Puromycin aminonucleoside nephrosis results in a marked increase in fractional clearance of albumin

TANYA M. OSICKA, ALEXANDRA R. HANKIN, AND WAYNE D. COMPER
Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia 3168

Puromycin aminonucleoside nephrosis results in a marked increase in fractional clearance of albumin. Am. J. Physiol. 277 (Renal Physiol. 46): F139–F145, 1999.—Puromycin aminonucleoside nephrosis (PAN) results in a marked increase in the fractional clearance of albumin. The increase in the fractional clearance of [3H]albumin to ~0.045, as measured both in vivo and in the isolated perfused rat kidney (IPK) with PAN, occurs without an accompanying equivalent increase in glomerular capillary wall size selectivity as previously measured with dextrans. This is very similar to the marked increase in albuminuria seen with kidneys treated with inhibitors of endocytosis by the tubular epithelium, particularly lysine (T. M. Osicka, L. M. Pratt, and W. D. Comper. Nephrology 2: 199–212, 1996). The similarity is further established that, like in the presence of lysine, [3H]albumin excreted in urine from rats with PAN is essentially intact whereas, in both in vivo and IPK control experiments, excreted [3H]albumin is heavily degraded. The same observations have also been made for [3H]-labeled anionic horseradish peroxidase. These observations suggest that the significant albuminuria that occurs in PAN is primarily post-glomerular basement membrane in origin.


The hypothesis to be tested in this study is that PA-induced albuminuria is consistent with the inhibition of the rapid transtubular retrieval pathway in a manner similar to that caused by other tubulotoxic agents like lysine. We determine the urinary excretion and fractional clearance of albumin in both control and

 fractional clearance (up to ~2.5-fold) in relation to control values (3, 32). The latter changes corresponding to the onset of large pores (13), however, do not relate to the vast increase seen with the fractional clearance of albumin as measured by immunoassay in PAN (~700 times that of the control) (44).

Measurement of the loss of apparent charge selectivity in PAN has been made with various charged transport probes including dextran sulfate (3) and horseradish peroxidase (HRP) (32). This prompted many ultrastructural studies yielding varying results, some demonstrating a decrease in glomerular basement membrane (GBM) anionic sites (7, 8, 30) in nephrotic rats, whereas others found no difference (1, 2, 24, 25, 48). Furthermore, the levels of the major contributor to fixed anion charge to the GBM, namely heparan sulfate, have been found to be essentially unchanged in PAN rats (27, 38).

The conflicting nature of these results can be rationalized to some extent from recent studies on charge and size selectivity of the GCW. We have demonstrated that charge repulsion between albumin and the negative charges of the GCW (charge selectivity) is low as measured by biophysical studies in model systems (9, 10, 40, 49) and by clearance studies in isolated perfused kidney (IPK) systems (4, 6, 11, 33, 34, 36, 42, 46, 47) and in vivo (4, 11). In particular, the fractional clearance of dextran sulfate has been shown to be controlled by glomerular endothelial cells, which are known to desulfate the molecule during renal passage (4, 11, 47). On the other hand, the apparent charge selectivity of HRP appears to be controlled by differential tubular uptake rather than charge-based discrimination at the GCW (34, 36). This means that the flux of albumin across the GCW will be determined by size selectivity alone. This has been confirmed when the glomerular sieving coefficient for albumin was measured in the IPK, where protein uptake by tubules was inhibited by various agents including lysine (36). It also means that the relatively large amount of albumin that is filtered across the GCW, as governed essentially by size selectivity, must be recovered. This retrieval pathway, which ensures the return of filtered albumin to the circulation, has recently been identified (16). Since the exact anatomical location of the pathway has not been determined, for the purposes of this study, it will be referred to as the tubular retrieval pathway.

The hypothesis to be tested in this study is that PA-induced albuminuria is consistent with the inhibition of the rapid transtubular retrieval pathway in a manner similar to that caused by other tubulotoxic agents like lysine. We determine the urinary excretion and fractional clearance of albumin in both control and
PAN kidneys using [3H]albumin in vivo using osmotic pump technology and in the IPK system. The structural integrity of filtered albumin in both the IPK and in vivo is assessed by gel chromatography, whereas the structural integrity of filtered anionic HRP is also examined in vivo. Proximal tubule function is assessed by the reabsorption of lysozyme in both control and PAN kidneys.

METHODS

Materials. Male Sprague-Dawley rats (300–350 g) were obtained from the Monash University Central Animal House. PA (i.e., 6-dimethylamino-9’-amino-3’-deoxyribosyl purine), type VI peroxidase (EC 1.11.1.7; 288 U/mg solid from horseradish), catalase (EC 1.11.1.6; 1,600 U/mg solid from bovine liver), lysozyme (EC 3.2.1.17; 30,000 U/mg solid from chicken egg white), sulfofolic acid, benzoylated dialysis tubing (mol wt cutoff of 2,000), and the amino acids lysine, tyrosine, serine, cysteine, aspartate, glutamate, asparagine, and glutamine were from Sigma Chemical (St. Louis, MO). BSA (fraction V), Micrococcus luteus (American Type Culture Collection catalog no. 4698), and superoxide dismutase (EC 1.15.1.1; 5,000 U/mg solid from bovine erythrocytes) were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Sephadex G-100, Sephadex G-25 in PD-10 columns, and blue dextran T2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Nembutal (60 mg/ml) was from Cera Chemicals (Hornsby, NSW, Australia). Synthamin (a source of amino acids) was from Travenol Laboratories (NSW, Australia). Sodium heparin was from Commonwealth Serum Laboratories (Melbourne, Australia). Mannitol was from CSR Chemicals (Rhodes, NSW, Australia). ALZET osmotic pumps (model 2001) were purchased from ALZA Scientific. Tritiated water (0.25 mCi/g) and [carbon-14]Julin (2.7 mCi/g) were obtained from DuPont ( Wilmington, DE) and sodium borotritiated dialysis tubing. The dialyzed preparation was applied to a PD-10 column immediately before use. The activity of [3H]albumin was 1.13 x 10^7 dpm/mg. They also contained thimerosal at 1/5,000 wt/vol to inhibit bacterial growth. Prior to implantation, the pump was incubated in PBS containing 0.02% azide at 37°C for 4 h. The pump was then wiped with 70% isopropanol and implanted subcutaneously in an Nembutal-anesthetized Sprague-Dawley rat (anesthetized for 2 h), or in one to the scapulae, using sterile technique. Rats were then placed in a metabolic cage with free access to food and water for 7 days. Blood samples were obtained from the rat tail at 24-h periods with corresponding urine collections. Both blood and urine samples were centrifuged at 3,000 rpm in a KS-5200C Kubota bench-top centrifuge for 10 min and then analyzed for radioactivity. Glomerular filtration rate (GFR) was determined by the creatinine assay (15).

Urinary degradation of [3H]albumin and [3H]aHRP in vivo. In vivo experiments were performed on male Sprague-Dawley rats 9 days after the administration of PA. The rats were injected with either 1 x 10^7 dpm/ml [3H]albumin or [3H]aHRP in 0.8 ml PBS in the tail vein and maintained in a metabolic cage with free access to food and water. Urine samples were collected after 2 h and were analyzed on a Sephadex G-100 column (1.6 x 60 cm) eluted with PBS at 20 ml/h at 4°C. One-milliliter samples were loaded, and 95 fractions of 1.65 ml each were collected. The void volume (Vo) was determined with tritiated water. The total volume (Vt) was determined with tritiated water. The determination of the relative amounts of intact and degraded albumin was made by determining the distribution of tritium-labeled material on Sephadex G-100.

Kidney perfusion. Male Sprague-Dawley rats were anesthetized by a 1-ml intraperitoneal injection of Nembutal (18 mg/ml). One millilitre containing 10% mannitol and 200 U sodium heparin was injected into the femoral vein. A laparotomy was performed, and the right ureter was cannulated with polyethylene tubing (PE-10; Dural Plastics and Engineering, Auburn, NSW, Australia). The right renal artery was cannulated via the superior mesenteric artery, and the kidney was removed by en bloc dissection. This whole procedure took no longer than 10 min. The perfusion pressure was maintained at 90–100 mmHg with a peristaltic pump monitored by a calibrated aneroid manometer while the flow rate was monitored by a ball flowmeter. Kidneys were perfused with 160 ml of recirculated filtered 5% BSA in Krebs-Henseleit buffer containing glucose, essential amino acids (34, 36, 45), and oxygen radical scavengers to prevent partial ischemia (43). The system was maintained at 37°C, and the perfusate was continually gassed with 95% O2-5% CO2. Perfusion was performed on rats 5 days after the administration of PA or PBS. The kidney was allowed to equilibrate for 10 min, and
urine and perfusate samples were collected after 40 and 60 min of perfusion. The perfusate contained $-1 \times 10^6$ dpm/ml $[^{3}H]$albumin. Tubular reabsorption of lysozyme was determined by an assay based on the lysis of M. luteus $(5)$. Determination of fractional clearance of intact albumin was made by determining the percentage of the intact molecule on Sephadex G-100 as described above.

Calculations. All quantitative data are expressed as means ± SD, where n represents the number of determinations.

RESULTS

Urinary protein excretion in vivo. The intravenous administration of PA to rats in a single dose of 15 mg/100 g body wt resulted in a significant increase in proteinuria from a control level of 19.5 ± 4.4 mg/24 h at day 0 to 183.9 ± 15.2 mg/24 h at day 5, with a further increase to 219.2 ± 22.7 mg/24 h at day 8 as detected by the sulfosalicylic acid turbidity assay. Importantly, these results show a similar trend as those found by others (14, 19, 21, 32, 39). In comparison, when detected by biuret assay, a significant increase in proteinuria from a control level of 105 ± 40 mg/24 h at day 0 to 205 ± 25 mg/24 h at day 5, with a further increase to 559 ± 212 mg/24 h at day 8 was detected. Administration of a lower dose of 10 mg/100 g body wt resulted in a similar proteinuria of 541 ± 86 mg/24 h at day 7. Control rats administered PBS at the same dose showed no increase in urinary protein excretion from days 0 to 8 but maintained a control level of ~100 mg/24 h (Fig. 1). Since urinary protein is excreted as mixture of intact and degraded molecules, we suggest that the discrepancy between the two assays is due to the fact that the biuret assay, which detects peptide bonds, will detect all urinary protein, whereas the sulfosalicylic acid assay, which detects only precipitated protein, may not detect low-molecular-weight peptides.

Fractional clearance in the IPK. Details of the characteristics of our IPK’s when studied over a 1-h period are shown in Table 1. All parameters measured demonstrate a steady-state, stable preparation over this time period. Tubular reabsorption of lysozyme was normal and similar to that obtained by others in vivo. The fractional clearance of albumin of 0.0075 ± 0.0039 as measured by radioactivity in control rats was ~20 times higher than that measured for intact albumin as albumin in the urine is significantly degraded (36). Table 1 also shows renal parameters for PAN kidneys perfused 5 days after PAN administration. Kidneys perfused 9 days after PAN administration failed to function successfully. Steady state was achieved within the 1-h perfusion period. Urine flow rate was significantly reduced from 0.148 ± 0.065 ml/min in control animals to 0.016 ± 0.012 ml/min in PAN rats. GFR was also significantly reduced from a control value of 0.828 ± 0.166 to 0.121 ± 0.082 ml/min in PAN kidneys. Similar reductions in GFR have been observed by others (32). Renal plasma flow resistance and filtration fraction were also significantly reduced in PAN compared with control rats. However, the fractional clearance of $[^{3}H]$albumin in PAN animals was increased approximately

<table>
<thead>
<tr>
<th>Table 1. Renal parameters associated with control and PAN IPK compared with IPK in presence of lysine and unpublished data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>*UFR, ml/min</td>
</tr>
<tr>
<td>*GFR, ml/min</td>
</tr>
<tr>
<td>RPF resistance, mmHg·min⁻¹·ml⁻¹</td>
</tr>
<tr>
<td>Filtration fraction, %</td>
</tr>
<tr>
<td>Lysozyme reabsorption, %</td>
</tr>
<tr>
<td>*FC of albumin, radioactivity</td>
</tr>
<tr>
<td>*FC of intact albumin, column</td>
</tr>
</tbody>
</table>

Values are means ± SD for renal parameters including urine flow rate (UFR), glomerular filtration rate (GFR), renal plasma flow (RPF), and fractional clearance (FC) associated with control and puromycin aminonucleoside nephrosis (PAN) isolated perfused kidneys (IPK) (n = 6 for all data) compared with IPK in presence of 150 mM lysine at 40–60 min (n = 4; Refs. 34 and 36), and unpublished data from M. Burne (n = 4) (+).
sixfold to 0.0451 ± 0.0168. This corresponds to an excretion rate of ~390 mg/24 h, which is similar to that estimated by the biuret assay described above. The fractional clearance of intact albumin as determined by size-exclusion chromatography in PAN increased over 100-fold compared with the control (Table 1).

Lysozyme reabsorption by the proximal tubule was reduced to 0% in PAN animals, indicating total lack of proximal tubule function. The renal parameters characterizing PAN kidneys were similar to those obtained with lysine present in the perfusate (Table 1), particularly in relation to tubular function as measured by lysozyme reabsorption and fractional clearance of albumin as measured by radioactivity and the specific analysis of intact albumin by gel chromatography.

Elution profiles from in vivo studies. [3H]albumin was injected directly into the tail vein of rats with and without PAN (9 days after PA administration), and urine was collected for a period of 2 h and applied to a Sephadex G-100 column. The original [3H]albumin prior to injection is shown in Fig. 2A. It elutes as a single major peak with a K_{av} value of 0.190 [K_{av} = (V_e - V_0)/(V_t - V_0) where V_e is the elution volume]. In urine obtained from control rats 2 h after being injected intravenously with [3H]albumin, there was the appearance of ~90% of low-molecular-weight material in the elution profile (Fig. 2B) compared with urine collected from PAN rats, which elutes essentially as the original material with no low-molecular-weight material (Fig. 2C) and with the absence of the dimer.

Similar experiments were performed with [3H]aHRP. The original [3H]aHRP is shown in Fig. 3A and elutes as a single peak with a K_{av} of 0.231. In urine obtained from control rats 2 h after being injected intravenously with [3H]aHRP, there was the appearance of ~35% of lower-molecular-weight material in the elution profile (Fig. 3B) compared with urine collected from PAN rats, which elutes essentially as the original material with no lower-molecular-weight material (Fig. 3C).

Fractional clearance in vivo. To obviate the marked decrease in GFR observed when PAN was induced with 15 mg/100 g, we performed the osmotic pump experiments with a reduced level of 10 mg/100 g of PA. This resulted in a considerably higher GFR after 9 days compared with 10.2 ± 0.32 µg·min⁻¹·rat kidney⁻¹. Fractional clearance of intact albumin was determined by radioactivity analysis was found to be 0.0023 ± 0.0009 in the control rat compared with 0.0419 ± 0.0134 in the PAN rat (Table 2). The fractional clearance of intact albumin as determined by size-exclusion chromatography was found to be 2.1 ± 0.6 × 10⁻⁴ in the control rat compared with 0.0419 ± 0.0134 in the PAN rat (Table 2). Note that the fractional clearance of intact albumin was determined by fractionating urine obtained from rats 2 h after the intravenous injection of [3H]albumin to obviate any artifacts associated with protein breakdown in urine collected in the metabolic cage container for 24 h.

**DISCUSSION**

Proteinuria is one of the hallmarks of renal dysfunction. It reflects an increase in urinary excretion of various plasma proteins, namely albumin and immunoglobulins. Our studies have demonstrated that changes in glomerular permeability, including “charge selectivity” (see introduction) and the onset of “large pores” (5), will not be responsible for the marked changes seen in albuminuric states. We have recently established two pathways associated specifically with the tubular handling of albumin that are important in governing albumin excretion (16, 36). The major capacity pathway (~2,000 µg·min⁻¹·rat kidney⁻¹) processes ~90% of the filtered albumin and returns this albumin, intact, back to the blood supply via the retrieval pathway. The small amount of filtered albumin that escapes the pathway may then undergo tubular uptake, where it is degraded by lysosomal enzymes (degra-
dation pathway) and where degradation products are regurgitated back into the tubular lumen. These degradation products are ultimately excreted in the urine (5, 34, 35). Albumin that undergoes the retrieval pathway is not degraded, which implies that this pathway occurs earlier in renal passage compared with the degradation pathway. Although the amount of albumin processed by the degradation pathway is relatively small, it serves as an important marker for normal tubular activity. When the tubules are not functioning, this degradation is inhibited, and paradoxically proteins are excreted in an undegraded form. Both pathways are affected by lysosomotropic agents that inhibit endocytosis by the tubular epithelium. The inhibition will result in a marked increase in the clearance of albumin without affecting size selectivity (retrieval pathway) as well as resulting in excreted proteins that are not degraded (degradation pathway). The findings of the present studies with PAN are consistent with the fact that PA acts primarily as an inhibitor of post-GBM cell uptake, which will affect not only the magnitude of albumin fractional clearance but the integrity of the molecules being excreted.

The marked increase in the fractional clearance of albumin in PAN kidneys to ~0.045 as measured in both in vivo studies and in the IPK confirms the earlier result obtained by Tencer et al. (44), who obtained a fractional clearance of 0.0383 in vivo as measured by a radioimmunoassay that detects only intact albumin (35). The marked changes in albumin clearance are accompanied by relative small changes in dextran clearance (3, 32), which is consistent and compelling evidence that other factors apart from changes in GCW size selectivity must be responsible for the albuminuria seen in PAN rats. This would also suggest that the morphological changes associated with glomerular epithelial cell including loss of organization and detachment (23, 32, 37) have no major effect on size selectivity and are probably not directly related to the etiology of proteinuria. Overall, there is notable similarity of these results to our lysine studies (34, 36), where no effect on size selectivity was observed, although there was a 20-fold increase in albumin clearance. This would represent evidence that the high-capacity retrieval pathway has been inhibited in PAN rats and be consistent with the more subtle, but distinct morphological change that occurs in the tubulointerstitium (31). The high fractional clearance of albumin would then give rise to a relatively high steady-state concentration of albumin near the proximal tubule, and this has been previously established by micropuncture studies of Lewy and Pesce (28), who measured concentrations of up to 2.4 mg/ml.

The abnormally low GFR that accompanies the perfusion of kidneys from PAN rats (Table 1) could be argued to affect albumin clearance compared with the control. A comparison of the relative reductions in GFR with PAN would suggest this not to be the case. Similar fractional clearances were obtained when we had a 52% reduction in vivo [which is comparable to the study of Tencer et al. (44)] and a 88% reduction in the IPK. This

Table 2. Renal parameters associated with control and PAN rats 7 days after implantation of osmotic pumps

<table>
<thead>
<tr>
<th></th>
<th>Control*</th>
<th>PAN (10 mg/100 g body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFR, ml/min</td>
<td>0.0059 ± 0.0017</td>
<td>0.0154 ± 0.0024</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>1.44 ± 0.39</td>
<td>0.683 ± 0.214</td>
</tr>
<tr>
<td>Protein excretion</td>
<td>105 ± 40</td>
<td>541 ± 86</td>
</tr>
<tr>
<td>(Biuret), mg/24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC of albumin (radio-activity)</td>
<td>0.0023 ± 0.0009</td>
<td>0.0419 ± 0.0134</td>
</tr>
<tr>
<td>FC of intact albumin (column)</td>
<td>2.1 × 10^-4 ± 0.6 × 10^-4</td>
<td>0.0419 ± 0.0134</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 experiments. *Control data are from Ref. 35.
is consistent with our previous findings, which demonstrated that the variation in the GFR in lysine-treated IPKs did not affect the high fractional clearance of albumin obtained in this system (Table 1).

Since the clearance of albumin is measured in relation to the transport of inulin, the low GFR may affect the ratio of the two modes of transport that will govern these molecules, namely convection and diffusion across the GCW. If a low GFR is the result of a decrease in the area available for transport, for a given pressure differential across the GCW, then the magnitude of GFR will not have an effect on the relative clearance. For this case, the transport of inulin and albumin will be governed primarily by convective transport across the GCW and by their frictional interaction with the GCW. In this convective regime the relative difference in transport between albumin and inulin will be minimized. The other extreme is when low GFR represents a low volume flow across the GCW, which may be the result of a low pressure differential or some other factor. Here, the relative role of diffusion compared with convection will be enhanced; but this means it will have a disproportionate effect on the diffusion of inulin such that the relative transport will not only be dependent on the relative frictional interaction with the membrane (as will occur in the convection mode) but also on the ratio of the diffusion coefficients. This situation will then favor the transport of inulin and create an apparent lower clearance for albumin in low GFR states. This is quite the opposite to what is observed experimentally, where there was a significantly enhanced fractional clearance of albumin in both PAN and lysine IPKs. In fact, the albumin clearances measured in these low GFR states may be apparently lower due to the influence of diffusion on the fractional clearance estimate. There is also no physical basis to suggest that alterations in the filtration fraction will give rise to any changes in the relative transport of albumin and inulin.

A new feature of renal handling of proteins that has been reported recently is that proteins in the urine from normal rat kidneys collected from the IPK or in vivo have been shown to be heavily degraded (33, 35, 36). This degradation is tubular in origin, as it can be inhibited by agents, like lysine, that inhibit endocytosis by the tubular epithelium. A striking feature of the results reported in this study is that the degradation for both albumin and aHRP is totally absent in urine collected from day 9 PAN animals, whereas it is markedly reduced for albumin from urine collected from day 5 IPKs. The similarity of the PAN results and that obtained with lysine treatment is extended further where loss of charge selectivity seen with anionic and neutral forms of HRP in PAN (32) has also been demonstrated with lysine treatment in IPK (34, 36).

These results would suggest that post-GBM processing of proteins has been severely affected in PAN rats. This has been confirmed by analysis of lysozyme reabsorption. Lysozyme is solely reabsorbed by the proximal tubule and is therefore indicative of tubular function. In the control IPK, ~50% of the lysozyme crossing the GCW was reabsorbed. However, lysozyme reabsorption was lacking in PAN kidneys, clearly demonstrating that the tubules are not functioning properly.

The evidence presented in this study would suggest that PA is affecting the post-GBM processing of albumin. We have previously identified a lysine-sensitive pathway that involves uptake of the albumin by the tubules and release of degradation products which are excreted in the urine. Lysine is known to inhibit this tubular uptake as albumin excreted is not significantly degraded in its presence. We have found very similar results with PAN rats, where degradation of albumin in vivo was negligible and limited in the IPK system.

In conclusion, the significant increase in the fractional clearance of albumin observed in PAN kidneys is similar to that observed with lysine. This would be consistent with inhibition of the rapid retrieval pathway for albumin that we have previously identified. We would suggest that the etiology of PAN is an oxygen free radical-mediated response directed toward tubular epithelium and its processing of albumin.

Address for reprint requests and other correspondence: W. D. Comper, Dept. of Biochemistry and Molecular Biology, Monash Univ., Clayton, Victoria, Australia 3168 (E-mail: wayne.comper@med.monash.edu.au).

Received 4 December 1998; accepted in final form 5 April 1999.

REFERENCES