Kidney International, Vol. 50, No. 2 (1996), pp. 424–431

Alterations of cathepsins B, H and L in proximal tubules from polycystic kidneys of the Han:SPRD rat

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Alterations of cathepsins B, H and L in proximal tubules from polycystic kidneys of the Han:SPRD rat. Abnormalities of tubular matrix metalloproteinases have been shown recently to occur early in the course of polycystic kidney disease (PKD). The present study was conducted to determine whether lysosomal cysteine proteinases were altered in proximal tubules from 2-month-old, heterozygous Han:SPRD rats. The activities of cathepsins B (−45%), H (−39%) and L (−37%) were significantly lower in proximal tubules from PKD rats as compared to healthy offspring. Enzyme proteins were also decreased (cath. B, 2.4 ± 0.7-fold; cath. H, 1.9 ± 0.6-fold; $N = 4$, $P < 0.05$), while mRNA levels for cathepsins B, H and L were not different. Tubular cystatin C, a major inhibitor of cathepsins, was normal with regard to protein and mRNA levels in PKD animals. The decrease in cathepsins in PKD was specific for tubules, as enzyme activities in glomeruli and liver tissue were unchanged and limited to the lysosomal compartment, since marker enzymes for cytoplasm, endoplasmatic reticulum and mitochondria were all normal. Intralysosomally, soluble enzymes like cathepsins and β-NAG were decreased, while membrane-bound acid phosphatase was unchanged. The presence of cathepsins could be demonstrated in cyst fluid from homozygous PKD rats and urinary excretion of cathepsins was enhanced in heterozygous animals. Taken together, these findings indicate that the reduction in tubular cathepsins B, H and L was neither due to decreased gene expression nor to up-regulation of specific inhibitors, but was likely due to enhanced apical secretion of these enzymes.

A number of studies on human and experimental forms of polycystic kidney disease (PKD) suggest that an abnormal matrix plays a key role in cystogenesis [1–3]. This in turn could lead to abnormal cell/basement membrane interactions [4, 5] that may be central to the pathogenesis of PKD. This assumption has been considerably strengthened recently by reports [6, 7] showing that the PKD1 gene encodes a high molecular weight protein of circa 500,000 kDa with multiple cell recognition domains. This novel protein which has been designated "polycystin," thus may be involved in cell-cell/matrix interactions.

In general, the turnover of extracellular matrix components depends on the balance between their synthesis and degradation. Thus, excessive matrix deposition may occur either when synthesis is enhanced and/or when the activity of proteinases is down-regulated. Recently, we [8] and others [9] have reported on alterations of matrix metalloproteinases (MMPs) and their inhibitors in tubular epithelium from polycystic kidneys.

Received for publication December 20, 1995
and in revised form February 26, 1996
Accepted for publication February 26, 1996

Apart from metalloproteinases the mammalian kidney contains high activities of cysteine proteinases, such as cathepsins B, H and L [10, 11], both within the glomerulus and proximal tubule. Intracellularly these enzymes are located within lysosomes. They have a relatively broad substrate specificity and an optimum at acidic pH [12]. Their ability to degrade intact basement membranes as well as isolated matrix components has been widely documented [10, 13–17]. Besides direct degradation of extracellular matrix, cathepsin B has been found to indirectly regulate matrix turnover by proteolytically activating prourokinase as well as latent forms of metalloproteinases [18, 19].

Abnormalities in lysosomal proteinases in human ADPKD epithelia have already been addressed in preliminary form by Wilson and Hartz [20, 21]. However, until now there has been no direct evidence for alterations in tubular cathepsins coming from any animal model of PKD. Thus, the objective of the present study was to investigate whether cathepsins B, H and L and their inhibitors were altered in proximal tubules from heterozygous PKD rats (Han:SPRD) in an early stage of cystogenesis.

Methods

Animals

Homozygously (cy/cy) and heterozygously (cy/+) affected male Han:SPRD rats as well as healthy littermates (+/+) were used in this study. This model of ADPKD is derived from a spontaneous mutation in the Sprague-Dawley strain and the disease is transmitted in an autosomal dominant pattern. Homozygous animals develop excessive cysts with gross renal enlargement and die early (after 4 weeks) from renal failure. By contrast, heterozygously afflicted rats suffer from slow, progressive renal cystic disease and death usually occurs after 12 to 21 months [22–24].

Heterozygously affected Han:SPRD rats (cy/+) were investigated at the age of two months. Age-matched, unaffected littermates (+/+) were used as control animals. The presence of enlarged kidneys was checked by abdominal palpation and further controlled by histopathology to identify affected animals. Cyst fluid was obtained from homozygous three-week-old Han:SPRD rats, because only homozygous animals develop cysts large enough to harvest quantitative amounts of cyst fluid [24]. All animal experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Isolation and characterization of glomeruli and proximal tubules

Animals were anesthetized with hexobarbital (150 mg/kg), the kidneys removed and glomeruli and tubules isolated according to

the method of Spiro [25]. In brief, renal cortex from one kidney of each animal was minced and resuspended in ice-cold saline. A sample of the total homogenate was reserved and the rest of the homogenate was passed through a stainless steel sieve (250 μm pore size, Linker, Kassel, Germany). The sieved material was suspended in ice-cold saline and poured over a 50 μm nylon sieve (Schweizerische Seidengazefabrik, Zurich, Switzerland), thereby allowing cell debris and small fragments to pass through, while retaining glomeruli and tubules. Separation of tubules from glomeruli was achieved by passage on a 150 μm nylon sieve. The tubuli which were retained by this sieve were resuspended on 50% Percoll and centrifuged at 12.200 g for 30 minutes at 4°C according to Vinay et al [26]. This preparation contained proximal tubules obtained from a single kidney after sieving and Percoll gradient separation.

The fraction containing proximal tubules was resuspended in 0.9% saline, washed three times and then examined by light microscopy. A sample from each preparation was also processed for histological evaluation after formalin fixation and periodic acid Schiff (PAS) staining. This allows for clear differentiation between proximal (granular yellow cytoplasm and pink brush border membrane) and distal tubular epithelia (clear cytoplasma, no brush border membrane) [26]. The purity of the proximal tubular preparation was 95%. Isolated proximal tubules were either used for RNA isolation or were disrupted by sonication (5 bursts, 2 seconds each) at 4°C for the measurements of enzyme activities.

Glomeruli were collected after passage through the 150 μm nylon sieve. Purity, controlled by light microscopy, was 90 to 95%. Glomeruli from each kidney preparation were counted thrice in a Fuchs-Rosenthal chamber. Isolated glomeruli were disrupted by sonication (3 bursts, 2 seconds each) at 4°C prior to the determination of cathepsin activities.

Preparation of liver homogenates

Portions of livers (1 g each) were homogenized in cooled isotonic saline using 10 strokes of a Potter homogenizer. The homogenates were sonicated (8 bursts, 2 seconds each) and then centrifuged at 10,000 g for 10 minutes. The supernatants were used for measuring cathepsin activities.

Cyst fluid samples

Cyst fluids from homozygous polycystic kidneys were obtained by puncture and were free of leukocytes. Proteolytic enzyme activities were measured in supernatants after centrifugation at 5,000 rpm for 10 minutes.

Urine samples

Urine was collected for 24 hours in metabolic cages, examined in order to exclude the presence of leukocytes and centrifuged for 10 minutes at 5,000 rpm. Prior to protein and activity measurements of cathepsins the supernatants were dialyzed for 24 hours at 4°C against four changes of a solution composed of 30 mM potassium citrate, pH 6.0/100 mM NaCl/0.1% Triton X-100, using dialysis sacks (Sigma, St. Louis, MO, USA) retaining proteins greater than 12 kDa. After dialysis, urine samples were concentrated 25-fold by microconcentrators (Microcon 10; Amicon, Beverly, MA, USA).

Cathepsin B, H and L activities

The activities of cathepsin B, H, and L were measured as previously described by Barrett and Kirschke [27] and Baricos et al [16] using fluorogenic peptidyl substrates (peptidyl-7-amido-4-methylcoumarins, AMC; Bachem, Heidelberg, Germany): Z-Arg-Arg-AMC for cathepsin B, H-Arg-AMC for cathepsin H and Z-Phe-Arg-AMC as the common substrate for cathepsin B and L in combination with 0.6 μM of the cathepsin L inhibitor Z-Phe-Phe-CHN_2 (Enzyme Systems Products, Dublin, CA, USA). Cathepsin L activity was calculated as the difference in Z-Phe-Arg-AMC hydrolysis in the presence and absence of the cathepsin L inhibitor. In the case of cathepsin H, 0.1 mM puromycin hydrochloride was used to inhibit arylamidases. Experiments addressing the specificity of these substrates, showed 100% inhibition of hydrolysis of each of the peptidyl 7-amino-4-methylcoumarin substrates by a 15 minute pre-incubation with 10 μM E-64 (cysteine proteinase inhibitor). The fluorescence of free AMC was determined by excitation at 360 nm and emission at 460 nm using a Luminescence Spectrometer (LS 50; Perkin Elmer, Langen, Germany). One unit of activity was defined as the production of 1 nm of AMC/min.

Western blot analysis

Prior to Western blot analysis of cathepsins B and H, homogenates of proximal tubules containing equal amounts of protein or samples of cyst fluid were semipurified as described previously [15, 28]. Briefly, homogenates of proximal tubules were extensively extracted with 0.2% (vol/vol) Triton X-100. The extracts as well as cyst fluids were autolysed at 40°C for one hour and the insoluble material was removed by centrifugation. Material precipitated by 30 to 70% ammonium sulphate was collected and dialyzed against 10 mM sodium acetate (pH 4.3) and subsequently concentrated 10-fold (Microcon 10, Amicon). To determine the rate of recovery, the activities of cathepsin B and H were measured before and after semipurification.

Prior to Western blot analysis of cystatin A and C, homogenates of proximal tubules containing equal amounts of protein were treated with alkali (pH 11.0) to inactivate cellular cysteine peptidases, mixed with equal volume of 0.1 M Tris-HCl buffer (pH 7.4) containing 1.0 M NaCl and 5% inhibitor cocktail concentrate (0.1 M benzamidinum chloride, 0.2 M EDTA, 2% (wt/vol) sodium azide) and applied to a papain affinity filtration cartridge (Sigma). After washing with 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM NaCl the bound material was eluted with 0.2 M trisodium phosphate, pH 12.7/0.5 M NaCl and immediately neutralized with 2 M Tris-HCl, pH 7.4 [29].

SDS polyacrylamide gel electrophoresis was carried out following the method of Laemmli [30] using 10% (wt/vol) gels for cathepsins B and H and 15% (wt/vol) gels for cystatins A and C. Proteins were transferred electrophoretically at 100 V for one hour to nitrocellulose membranes using the Bio-Rad Mini Trans-Blot Apparatus as recommended by the manufacturer. The membranes were blocked by incubation with 1% (wt/vol) BSA in PBS containing 0.1% (vol/vol) Tween 20 (PBST) for two hours. After washing in PBST for 25 minutes, the nitrocellulose was incubated over night at 4°C in PBS containing 0.05% Tween 20 and 1% BSA (PBSTB) with the following primary antibodies:

(1) Rabbit polyclonal antibody to recombinant rat cathepsin B (provided by Dr. J.S. Mort, Montreal, Canada) in a dilution of 1:400.

(2) Rabbit polyclonal antibody to human cathepsin H (RD Laboratories, Diessen, Germany) in a dilution of 1:200.
(3) Rabbit polyclonal antibody to rat cystatin A (provided by Dr. K. Fukuyama, San Francisco, CA, USA) in a dilution of 1:50 to 1:300.
(4) Rabbit polyclonal antibody to human cystatin C (RD Laboratories, Diessen, Germany) in a dilution of 1:200.
(5) Rabbit polyclonal antibody to human cathepsin L (RD Laboratories, Diessen, Germany).

Thereafter, the nitrocellulose strips were washed for one hour in PBST and incubated in PBSTB containing the second antibody in a dilution of 1:2000 (anti-rabbit IgG, peroxidase-linked, species-specific whole antibody) from Amersham (Amersham, UK) for one hour. The strips were then extensively washed in PBST and incubated in ECL Western blotting detection reagent (Amersham) and exposed to Kodak X-OMAT AR film.

Northern blot analysis

Northern blots were performed on total RNA extracted from proximal tubules isolated from single animals with the acid guanidinium thiocyanate phenol chloroform extraction method [31]. Total RNA was quantitated by UV spectrophotometry at 260 nm and 280 nm. RNA samples (20 to 30 μg per lane) were denatured and electrophoresed through 1.2% agarose formaldehyde gels and then transferred by pressure blotter (Posi Blot, Stratagene, La Jolla, CA, USA) onto nylon filters (GeneScreen, DuPont-NEN, Boston, MA, USA). The RNA was fixed to the filters by ultraviolet irradiation for two minutes at 254 nm (UV Stratalinker, Stratagene).

All probes were radiolabeled with [^{32}P]dCTP (3.000 Ci/mmol) using the DECAprime DNA labeling kit (Ambion, Austin, TX, USA). Membranes were prehybridized at least for two hours at 42°C in 5 × SSPE/50% deionized formamide/5× Denhard's/0.5% SDS/50 μg/ml herring sperm DNA. Hybridization was carried out overnight at 42°C. The filters were washed twice for 15 minutes in 2× SSPE at room temperature and then agitated in 2× SSPE/2% SDS at 65°C for 30 minutes and exposed to Kodak X-OMAT AR film at −80°C for one to two days. After autoradiography, the filters were stripped and hybridized according to the same procedure with a human GAPDH cDNA probe (1.2 kb Pst I restriction fragment of pBR 322). A 0.24 to 9.5 kb RNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used as reference for RNA size. The autoradiographs were digitized using an EPSON GT 6000 scanner (Epson, Tokyo, Japan). For all RNA samples the density of each individual RNA band was normalized for that of a GAPDH mRNA band to correct for the difference in RNA loading and/or transfer. Quantitative data were presented only on signals in the linear range of the film and the densitometer.

The plasmid containing cDNA for rat cathepsin B was provided by Dr. S.J. Chan (Howard Hughes Medical Institute, Chicago, IL, USA) and cDNA for murine cathepsin L was a gift from Dr. G.G. Sahagian (Tufts University, Boston, MA, USA). The plasmid containing cDNA for human cathepsin H was provided by Dr. J. Dodt (University of Darmstadt, Germany) and for murine cystatin A by Dr. K.A. Siminovitch (University of Toronto, Canada). cDNA for murine cystatin C was obtained from ATCC (Rockville, MD, USA).

Restriction fragments isolated from these plasmids were as follows: EcoRI 2.0 kb fragment for cathepsin B; Pst I-BamHI 1.4 kb fragment for cathepsin L; EcoRI-PstI 1.1 kb fragment for cathepsin H; XhoI 0.4 kb fragment for cystatin A; EcoRI 0.67 kb fragment for cystatin C.

Table 1. Body, single kidney weights and renal function in heterozygous PKD and control rats

Han:SPRD animals	Heterozygous PKD	Controls
Body weight *g*	329 ± 7	337 ± 8
Kidney weight *g*	2.3 ± 0.17[a]	1.1 ± 0.06
Serum creatinine *mg/dl*	0.56 ± 0.03	0.52 ± 0.02
Creatinine clearance *μl/min/100 g*	481 ± 22	496 ± 21

Data are given as means ± SEM from 9 animals in each group.
[a] $P < 0.05$ for heterozygous PKD vs. healthy offspring (2-months-old)

Analytical methods

Each preparartion of isolated proximal tubules and samples of total cortical homogenates from one kidney were assayed for fructose-1,6-diphosphatase activity according to the method of Pontremoli et al [32]. Acid phosphatase [33], N-acetyl-glucosaminidase [34], glucose-6-phosphatase [35], cytochrome C oxidase [36] and lactate dehydrogenase [37] were assayed in the isolated proximal tubules by established methods. Results were expressed as U (nM of substrate converted per min) per tubular preparation (proximal tubules isolated from one kidney).

Tissue protein content was determined according to the method of Smith, Krohn and Hermanson [38]. Analysis of serum and urine samples for creatinine was performed using a Technicon autoanalyzer (Bayer Diagnostics, Munich, Germany).

Statistics

Results were expressed as means ± SEM. Statistical analysis was performed using the unpaired Student's *t*-test. Significance was accepted at the 5% level.

Results

Kidney weight and function in heterozygous PKD rats

The body weight was comparable in two-month-old polycystic (329 ± 7 g) and control animals (337 ± 8 g). Kidney weight was considerably higher in PKD rats (2.3 ± 0.2 g) than in control animals (1.2 ± 0.1 g) and the enlarged kidneys could be readily detected by abdominal palpation. Polycystic animals displayed normal glomerular filtration rates, as measured by creatinine clearance (Table 1).

Characterization of isolated proximal tubules

Light microscopy of PAS-stained preparations revealed that proximal tubules were isolated to a purity of 95% and no glomeruli were detectable. To demonstrate that the isolation procedure resulted in comparable preparations from both PKD and control animals, the activity of fructose-1,6-diphosphatase, a specific marker for proximal tubular epithelial cells [26], was measured both in the kidney cortex homogenate and in the final proximal tubule preparation. Enzyme activity in the cortical homogenate was comparable in PKD and control rats (PKD 283.5 ± 21.4 vs. controls 276.3 ± 24.2 U/kidney cortex; $N = 9$). In parallel, isolated tubules from the initial homogenate also displayed more or less identical fructose-1,6-diphosphate activities

Table 2. Cysteine proteinase activities in proximal tubules from heterozygous PKD and control rats

	Cathepsin B	Cathepsin H	Cathepsin L
	U/tubular preparation		
PKD	4.45 ± 0.23[a]	2.98 ± 0.21[a]	8.19 ± 0.70[a]
Controls	8.04 ± 0.49	4.88 ± 0.19	12.92 ± 0.71

Data are given as means ± SEM from 9 animals in each group. Tubular preparations represent proximal tubules from one kidney.
[a] $P < 0.05$ for PKD animals vs. healthy offspring (2-months-old)

(PKD 218.3 ± 23.2 vs. controls 216.1 ± 20.9 U/tubular prep.; $N = 9$). This represented a 77.0% and 78.2% recovery, respectively and indicated that the isolation resulted in comparable tubular preparations both from normal and diseased kidneys. Furthermore, the amount of protein in both preparations was comparable (PKD 5.9 ± 0.7 vs. controls 6.3 ± 0.5 mg per tubular preparation; $N = 9$).

Cathepsins B, H and L in proximal tubules from PKD and control rats

The activities of cathepsin B (−45%), cathepsin H (−39%) and cathepsin L (−37%) were significantly reduced in proximal tubules from PKD as compared to control animals (Table 2). This could be due either to lower enzyme proteins or due to enhanced levels of inhibitor proteins. Thus, Western blot analysis was performed using polyclonal antibodies to recombinant rat cathepsin B or human cathepsin H. Prior to electrophoresis, homogenates of proximal tubules containing equal amounts of protein were semipurified. The recovery of cathesin B and H activities, measured before and after purification, was comparable both in PKD and the control groups (cathepsin B, PKD, 78.7 ± 8.1%, control, 73.9 ± 7.4%; cathepsin H, PKD, 69.9 ± 7.1%, control, 71.5 ± 8.6%; $N = 4$) and thus allowed semiquantitative evaluation of specific protein bands.

As can be seen in Figure 1, antiserum to cathepsin B recognized two protein bands of Mr 31 and 29 kDa, respectively, while cathepsin H gave a single band of Mr 28 kDa in proximal tubules both from PKD and control rats. These Mr values are consistent with those reported in the literature [39–41]. Densitometric evaluation showed that tubular cathepsin B and H proteins from PKD rats were 2.4 ± 0.7-fold and 1.9 ± 0.6-fold ($N = 4$, $P < 0.05$) lower as compared to control animals. To demonstrate cathepsin L protein in isolated tubules, a polyclonal antiserum to human cathepsin L was used both in ELISA technique and Western blot analysis. However, there was no cross-reactivity with rat cathepsin L and therefore, we could not obtain data on cathepsin L protein.

Tubular mRNA expression of cathepsins B, H and L

Northern blot analyses of cathepsin B, H and L were performed in proximal tubules from PKD and control rats (Fig. 2). Densitometric evaluation of of signals for cathepsins B, H and L mRNAs (each mRNA was probed 3 times) revealed no difference between proximal tubules from PKD and those from control animals.

Marker enzymes for subcellular comparments of proximal tubules

To examine whether proximal PKD tubules displayed more wide spread disturbancies of other enzyme systems (as oppossed to a more specific alteration in lysosomal cathepsins) other

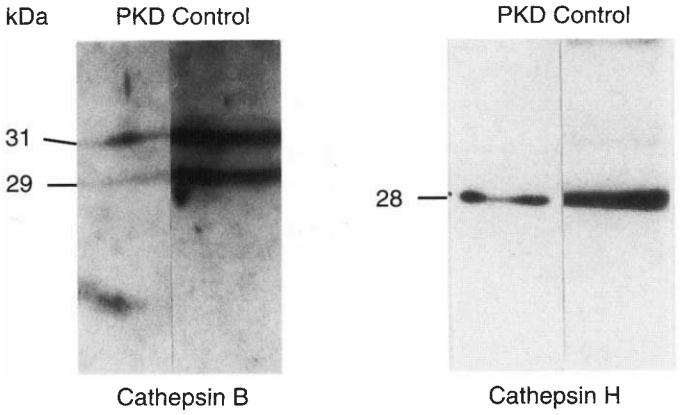

Fig. 1. *Western blot analysis of cathepsins B and H in proximal tubules isolated from PKD and control rats.* Polyclonal antibody to rat cathepsin B recognized 2 bands of Mr 31 and 29 kDa, while polyclonal antibody to human cathepsin H recognized a single band of Mr 28 kDa. Enzyme proteins, measured by densitometry was for cathepsin B 2.4 ± 0.7-fold and for cathepsin H 1.9 ± 0.6-fold lower ($N = 4$; $P < 0.05$) in tubular preparations of PKD animals.

lysosomal enzymes (β-NAG and acid phosphatase) and enzymes from other subcellular compartments, such as mitochondria (cytochrome c oxidase), endoplasmatic reticulum (glucose-6-phosphatase) and cytosol (lactate dehydrogenase) were determined. There was 1.7-fold reduction in β-NAG activity in proximal PKD tubules, while acid phosphatase activity was unchanged. In terms of cytochrome c oxidase, glucose-6-phosphatase and lactate dehydrogenase there was no difference between tubules from PKD and control animals (Table 3).

Cathepsin activities in isolated glomeruli and liver samples

To examine whether alterations of cathepsin activities occur only in proximal tubules in PKD animals and are specific for those cells involved in cystogenesis or can be observed in other parts of the kidney or other tissues as well, activities of these enzymes were measured in isolated glomeruli and liver samples. Both glomeruli and liver are not affected in the male, heterozygous Han:SPRD rat.

The amount of isolated glomeruli obtained from one kidney was comparable in both groups (PKD 24,183 ± 1,863 vs. controls 25,014 ± 1,524 glomeruli/kidney; $N = 9$). Liver weights in both groups were similar (PKD 11.6 ± 1.1 vs. controls 12.0 ± 1.2 g; $N = 9$). There were no differences in the activities of cathepsins B, H and L neither in glomeruli nor in liver samples obtained from PKD or control animals (Table 4).

Cathepsin B, H and L in cyst fluid from homozygous PKD rats

Cyst fluid from homozygous PKD rats displayed considerable activities of cathepsin B (31.6 ± 6.1 mU/ml), cathepsin H (173.2 ± 16.1 mU/ml), as well as cathepsin L (12.9 ± 2.4 mU/ml). Western blotting using polyclonal antibodies to cathepsins B and H recognized two protein bands of Mr 31 and 29 kDA in case of cathepsin B and a single 28 kDa band for cathepsin H (Fig. 3).

Urinary excretion of cathepsins in PKD and control rats

As can be seen in Table 5, urinary excretion of cathepsins B, H and L activities in PKD rats was 72%, 92% and 79% higher

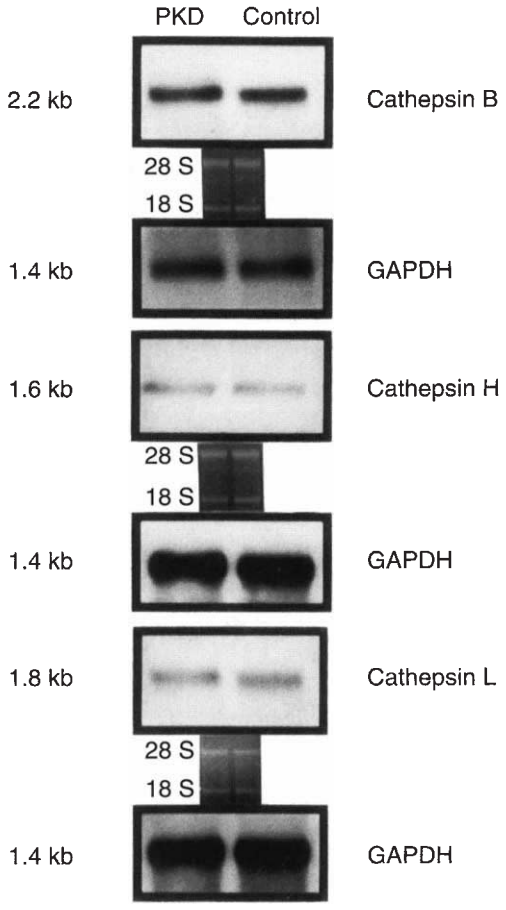

Fig. 2. *Northern blot analysis (N = 3) of tubular cathepsin B, H and L mRNAs of PKD and control rats.* As measured by densitometry, the levels of mRNA coding for cathepsin B were identical in PKD and healthy controls. Expression of GAPDH mRNA in the same membrane is shown on the bottom panel. The 18S and 28S ribosomal RNA transcripts detected by ethidium bromide staining are shown on the middle panel to indicate equality of loading and integrety of RNA.

compared to healthy control animals. Based on our observation that creatinine clearances were identical in PKD and control rats (Table 1), enzyme activities were given as activity excreted over 24 hr (mU/24 hr).

Western blot analysis of cathepsin B and H in urine samples of PKD animals demonstrated the presence of cathepsin B as two protein bands of 31 and 29 kDa and a single cathepsin H band of 28 kDa. By contrast, cathepsins B and H were hardly detectable by Western blotting in the urine of healthy control animals (Fig. 4).

Cystatins in proximal tubules from heterozygous PKD and healthy control rats

Since alterations of cathepsin activities might be due to changes in the expression of their specific inhibitors, cystatin A and C were analyzed in proximal tubules in terms of enzyme protein and mRNA levels. Western blotting for cystatin C following semipurification of equal amounts of protein from proximal tubular homogenates revealed a band of 20 kDa (Fig. 5) in accordance with what has been described previously in homogenates of the whole rat kidney [42]. Densitometric evaluation of this protein band ($N = 3$) did not show any difference in cystatin C protein content in proximal tubules from PKD and control rats.

Table 3. Activities of marker enzymes for subcellular compartments of proximal tubules from heterozygous PKD and control rats

	PKD	Controls
	U/tubular preparation	
Lysosomes		
β-NAG	2.73 ± 0.35[a]	4.53 ± 0.50
Acid phosphatase	8.79 ± 0.71	8.98 ± 0.95
Mitochondria		
Cytochrome c oxidase	23.4 ± 5.4	21.6 ± 5.0
Endoplasmatic reticulum		
Glucose-6-phosphatase	36.2 ± 6.4	39.4 ± 5.8
Cytosol		
Lactate dehydrogenase	42.2 ± 7.3	43.0 ± 6.5

Data are given as means ± SEM from 9 animals in each group. Tubular preparation represents proximal tubules from one kidney.
[a] $P < 0.05$ for PKD vs. healthy offspring (2-months-old)

In accordance with enzyme protein levels, mRNAs for cystatin C in both PKD and control tubules, analysed by Northern blotting (Fig. 6) and subsequent densitometry ($N = 3$), were comparable. Cystatin A was not detectable in proximal tubules from PKD and control animals neither as enzyme protein nor in terms of mRNA.

Discussion

Our findings provide evidence for a reduction of the cysteine proteinases cathepsin B, H and L both in terms of activity and enzyme protein in proximal tubules isolated from PKD rats, while mRNA expression for these enzymes was unchanged. Cystatin C, a major inhibitor of lysosomal cysteine proteinases, apparently did not contribute to the reduced cathepsin activities, since its protein levels and expression of mRNA were not altered.

This decrement in proteinase activity appears to be specific for proximal tubules, since renal glomeruli and liver slices displayed no such alterations. Moreover, these disturbances were limited to lysosomal hydrolases, because marker enzymes for other intracellular compartments (cytosol, mitochondria and endoplasmatic reticulum) were unchanged in proximal tubules from PKD animals. Finally, the alterations in lysosomal enzymes affected only cathepsins B, H and L as well as β-NAG, while lysosomal acid phosphatase was unchanged in PKD rats. Luminal secretion of tubular cathepsins appeared to be enhanced, as cathepsins could be demonstrated in cyst fluid and the urinary excretion of these enzymes was found to be increased.

In general, there are only a few preliminary reports which address the potential involvement of lysosomal proteinases in the evolution of PKD [20, 21]. Using kidney homogenates from ADPKD patients, Wilson and Hartz [20] found a deficit in lysosomal proteinases measured against azocasein as a substrate. They were able to confirm this finding when they determined cathepsin B, H and L activities by using specific substrates in cultured human cyst epithelia.

These authors [20, 21] hypothetized that the decrement in lysosomal enzyme activity might be due to a central error in protein targeting in cyst epithelia. This would fit quite well to our findings in kidney tubules from the heterozygous Han:SPRD rat, which suggested enhanced secretion of cathepsins into the lumen

Table 4. Cathepsin activities in isolated glomeruli and liver samples from heterozygous PKD and control rats

	Cathepsin B	Cathepsin H	Cathepsin L
Glomeruli *mU/10³ glomeruli*			
PKD	39.4 ± 3.2	22.6 ± 1.6	24.0 ± 2.0
Controls	42.3 ± 4.2	21.6 ± 1.9	25.1 ± 3.0
Liver *mU/mg protein*			
PKD	385 ± 29	180 ± 19	542 ± 61
Controls	363 ± 31	163 ± 20	569 ± 59

Data are given as means ± SEM from 9 animals in each group.

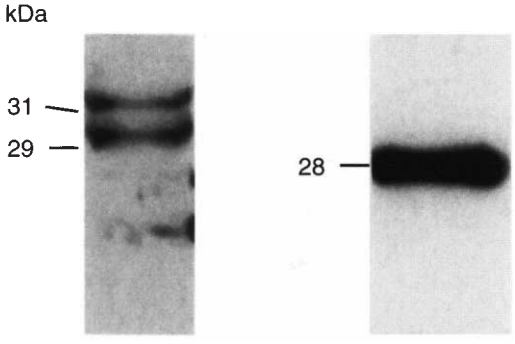

Fig. 3. *Western blot analysis (N = 4) of cyst fluid from homozygous PKD rats.* Polyclonal antibody to rat cathepsin B recognized 2 bands of Mr 31 and 29 kDa, while polyclonal antibody to human cathepsin H bound to a single band of Mr 28 kDa.

Table 5. Urinary excretion of cathepsin activities in heterozygous PKD and control rats

	Cathepsin B	Cathepsin H	Cathepsin L
	mU/24 hr		
PKD	115.9 ± 6.8[a]	110.2 ± 9.0[a]	153.6 ± 13.1[a]
Controls	67.3 ± 7.1	57.4 ± 5.2	85.8 ± 9.4

Data are given as means ± SEM from 9 animals in each group.
[a] $P < 0.05$ for PKD vs. healthy offspring (2-months-old)

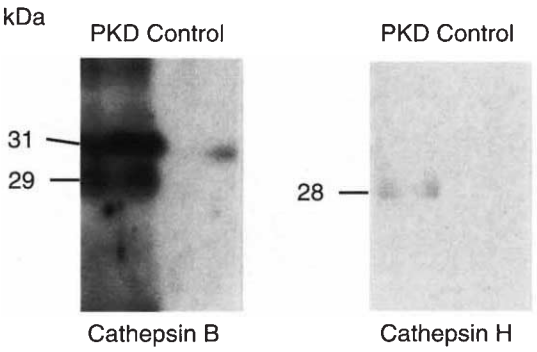

Fig. 4. *Western blot analysis (N = 4) of urinary cathepsins B and H from PKD and control rats.* Cathepsin B (bands of 31 and 29 kDa) and H (28 kDa band) proteins in urine from PKD rats can be clearly recognized. In the urine from healthy controls cathepsins B and H were almost not detectable.

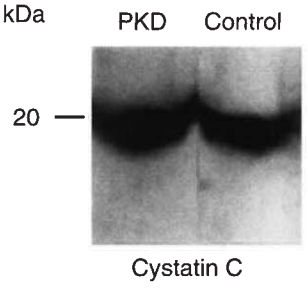

Fig. 5. *Western blot analysis (N = 3) of cystatin C in proximal tubules isolated from PKD and control rats.* Polyclonal antibody to human cystatin C recognized a single band of 20 kDa. Densitometric evaluation indicated that there was no difference between PKD and control animals.

in terms of activity and enzyme protein, while the respective mRNA levels remained unchanged. Alterations in the polarized transport of specific proteins have indeed been shown to occur in PKD epithelia. Thus, the Na/K-ATPase which normally is inserted into the basolateral membrane has been found to be mislocated to apical plasma membranes [43].

Besides enhanced secretion of lysosomal cysteine proteinases, reduced cathepsin activities might also have been due to an increase in the specific intracellular inhibitors. In general, cysteine proteinases are controlled by a class of inhibitors referred to as cystatins [44]. Cystatin C is expressed in many human tissues including the kidney and can be found in the circulation [45], while cystatin A is selectively expressed in epithelial cells and polymorphonuclear leukocytes [46]. In the present experiment, we studied mRNA and protein levels of cystatins A and C in isolated proximal tubules from PKD rats. In terms of cystatin C, both mRNA and protein levels were not altered in kidney tubules from PKD animals. Cystatin A, on the other hand, could not be demonstrated neither by Northern nor by Western blotting in tubules from normal or PKD animals. Taken together, these results suggest that intracellular cysteine proteinase inhibitors appear unlikely to have contributed to the reduction in cathepsin activities in PKD tubules.

Interestingly, not all lysosomal enzyme activities were found to be reduced in PKD tubules, since the activity of lysosomal acid phosphatase was normal. This may point to a possible mechanism by which these disturbances in lysosomal enzymes are brought about. Acid hydrolases are synthetized in the endoplasmatic reticulum and then transported to the Golgi apparatus, where their oligosaccharide side chains are modified to contain mannose-6-phosphate. In the trans-Golgi network hydrolases then bind to the mannose-6-phosphate receptor and are transferred to endosomes. Here the hydrolases dissociate from the receptor and subsequently reach the lysosome. Lack or alteration of the mannose-6-phosphate receptor results in extracellular secretion of newly synthetized hydrolases [46]. Among these hydrolases, acid phosphatase resembles lysosomal membrane proteins which are, in contrast to soluble proteins of the lysosomal matrix (cathepsins B, H and L and β-NAG), routed from the Golgi complex to lysosomes independently of the mannose-6-phosphate receptor [47, 48].

Together with ultrastructural findings by Carone, Bacallao and Kanwar [49] that the Golgi complex is altered in tubular epithelium from polycystic kidneys, the above-mentioned considerations

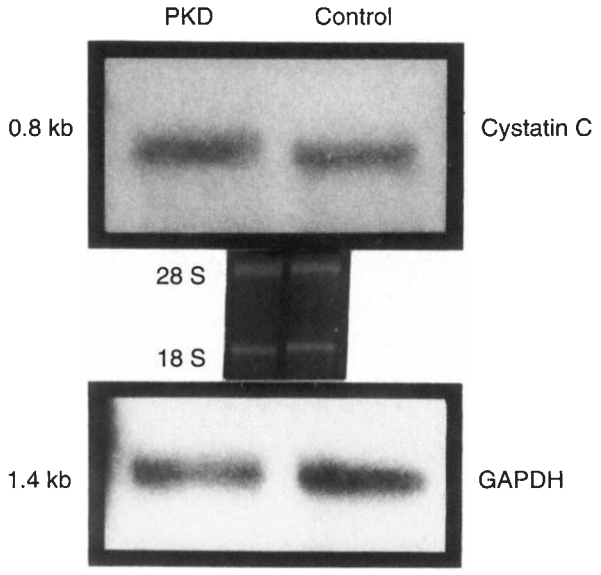

Fig. 6. *Northern blot analysis (N = 3) of tubular cystatin C mRNA of PKD and control rats.* The levels of mRNA for cystatin C, measured by densitometry, did not differ between both groups. The middle panel shows ethidium bromide staining of 18S and 28S ribosomal RNA transcripts. Expression of GAPDH mRNA in the same membrane is shown on the bottom panel.

and our findings that there are differences in the handling of lysosomal acid hydrolases in PKD tubules suggest that trans-Golgi network signal mechanisms may be defective in tubular PKD epithelium leading to enhanced secretion of lysosomal hydrolases instead of storage within lysosomes.

Finally, the question remains whether a reduction in the activities of cathepsins does infact translate into alterations of extracellular matrix turnover in the kidney in PKD. While there is no direct evidence presently available, several observations suggest a close relationship. Abnormal matrix turnover occurs early during the course of human ADPKD [2, 3] as well as in the Han:SPRD rat model [24] and cathepsins B and L have been shown to degrade collagen IV, laminin and fibronectin *in vitro* not only at acid but also at neutral pH [50]. Therefore, decreased levels of cathepsins could lead to accumulation of matrix components.

In addition, in our experiment alterations of cathepsins were limited to proximal tubules. Glomeruli and liver tissue of the PKD animals displayed normal cathepsin activities. As cyst formation in the male Han:SPRD rat predominantly occurs in proximal tubules [24], while glomeruli [51] and liver are unaffected [52], it is conceivable that the reduction in tubular cathepsins may play a role in cyst formation in PKD.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft and the Heinrich-Hertz Foundation. We thank Dr. J.S. Mort for providing us with the antibody to rat cathepsin B, as well as Dr. K. Fukuyama for the antibody to rat cystatin A. We are grateful to Dr. S.J. Chan, Dr. G.G. Sahagian, Dr. J. Dodt, and Dr. K.A. Siminovitch for providing us with the cDNAs for the cathepsins B, L and H as well as cystatin A. The technical assistance of Mrs. S. Meisinger is appreciated.

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