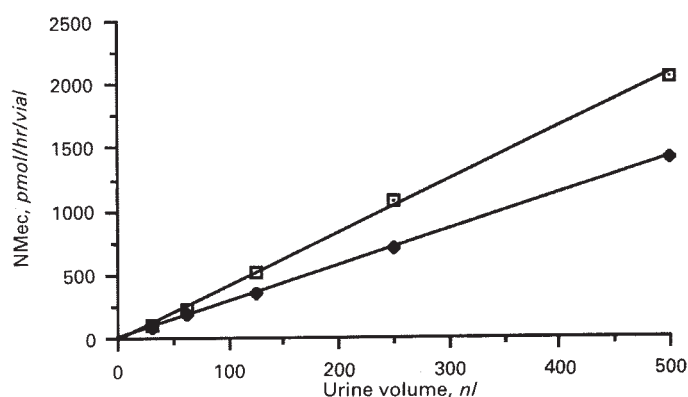
Fig. 5. *Assay of cathepsin B and L activity in urine.* Relationship between liberated reaction product (NMec) and incubated urine volume. Each symbol represents values from one rat. The correlation coefficient was 0.99 for both urines.

and L activity in urine was completely reversible by 24 hours of dialysis (Fig. 9).

Gentamicin in kidney cortex

After one injection of gentamicin at a dose of 100 mg/kg body weight, the concentration of gentamicin in the kidney cortex was 313 $\pm$ 70 μg/g wet weight ($N = 6$). After four injections at 24 hour intervals, the concentration amounted to 1288 $\pm$ 200 μg/g wet weight ($N = 6$).

Discussion

Gentamicin injections were accompanied by reduction of cathepsin B and L activities in the third mm of S1 segments and in both S2 segments of the proximal tubule as early as 24 hours following the first injection, while the control values of cathepsin were similar to previously reported data [18]. The pattern of cathepsin inhibition is in good agreement with results from autoradiographic studies of gentamicin uptake by the rat proximal tubule [6, 7], showing significant accumulation of gentamicin only in S1 and S2 segments. A slight decrease in cathepsin B activity was described recently in homogenates of kidney cortex at six days following daily gentamicin injections of 50 mg/kg [20]. Comparisons are difficult to make since the applied substrate used for cathepsin B was less specific [21], the gentamicin dose was lower, and our analysis was far more sensitive due to the determination of cathepsin activities in single segments.
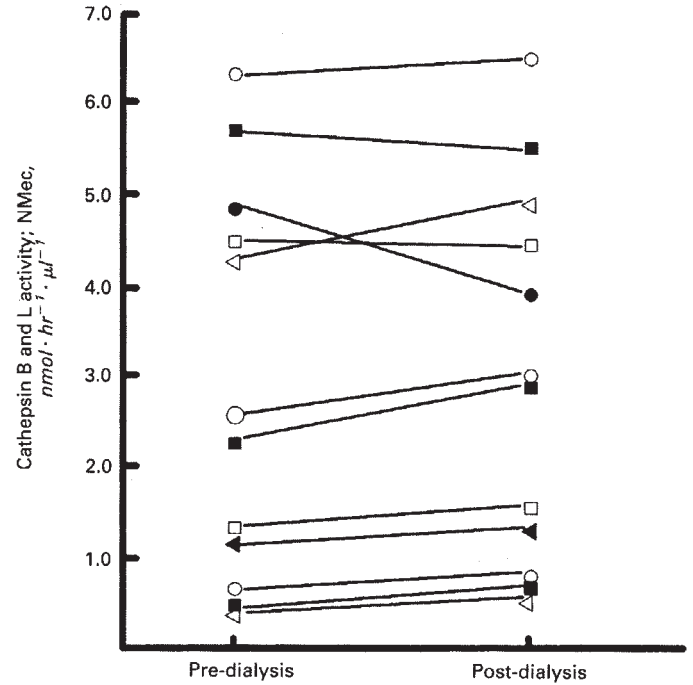
Factors that must be considered in the interpretation of our data, in addition to gentamicin accumulation, include changes in the level of proteinuria, altered structure of the lysosomes, necrosis of proximal tubule cells, and increased exocytosis of lysosomes containing cathepsins. The level of urinary protein excretion affects cathepsin activities in the proximal tubule [18]. However, such a mechanism is unlikely in the present experiments since protein excretion remained unchanged following gentamicin injection. Within 48 hours following gentamicin administration an increase in the number and size of lysosomes which contain both myeloid bodies and amorphous material is observed in the proximal tubule [22–24]. These changes per se have to be considered as causal factors for the observed decrease in cathepsin activity. To evaluate this possibility we
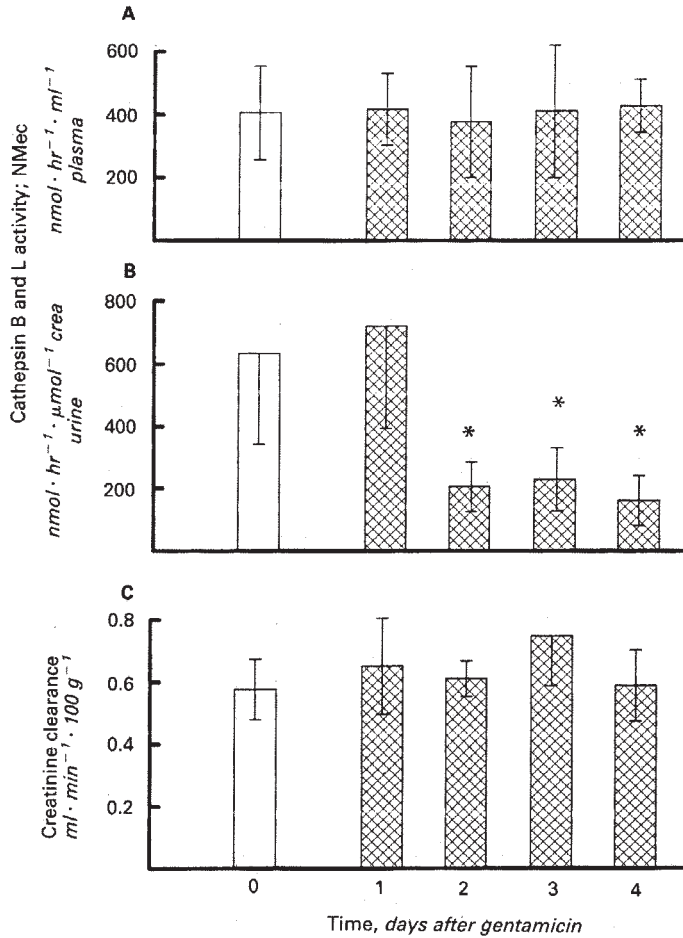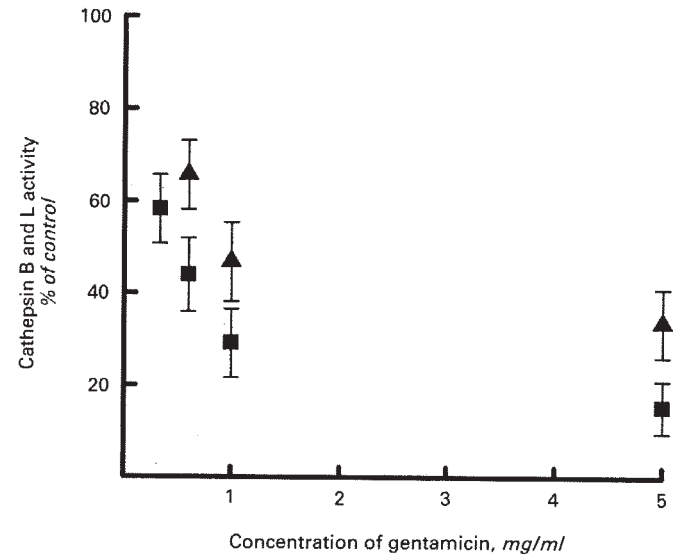
Fig. 6. *Cathepsin B and L activities in plasma and urine.* Open bars are values from rats 24 hours prior to gentamicin injection. Cross hatched bars represent data from the rats following one, two, three, and four injections of gentamicin. $N = 12$. The values are means ± SD. Asterisks indicate $P < 0.05$ vs. preinjection value. The lower panel shows the creatinine clearance of the same animals.

Fig. 7. *Cathepsin B and L activities in urine of 12 rats after four gentamicin injections.* Values were measured immediately prior to and after 24 hours of dialysis. The pre-dialysis and post-dialysis values were not different ($P > 0.05$). The enzyme activity is expressed in nmol reaction product (NMec) released per hour incubation per μl.

Fig. 8. *In vitro inhibition of cathepsin B and L activity by gentamicin.* Triangles represent urine cathepsin activities from eight rats. Squares represent enzyme activities in S1 segments from four rats. In each rat three to five segments were analyzed for each gentamicin concentration. Values are mean ± SD.

measured cathepsin activity in dextran-injected rats. Dextran is filtered by the glomerulus, is partly reabsorbed by proximal tubule cells, and induces an increase in the size and number of lysosomes [25]. In contrast to gentamicin, dextran induced an increase in proximal tubule cathepsin activities. Therefore, it appears unlikely that altered structure of lysosomes per se is the primary event leading to decreased cathepsin activity, although admittedly the changes in lysosomal ultrastructure caused by gentamicin are different from those following injection of dextran.

Following the initial lysosomal alterations the morphologic changes in proximal tubules progress to frank cellular necrosis [2, 22]. The observed decrease in cathepsin B and L activity could be related to cell necrosis. However, the reduction of cathepsin activities as early as 24 hours following a single injection of gentamicin argues strongly against necrosis as a causal factor since overt cell necrosis has not been described in the proximal tubule at that early time. Furthermore, a loss of tubular cathepsins by cell necrosis as well as a loss by an increased rate of exocytosis of lysosomes containing cathepsins
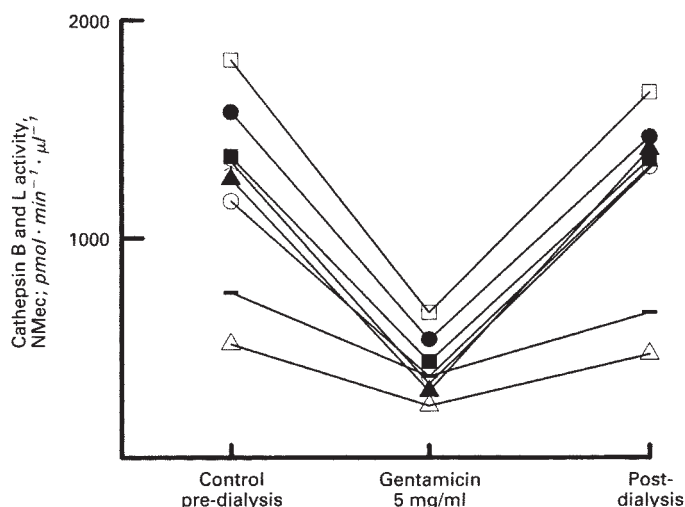
seems unlikely since the urine cathepsin excretion was actually reduced in rats following gentamicin injection. Increased urine excretion of cathepsins would be a prerequisite for both mechanisms. An additional argument against cell necrosis and exo-

Fig. 9. *Reversible inhibition of urine cathepsin B and L activity by gentamicin.* Urine samples from eight control rats were analyzed without dialysis (**A**), after one hour incubation with 5 mg/ml of gentamicin (**B**), and after incubation with gentamicin followed by 24 hours of dialysis (**C**). Pre- and post-dialysis values were not different. The inhibition of cathepsin B and L activity by gentamicin was significant ($P < 0.001$ vs. pre- and post-dialysis).

cytosis is the finding of an unchanged acid phosphatase activity following gentamicin. Both mechanisms should lead to reduced acid phosphatase activity. Therefore, the data support the interpretation that gentamicin itself induced the decrease in cathepsin B and L activities.

The finding of a similar reduction in tubular cathepsin activities following one and four injections (Fig. 1) in spite of a fourfold increase in gentamicin concentration in the kidney cortex does not argue against a causal relationship between gentamicin accumulation and cathepsin reduction. It is conceivable that the concentration following one injection was sufficient for a maximum inhibitory effect, and an increase in gentamicin concentration may not further decrease cathepsin activities. It is also conceivable that the intralysosomal concentration of gentamicin was similiar on the first and the fourth day following gentamicin injection, since the increase in kidney cortex concentration could be mainly due to an increase in the number and size of the lysosomes in the proximal tubules.

Gentamicin may reduce cathepsin B and L activity by two mechanisms, enzyme inhibition and decreased generation of active intralysosomal enzyme. Direct enzyme inhibition is the less likely mechanism. Although we found in vitro inhibition of cathepsin B and L activity in tubule segments as well as in urine, this inhibition was reversible by 24 hours of dialysis. The assay procedure for cathepsins in tubule segments included a dilution step of approximately 1:5000, since one tubule segment with about 0.01 μl volume was dissolved in 50 μl incubation solution. This dilution should be sufficient to abolish all reversible inhibition. Hence, reversible inhibition of cathepsin B and L activity, as demonstrated for urine cathepsin, is unlikely to explain the reduced enzyme activities. On the other hand, direct inhibition may be present in vivo, and the true in vivo cathepsin B and L activity could be considerably lower than

measured in the present study. This may be of functional importance.

The likely explanation for the reduced cathepsin activity is an inhibition in the generation of active intralysosomal cathepsins by gentamicin. The process of generation of active lysosomal enzymes involves biosynthesis of precursors on membrane-bound polysomes, modifications at the Golgi, and transport to and uptake into the lysosomes where proteolytic processing generates mature active enzymes [26, 27]. It is possible that one or more of these steps may be inhibited by gentamicin. For instance, inhibition of microsomal protein synthesis in the kidney cortex has been described two days following gentamicin injection [28]. It is conceivable, however, that intralysosomal proteolytic activation or precursor uptake into lysosomes or both are also inhibited by gentamicin.

The functional relevance of decreased cathepsin activities may be twofold. The inhibition of cathepsins reduced cellular protein degradation in other organs by up to 70% [12–15]. Hence, a decrease in proximal tubule protein catabolism by gentamicin seems likely. This hypothesis is supported by the finding that the degradation of low molecular weight proteins which are filtered by the glomerulus and reabsorbed by the proximal tubule, was reduced in kidney cortex from rats injected with gentamicin [29]. Degradation to amino acids occurs in the lysosomes by proteases including cathepsin B and L. Furthermore, cathepsin B and L, as well as other cysteine-proteinases are involved in the proteolytic activation of lysosomal enzyme precursors [26, 27], and a decrease in cathepsin activity may lead to decreased activities of other lysosomal enzymes. Therefore, the accumulation of phospholipids in renal cortex following gentamicin administration and the appearance of myeloid bodies in proximal tubule lysosomes, which have been attributed to reduced activities of phospholipases and sphingomyelinase [20, 30], may be the indirect result of cathepsin inhibition by gentamicin.

The link between renal functional deterioration in gentamicin nephrotoxicity, reduced protein catabolism and reduced activity of other lysosomal enzymes remains to be determined. It may be similiar to lysosomal storage disease where the reduced activity of one lysosomal enzyme causes serious morbidity.

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