Increased cathepsin D-like activity in cortex, tubules, and glomeruli isolated from rats with experimental nephrotic syndrome

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We have examined the activity and distribution of cathepsin D (EC 3.4.23.5), a major renal lysosomal endoproteinase, in the various anatomical and functional areas of normal rat kidney. Cathepsin D-like activities ($\Delta A_{280}/h$ per mg of protein) in normal rat tissues were: cortex, 0.78 ± 0.05 , $n = 37$; medulla, 0.62 ± 0.03 , $n = 12$; papilla, 0.63 ± 0.04 , $n = 12$; tubules, 0.74 ± 0.04 , $n = 28$; glomeruli, 0.59 ± 0.03 , $n = 28$; and liver, 0.41 ± 0.02 , $n = 28$. Enzyme activity was maximal at pH 3.0–3.5 and inhibited more than 90% by pepstatin ($6.7 \mu\text{g/ml}$), suggesting that the enzyme is cathepsin D. In subsequent experiments we measured cathepsin D-like activity in cortex, tubules and glomeruli isolated from rats with puromycin aminonucleoside (PAN)-induced nephrotic syndrome. Treated animals (15 mg of PAN/100 g body wt., intraperitoneally) developed proteinuria beginning 4 days after injection and exceeding 900 mg/24 h on day 9. In two separate experiments involving 52 animals we observed a significant increase in cathepsin D-like activity in cortex (+82.7%), tubules (+109.6%) and glomeruli (+54.7%) isolated from PAN-treated rats killed during marked proteinuria (day 9, mean total urinary protein excretion: 937 ± 94 mg/24 h). This increase was observed whether the activity was expressed per mg of DNA or per mg of protein. Increased cathepsin D-like activity was first observed in cortex and tubules coincident with the onset of proteinurea (day 4, mean total urinary protein excretion: 112 ± 23 mg/24 h). In contrast with the significant elevation of renal cathepsin D-like activity, the activity (nmol/h per mg of protein) of α -L-fucosidase (EC 3.2.1.51), a non-proteolytic enzyme, was markedly decreased in the identical samples used for the measurement of cathepsin D-like activity: cortex (−46.4%); tubules (−46.1%); and glomeruli (−38.5%). In addition to changes in renal enzyme activities, PAN-treated rats excreted large amounts of cathepsin D-like activity in their urine (beginning on day 3) compared with nearly undetectable cathepsin D-like activity in the urine from control rats. The significant increases in glomerular and tubular cathepsin D activity may reflect an important role for this enzyme in the pathophysiology associated with PAN-induced nephrotic syndrome.

The kidney is known to play an important role in the catabolism of a variety of peptides and plasma proteins. Higher M_r proteins are internalized by the epithelial cells of proximal tubules and subsequently degraded within the lysosomes (Bourdeau & Carone, 1974). Increased activity of this lysosomal pathway has been observed in various types of experimental and human renal disease associ-

ated with increased excretion of urinary protein (Straus, 1957; Mendel, 1961; Katz *et al.*, 1983; Jensen *et al.*, 1967; Exaire *et al.*, 1972). Although a variety of proteolytic enzyme activities have been described in the kidney (Straus, 1956; Shibko & Tappel, 1965; Thomas *et al.*, 1980; Kirschke *et al.*, 1980; Lovett *et al.*, 1983; Gossrau, 1983), only limited information is available concerning the specific lysosomal enzymes associated with the increased protein catabolism which occurs in experimental renal disease (Straus, 1957; Maack, 1967; Gang & Mautner, 1972; Olbricht *et al.*,

Abbreviations used: PAN, puromycin aminonucleoside [6-dimethylamino-9-(3'-amino-3'-deoxyribosyl)-purine]; HBSS, Hanks' balanced salt solution.

1983). Cathepsin D is a well-characterized lysosomal endoproteinase of the aspartic acid type distinguished from most other proteinases by its pH optimum and sensitivity to pepstatin (Barrett, 1977). In rat kidney, cathepsin D appears to account for a large fraction of the total proteinase activity (Shibko & Tappel, 1965; Kirschke *et al.*, 1980).

PAN-induced nephrotic syndrome is a well-characterized model of renal disease. However, previous studies of enzymic changes in kidney tissues associated with PAN-induced nephrotic syndrome have emphasized the role of non-proteolytic enzymes (Dubach & Recant, 1960; Kissane & Hoff, 1962; Nagle *et al.*, 1972; Bhuyan *et al.*, 1980; Velosa *et al.*, 1981). In the present study we have measured the activity of cathepsin D, and, for comparison, a non-proteolytic enzyme, α -L-fucosidase, in cortex, medulla, papilla, tubules and glomeruli isolated from normal rat kidney. In addition we have measured the activity of these enzymes in cortex, tubules and glomeruli isolated from rats with PAN-induced nephrotic syndrome.

Experimental

Materials

Male Sprague-Dawley rats (250–300g) were obtained from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. Haemoglobin (substrate, powder, type II), PAN, 4-nitrophenyl α -L-fucoside, pepstatin A, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Nitex sieves were purchased from Tetko, Elmsford, NY, U.S.A. All other chemicals were of reagent grade or higher.

Methods

Preparation of tissue homogenates. Rats were anaesthetized with pentobarbital (50mg/kg). A polyethylene catheter (PE 100) was inserted into the lower abdominal aorta immediately above the bifurcation and 2–3ml of blood drawn into a heparinized syringe. Subsequently the aorta was clamped above the renal arteries, the renal veins cut and the kidneys perfused with ice-cold HBSS. During perfusion of the kidneys a small piece of liver (approx. 250mg) was removed and used for preparation of a 10% (w/v) homogenate as described below. After blanching the kidneys were rapidly removed and decapsulated. All subsequent steps were carried out at 0–4°C. Homogenates (10%) of cortex, medulla, papilla, and liver were prepared in 0.15M-potassium acetate buffer, pH 4.4, containing 0.05% Triton X-100 (buffer). The homogenates were sonicated once for 5s at 0–4°C with a Branson Sonifier (model no. 575, setting no. 7) and a small portion solubilized in 0.1M-

NaOH for protein determination. The remaining homogenate was divided into small portions, quick-frozen in liquid N₂ and stored at –80°C.

Isolation of glomeruli and tubules. Glomeruli and tubules were isolated by a combination of differential centrifugation and sieving as described previously (Shah *et al.*, 1979) with minor modifications. After perfusion a small piece of cortex from each animal was immediately fixed in 10% (v/v) neutral buffered formalin for colloidal-iron staining. Kidneys were rapidly removed, each cortex carefully dissected and a small piece (approx. 100mg) homogenized in 1.0ml of buffer. The remaining cortex was minced and gently pressed through a stainless-steel sieve (250 μ m pore size). The resulting suspension of cortical tissue was washed several times in HBSS by repeated centrifugation (200g, 2min) to eliminate small fragments in the supernatant. The pellet was resuspended in HBSS and sequentially passed through Nitex nylon sieves of 390, 250 and 180 μ m-pore-size. The particles retained on the 390 and 250 μ m-pore-size sieves, consisting primarily of fragments of the proximal tubules, were pooled producing a tubular fraction. This tubular fraction was washed again on the 250 μ m-pore-size nylon sieve with HBSS to decrease glomerular contamination to negligible levels. The washed tubules were collected by centrifugation and resuspended in 6.0ml of buffer. After gentle but thorough mixing, 2.0ml of this preparation was sonicated as described above and a small portion solubilized in 0.1M-NaOH for subsequent protein determination. The remainder was divided into small portions, quick-frozen in liquid N₂ and stored at –80°C.

To obtain glomeruli the suspension passing through the 180 μ m-pore-size sieve was passed through a Nitex no. 25 screen cloth (pore size ~60 μ m), which retains glomeruli. Glomeruli prepared in this way were well preserved and without capsules. The purity of each preparation of glomeruli was evaluated by counting under the optical microscope. All preparations used in the present study were more than 90% pure. After the determination of purity, glomeruli were collected by centrifugation (200g, 2min), resuspended in 0.5ml of buffer, sonicated as above, and a small portion solubilized in 0.1M-NaOH for subsequent protein determination. The remainder was divided into small portions, frozen in liquid N₂ and stored at –80°C.

Induction of PAN nephrotic syndrome. Rats were housed in metabolic cages at least 3 days before injection (day 0) with free access to standard rat chow and water. Nephrotic syndrome (proteinuria) was induced by a single intraperitoneal injection of PAN in sterile saline (15mg/100g body

wt. as previously described; Velosa *et al.*, 1981; Abboud *et al.*, 1982). Control animals were injected with a corresponding amount of sterile saline. Urine was collected daily, and urinary protein was measured by using the Bio-Rad Protein Assay as described by the manufacturer.

Enzyme assays. Cathepsin D (EC 3.4.23.5) activity in the various tissue preparations was measured as described by Barrett & Heath (1977) with minor modifications. Each 1.5 ml microcentrifuge tube contained 50 μ l of 8% (w/v) dialysed haemoglobin (in water), 50 μ l of 1.0 M-formate buffer, pH 3.0, and exactly 100 μ g of tissue protein in a final volume of 0.2 ml. The tubes were capped, gently mixed and incubated for 60 min at 45°C. The reaction was terminated by immersing the tubes in ice/water, followed by the addition of 1.0 ml of 3.0% (w/v) trichloroacetic acid. The tubes were mixed well, left for 5–10 min in ice/water, then centrifuged at 10000g for 3 min in a Brinkman Microfuge. The clear supernatant was carefully removed and the A_{280} measured in a Varian DMS 90 spectrophotometer. Duplicate enzyme blanks (tissue homogenate added after the addition of 3.0% trichloroacetic acid) were run simultaneously for each tissue. Cathepsin D-like activity was calculated by subtracting the mean A_{280} of the appropriate duplicate enzyme blanks from the mean A_{280} of triplicate experimental tubes and expressed as $\Delta A_{280}/h$ per mg of protein. Urinary cathepsin D-like activity was measured using 100 μ l of urine (previously dialysed overnight against at least 100 vol. of phosphate-buffered saline, pH 7.4) in place of the tissue homogenate. Controls were as described above. Cathepsin-D like activity was calculated as described above (A_{280} of triplicate experimental tubes minus A_{280} of duplicate blanks) and expressed as total urinary cathepsin-D like activity (mean $\pm$ S.E.M.) per 24 h urine sample.

α -L-Fucosidase (EC 3.2.1.51, α -fucosidase) activity of the various tissue preparations was measured as described by Barrett & Heath (1977) with minor modifications. Each 1.5 ml microcentrifuge tube contained 0.2 ml of 0.3 M-sodium citrate/citric acid buffer, pH 5.5, 0.2 ml of 4-nitrophenyl α -L-fucoside (15 mM) in water, and 100 μ g of tissue protein in a final volume of 0.6 ml. Each tube was capped, mixed gently and incubated at 37°C for 2 h. The reaction was terminated by immersing the tubes in ice/water, followed by the addition of 0.6 ml of 3.3% (w/v) trichloroacetic acid. The tubes were mixed well, left in ice/water for 5–10 min, then centrifuged at 10000g for 3 min in a Brinkman Microfuge. A portion (0.8 ml) of the clear supernatant was carefully transferred to a second tube containing 0.4 ml of 0.5 M-bicarbonate/0.5 M carbonate buffer, mixed well and the A_{420} was

measured in a Varian DMS 90 spectrophotometer. Duplicate enzyme blanks (tissue homogenate added after the addition of trichloroacetic acid) were run simultaneously for each preparation. α -Fucosidase activity was calculated by subtracting the mean A_{420} of duplicate enzyme blanks from the mean A_{420} of triplicate experimental samples. Results are expressed as nmol/h per mg of protein, a molar absorption coefficient of 18100 litre $\cdot$ mol $^{-1}\cdot$ cm $^{-1}$ for the 4-nitrophenyl anion (Borooah *et al.*, 1961) being used.

Other analytical methods. The protein content of tissue samples was determined, after solubilization of the tissue in 0.1 M-NaOH, by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, type V) as the reference standard. Total plasma protein was measured by the method of Lowry *et al.* (1951) in trichloroacetic acid precipitates of serum that had been resolubilized in 0.1 M-NaOH. Plasma creatinine was determined by using a Beckman Creatinine Analyzer 2. DNA was measured by the method of Fiszer-Szafarz *et al.* (1981) with calf thymus DNA as the standard. Colloidal-iron staining of glomerular polyanion (sialoglycoprotein) was performed essentially as described by Mowry (1963).

Results and discussion

Since the distribution and activity of cathepsin D in kidney had not been previously reported, we first examined cathepsin D-like activity in the major anatomical subdivisions of the kidney: cortex, medulla and papilla. For comparison we also measured cathepsin D activity in liver obtained from the same animals (Table 1). Our data, including pH optimum (3.0–3.5), inhibition by pepstatin (>90%, 6.7 μ g/ml) and high activity with haemoglobin as a substrate, indicate that the enzyme activity measured is cathepsin D (Barrett, 1977). However, we prefer the term 'cathepsin D-like activity' pending further characterization. Highest cathepsin D-like activity was found in renal cortex, with slightly lower activity in medulla and papilla (Table 1). These results are in general agreement with those previously published by Shibko & Tappel (1965), who observed approximately equal activities of 'cathepsin' in homogenates of whole kidney, medulla and papilla. The activity of α -fucosidase, a non-proteolytic enzyme, was also measured in the same fractions. α -Fucosidase activity was also highest in cortex. However, in contrast with cathepsin D, medulla and papilla had only one-third the α -fucosidase activity of cortex (Table 1).

Because of the major role of the proximal tubule in renal protein catabolism we then measured

cathepsin D-like activity in tubules and, for comparison, glomeruli isolated from normal rat kidney. As shown in Table 1, tubules contained slightly higher cathepsin D-like activity compared with glomeruli isolated from the same cortex. α -Fucosidase activity of tubules was markedly higher (3.5-fold) than that of glomeruli. The lower cathepsin D-like and α -fucosidase activity in glomeruli compared with tubules and cortex is in good agreement with data previously reported for α -fucosidase (Velosa *et al.*, 1981) and other lysosomal enzymes, which, with the exception of acid phosphatase, appear to have lower activity in glomeruli (Taylor *et al.*, 1971; Velosa *et al.*, 1981; Lovett *et al.*, 1982).

We next examined renal cathepsin D-like activity in PAN-induced nephrotic syndrome. In keeping with previously published data (Abboud *et al.*, 1982; Mayer *et al.*, 1981), preliminary experiments established that, after the intraperitoneal injection of PAN (day 0), increased protein excretion begins after 3–4 days and reaches a maximum on day 8–10. We refer to these times as onset and peak proteinuria respectively. In the present study, rats injected with a single dose of PAN (15mg/100g body wt., intraperitoneally) developed proteinuria beginning 4 days after injection and exceeding 900mg/24h by day 9, when all of the animals were killed (Fig. 1). This proteinuria was accompanied by hypo-proteinaemia, ascites, lipaemia, and loss of glomerular colloidal-iron staining as previously described (Michael *et al.*, 1970; Table 2).

In the first series of experiments, cathepsin D-like activity and α -fucosidase activity were

measured in homogenates of renal cortex, tubules and glomeruli obtained from animals killed during peak proteinuria (day 9). It should be emphasized that control and experimental animals were killed on the same day and that enzyme assays from control and experimental animals were run simultaneously. As shown in Fig. 2, we observed a significant increase in cathepsin-D like activity in cortex, tubules and glomeruli isolated from rats killed during the peak of proteinuria (day 9). This increase was observed whether expressed per mg of protein or per mg of DNA (results not shown).

In contrast with this increase in cathepsin D-like activity, we observed a marked decrease in α -fucosidase activity in the identical preparations of cortices, tubules and glomeruli used for the measurement of cathepsin D-like activity (Fig. 2). A decrease in cortical and glomerular α -fucosidase activity as well as a decrease in several other lysosomal enzymes has previously been reported in PAN-induced nephrotic syndrome (Velosa *et al.*, 1981). Thus the increase in cathepsin D-like activity does not appear to be the result of a generalized increase in lysosomal enzyme activity or non-specific response to PAN.

The relationship between changes in renal cathepsin D-like activity and proteinuria was further investigated in a second series of experi-

Table 1. Cathepsin D and α -fucosidase activity in normal rat kidney and liver

Killing of the animals, tissue processing and enzyme assays are described in the Experimental section. Medulla and papilla were always assayed simultaneously with the corresponding cortex. The same homogenate was used for both cathepsin D and α -fucosidase measurements. Enzyme activities are expressed as means $\pm$ S.E.M. of triplicate determinations for the number of animals shown in parentheses.

Tissue	Cathepsin D activity ($\Delta A/h$ per mg of protein)	α -Fucosidase activity (nmol/h per mg of protein)
Cortex	0.78 ± 0.05 (37)	211.3 ± 15.0 (20)
Medulla	0.62 ± 0.03 (12)	78.7 ± 6.2 (12)
Papilla	0.63 ± 0.04 (12)	78.7 ± 2.7 (12)
Tubules	0.74 ± 0.04 (28)	225.7 ± 11.0 (13)
Glomeruli	0.59 ± 0.03 (28)	72.4 ± 4.7 (13)
Liver	0.41 ± 0.02 (28)	22.7 ± 2.2 (14)

Fig. 1. Total urinary protein excretion (mg/24h) in control (saline-injected, $\circ$) and PAN-treated rats ($\bullet$)

Rats were housed in individual cages with free access to food and water. Urinary protein was determined by the Bio-Rad Protein Assay. Values are expressed as means $\pm$ S.E.M. of duplicate determinations for eight animals in each group. Significantly different from controls: * $P \leq 0.01$; † $P \leq 0.001$ (unpaired *t* test).

Table 2. Physiological data for expt. 1

Rats were housed in individual cages with free access to food and water. After a 2–3-day adaptation period, animals were injected (day 0) with saline (controls) or PAN on a staggered schedule to allow the simultaneous killing of two controls and two PAN-treated rats each day. Urine was collected every 24h. Rats were killed 9 days after injection. Values shown represent means $\pm$ S.E.M. for the number of animals shown in parentheses. Details are given in the Experimental section.

Parameter measured	Day of Measurement	Experimental group:		
		Control	n	PAN-treated
Weight	0	283 $\pm$ 3.5	(8)	285 $\pm$ 3.3
	9	333 $\pm$ 9.3	(8)	302 $\pm$ 6.6
Urinary protein (mg/24h)	9	34.0 $\pm$ 3.6	(8)	982 $\pm$ 134
Serum protein (g/100ml)	9	4.87 $\pm$ 0.11	(8)	3.55 $\pm$ 0.18
Serum creatinine (mg/100ml)	9	0.34 $\pm$ 0.06	(8)	0.47 $\pm$ 0.08
Lipaemia*	9	0/8		8/8
Ascites*	9	0/8		8/8

* Occurrence/total number of animals.

Fig. 2. Cathepsin D activity (a) and α -L-fucosidase activity (b) in renal fractions prepared from rats 9 days after injection with saline (controls, $\square$) or PAN ($\blacksquare$) Results are means $\pm$ S.E.M. of triplicate determinations for eight animals in each group. Significantly different from appropriate control: * $P \leq 0.01$; † $P \leq 0.001$ (unpaired t test). See the Experimental section for details.

ments in which cathepsin D-like activity was measured at the onset of proteinuria (day 4) as well as at the peak of proteinuria (day 9). Rats were treated exactly as described in the first experiment, except that injection schedules were arranged to allow killing and tissue processing of one animal

Fig. 3. Cathepsin D activity in renal fractions prepared from rats 4 days and 9 days after injection with saline (controls, $\square$) or PAN ($\blacksquare$) Results are means $\pm$ S.E.M. of triplicate determinations for eight (cortex) or ten (tubules and glomeruli) animals. Significantly different from appropriate controls: * $P \leq 0.001$; † $P \leq 0.02$ (unpaired t test). See the Experimental section for details.

from each group (day 4 and day 9, control and experimental) on the same day. After all the animals had been killed, cathepsin D-like activity was then simultaneously measured in control and experimental tissue fractions. No significant changes in cathepsin D-like activity were observed in day-4 controls versus day-9 controls (Fig. 3). Coincident with the onset of protein excretion (day 4, mean total urinary protein excretion: 112 ± 23 mg/24h, $n = 10$) we observed a moderate increase in cathepsin D-like activity in both cortex and tubules of PAN-treated rats (Fig. 3). In common with the experiment above (Fig. 2), a marked increase in cathepsin D-like activity was observed in cortex, tubules and glomeruli isolated

from PAN-treated rats killed at the peak of urinary protein excretion (day 9, mean total urinary protein excretion: 901 ± 63 mg/24h, $n = 9$). This increase was significant ($P \leq 0.01$) when compared with results for both 4-day PAN-treated and 9-day control animals.

Cathepsin D-like activity was also measured in urine samples obtained from control and PAN-treated rats. Urinary enzyme activity was optimal at pH 3.0–3.5 and completely inhibited by pepstatin ($6.7 \mu\text{g/ml}$; result not shown), suggesting that this activity is also cathepsin D. However a role for other aspartic proteinases such as uropepsinogen (EC 3.4.23.-) in this urinary cathepsin D-like activity can not be ruled out on the basis of these data. As shown in Fig. 4, cathepsin D-like activity was hardly detectable in dialysed urine samples from control rats. In contrast, relatively large amounts of cathepsin D-like activity were measured in dialysed urine samples obtained from PAN-treated animals. This increase in urinary cathepsin D-like activity was coincident with the onset of proteinuria (day 4) and continued to increase up to day 9, when the animals were killed. These findings are in agreement with those of Gang & Mautner (1972), who reported increases in

the urinary excretion of alkaline and acid proteinases in rats treated daily with subcutaneous injections of PAN (1.5 mg/100g body wt.). However, in contrast with the present work, those workers observed no change in the acid-proteinase activity in kidneys obtained from PAN-treated rats.

In summary, in two separate experiments we have observed a significant increase in cathepsin D-like activity in cortex, tubules and glomeruli isolated from rats with PAN-induced nephrotic syndrome killed at the time of peak proteinuria. In contrast, the activity of α -fucosidase, a non-proteolytic enzyme, was significantly decreased in these same renal fractions. Increased renal cathepsin D-like activity was first observed coincident with the onset of proteinuria (day 4) in cortex and tubules, and was accompanied by a significant increase in the excretion of urinary cathepsin D-like activity. The largest increase in cathepsin D-like activity was observed in the tubular fraction, a result in keeping with the specialized function of the proximal tubule in the catabolism of filtered protein. However, the significant increase in cathepsin D-like activity of glomeruli, coupled with the previous observation of Caulfield *et al.* (1976), showing increased numbers of lysosomes in glomerular epithelial cells in response to PAN-induced nephrotic syndrome, suggests a potentially important role for cathepsin D in PAN-induced glomerular injury.

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Fig. 4. Urinary cathepsin D-like activity

Values represent cathepsin D-like activity (mean $\pm$ S.E.M.)/24 h urine sample ($\Delta A_{280}/100 \mu\text{l} \times 10 \times 24$ h urine volume) for 8–13 rats in each group. Significantly different from control: $P \leq 0.01$, $\dagger P \leq 0.001$ (unpaired *t* test). Further details are given in the text.

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