Alterations in renal degradation of albumin in early experimental diabetes in the rat: a new factor in the mechanism of albuminuria

Melissa J. BURNE, Sianna PANAGIOTOPoulos*, George JERUMS* and Wayne D. Comper
Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia, and *Endocrine Unit, Austin and Repatriation Medical Centre, Department of Medicine, University of Melbourne, Heidelberg, Victoria 3084, Australia

**ABSTRACT**

1. Albumin is normally excreted as a mixture of intact protein and fragments that are produced during renal passage. The purpose of this study was to investigate the ratio of intact versus degraded forms of excreted albumin to ascertain whether changes in this ratio could account for the apparent increase in albumin excretion seen in diabetes, as measured by standard radioimmunoassay techniques.

2. Four-week male Sprague–Dawley rats with streptozotocin-induced diabetes and age-matched control rats were intravenously injected with [3H]albumin. Urine collected over 2 h was analysed by size exclusion chromatography and radioimmunoassay. A standard radioimmunoassay found a 7-fold increase in albumin excretion rate in diabetic rats, whereas there was only a 2-fold increase in albumin excretion (intact plus fragments). Urine analysed by size exclusion chromatography showed severe degradation for control rats (4 ± 2% monomer); in diabetic rats there was a significant amount of monomer albumin excreted, along with moderately degraded and heavily degraded albumin (17 ± 5%).

3. This study has shown that the radioimmunoassay, which specifically detects intact albumin, considerably underestimates the amount of total urinary albumin which consists of intact and degraded material. The increase in albumin excretion rate observed in diabetes as measured by radioimmunoassay is mainly due to a change in the amount of intact albumin excreted and this is specifically due to the inhibition of albumin degradation at a post-glomerular site and not due to the onset of any type of glomerular 'shunt' pathway.

**INTRODUCTION**

An increase in albumin excretion rate leading to microalbuminuria is widely acknowledged as the earliest index of diabetic nephropathy [1] and as a risk factor for the development of overt diabetic renal disease [2] and macrovascular disease [3]. The mechanisms underlying the increase in albumin excretion rate have not been clarified, but both functional and structural changes in the kidney have been proposed as possible contributors. These include early alterations in glomerular filtration rate [4], intraglomerular pressure [5], glomerular size [6], glycosylation of membrane proteins [7] and circulating albumin [8], and changes in the composition of glomerular extracellular membrane material leading to glomerular basement membrandhickening [9], loss of heparan sulphate [10] and alterations in epithelial slit pores may also contribute to increases in albumin excretion rate in diabetic nephropathy [11].

It has long been established that low-molecular-mass proteins such as lysozyme, growth hormone and insulin (< 20000 Da) are reabsorbed by the tubular epithelium...
and are catabolized in normal kidneys [12,13]. However, the integrity of high-molecular-mass proteins (> 40000 Da) which are excreted by the kidney has not been investigated until recently. We have identified that proteins, including albumin, are heavily degraded during renal passage by post-glomerular cells [14–16]. These fragments, which are mostly produced in lysosomes of the proximal tubule, are regurgitated back into the tubular lumen with subsequent excretion in the urine within a matter of minutes. This is in agreement with other studies which have demonstrated that proteins may be taken up by lysosomes or cells in culture and regurgitated into the medium as peptide fragments within an hour [17,18].

Studies both in vivo and in the isolated perfused kidney system have demonstrated that albumin is degraded by the kidney to peptides that are exclusively excreted in the urine. The return of these degradation products to the circulation has not been detected [14–16]. Furthermore, the extrarenal contribution to the excretion of albumin degradation products is minimal. In experimental puromycin aminonucleoside nephrosis or lysine administration the degradation of albumin is inhibited [14]. This inhibition results in a marked increase in the renal clearance of intact albumin without affecting glomerular size selectivity [16]. Lysine infusion is known to prevent tubular uptake of proteins [16,19].

The ratio of intact and degraded albumin is therefore a potentially important marker in renal pathology but this parameter has not been previously addressed because of methodological limitations. The aim of this study was to investigate the ratio of intact versus degraded albumin in early experimental diabetes and to ascertain whether changes in this ratio could account for initial increases in albumin excretion as measured by standard radioimmunoassay (RIA) techniques.

METHODS

Materials

Male Sprague-Dawley rats (200-250 g) were obtained from the Monash University Central Animal House. Streptozotocin (STZ) was purchased from I CN Biomedicals Inc. (A urora, O H, U.S.A.). N embutal (60 mg/ ml) was from C era Chemicals Pty. Ltd. (H ornsby, N SW, A ustralia). Bovine serum albumin (BSA) Fraction V was purchased from Boehringer Mannheim GmbH Biochemica (M annheim, Germany). Rat serum albumin standard was purchased from Sigma Chemical Co. (St Louis, M O, U.S.A.). Sephadex G-100 and blue dextran T2000 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Sodium boro-[¹⁴C]hydride (132 mCi/mg) was obtained from Amersham International (B ucks, U.K.). Sodium-[¹³¹I]iodide was purchased from Australian Radioisotopes (L ucks Heights, N SW, A ustralia). Tritiated water (0.25 mCi/g) was from D u Pont (W ilmington, D etroit, U.S.A.). Rabbit antiserum to rat albumin antibody and sheep anti-rabbit antibody were from C appel Research Products (D urham, N C, U.S.A.).

Table 1 Physiological characteristics of STZ-diabetic rats compared with control rats at 4 weeks (n = 6)

<table>
<thead>
<tr>
<th>Control rats</th>
<th>Diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>368.0 ± 12.0</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.6 ± 0.08</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>Urine volume (ml/24 h)</td>
<td>11.8 ± 1.3</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min)</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Urinary albumin (RIAT/mg/24 h)</td>
<td>0.36 × 10⁻¹</td>
</tr>
<tr>
<td>Urinary total protein (mg/24 h)</td>
<td>18.0 ± 5.2</td>
</tr>
<tr>
<td>Urinary albumin/total protein ratio</td>
<td></td>
</tr>
</tbody>
</table>

Chasched from Australasian Radioisotopes (L ucks Heights, N SW, A ustralia). T ritiated water (0.25 mCi/g) was from D u Pont (W ilmington, D etroit, U.S.A.). Rabbit anti-serum to rat albumin antibody and sheep anti-rabbit antibody were from C appel Research Products (D urham, N C, U.S.A.).

Induction of diabetes

Male Sprague-Dawley rats (8 weeks old), starved overnight, were injected via the tail vein with STZ, dissolved in citrate buffer (pH = 4.5) at a concentration of 50 mg/kg body weight. A matched control rats were injected with citrate buffer alone. The diabetic rats also received 4 i.u. of insulin 3 days a week to maintain body weight without normalizing hyperglycaemia. The rats were given free access to food and water. The diabetic state was confirmed by measurement of plasma glucose (> 15 mmol/l), urine volume (ml/24 h) and body weight (g) as depicted in Table 1. After 4 weeks of diabetes, urine was collected for 24 h while the rats were in individual metabolic cages with free access to food and water. Albumin excretion rate was determined using a RIA for rat albumin [20] and total protein excretion rate was determined by the Biuret assay [21] (using BSA as a standard). Glomerular filtration rate was determined using technetium⁹⁹m diethylenetriaminepenta-acetic acid as previously described [22].

Radioimmunoassay

The RIA utilized [¹²⁵I]-labelled rat serum albumin, prepared by the Chloramine-T method [23], rabbit antiserum to rat albumin and sheep anti-rabbit antibodies. The
Diabetic microalbuminuria

The urinary albumin concentration measured by this double-antibody RIA had an interassay coefficient of variation of 7% at a concentration of 180 ng/ml. The detection limit of the assay was 31.2 ng/ml [20]. The standard curve was prepared using a rat serum albumin standard (1 mg/ml) which was diluted to give a range of 4000 to 31.2 ng/ml.

**Processing of [³H]albumin in vivo**

Albumin was labelled with tritium by the reductive methylation method [24]. This involved a brief exposure to sodium boro-[³H]hydride and extensive dialysis for 4 to 5 days against PBS (pH = 7.4) consisting of 136.9 mmol/l NaCl, 2.68 mmol/l KCl, 8.1 mmol/l Na₂HPO₄ and 1.5 mmol/l KH₂PO₄. The dialysed preparation was applied to a PD-10 column immediately before use to remove any free tritium and purified by applying the sample on a Sephadex G-100 column and collecting the peak fractions.

In vivo experiments were performed by injecting approximately $1 \times 10^7$ d.p.m./ml [³H]albumin into the tail vein of control and diabetic ether anaesthetized (for approximately 1 min) Sprague-Dawley rats which were maintained in individual metabolic cages for 2 h with free access to food and water. The urine collected during this period was analysed by size exclusion chromatography using a G-100 column. The column was eluted with PBS (pH = 7.4) at 20 ml/h at 4°C. 50 ne-hundred fractions of 1.7 ml were collected. The void volume ($V_v$) of the column was determined with blue dextran T2000 and the total volume ($V_t$) with tritiated water.

**Counting of radioactivity**

Tritium radioactivity was determined in 1-ml aqueous samples with 3 ml of scintillant and measured on a Wallac 1410 liquid scintillation counter (Wallac, Turku, Finland) and $^{125}$I radioactivity was recorded by a Packard crystal 5412 gamma counter.

**Calculations**

All quantitative data are expressed as means ± S.D. where $n$ represents the number of determinations, except for albumin excretion as determined by RIA which is expressed as geometric mean × 1/1 tolerance factor. The significance of differences between means was analysed by unpaired Student’s t-test.

**RESULTS**

**Albumin excretion rate as determined by radioimmunoassay**

Table 1 shows the values for albumin excretion rate in STZ-diabetic rats compared with control rats as determined by RIA. Total protein as measured by the Biuret assay (which measures peptide bonds) is also given in Table 1. It is evident that the STZ-diabetic rat is proteinuric as there is a 3-fold increase in total protein excretion compared with the control. This increase is accompanied, however, by a disproportionate 7-fold increase in the excretion of RIA-detectable albumin. Since the RIA only measures intact albumin [15] the increase in urinary albumin (RIA) excretion (Table 1) suggests that relatively more intact albumin is being excreted in the diabetic rat. This was then tested through the analysis of urine by size exclusion chromatography.
Analysis of the degree of degradation of excreted albumin in STZ-diabetic and normal rats as determined by size exclusion chromatography and radioimmunoassay

The chromatographic profiles of urine obtained in the 2 h after the intravenous injection of $^{3}H \text{albumin}$ are shown in Figure 1. In the control rats (Figure 1a) there was severe degradation of $^{3}H \text{albumin}$ in the urine to peptides with molecular masses $< 10 \text{kDa}$. Only small quantities of albumin were retained in its monomer form (peak corresponding to fractions 33–37). The percentage monomer $^{3}H \text{albumin}$ is $4 \pm 2\% \ (n = 6)$ as calculated from the area under the peak corresponding to the position of the ultra-pure monomer-labelled albumin which eluted at fractions 33–38 as shown by the dashed line in Figure 1a. This profile of the labelled albumin has been shown to be similar to that obtained previously for rat and bovine $^{3}H \text{albumin}$ [14] and for unlabelled albumin [14]. In the same profile in Figure 1a the RIA failed to detect any degradation products of rat albumin present in the urine but only registered intact albumin between fractions 33 and 37 which falls within the range of the ultra-pure monomer peak. In the STZ-diabetic rats (Figure 1b), degradation of $^{3}H \text{albumin}$ also occurred but there was a considerable amount of monomer albumin, aggregated albumin and moderately degraded albumin present in the urine (% monomer $^{3}H \text{albumin}$ is $17 \pm 5\% \ (n = 6), P < 0.05$). The corresponding RIA of the same profile again yielded only intact albumin in fractions 32–40 (Figure 1b). On the basis of these results, albumin excretion (including fragments) was calculated (Table 1) and this showed an increase in diabetic rats of just over 2-fold compared with control rats.

**DISCUSSION**

We have demonstrated in this study that a standard RIA which specifically detects intact albumin [15] considerably underestimates the amount of total urinary albumin, which consists of intact plus degraded forms of albumin. Increases in albumin excretion rate in early diabetes are demonstrated in this study to be mainly ($> 95\%$ taking into account the increase in glomerular filtration rate by a factor of 1.3) due to the disproportionate increase in the excretion of intact albumin.

What is the mechanism of this increased appearance of intact albumin in diabetic urine? It should be noted at the outset that we have not observed any form of degradation product of albumin in the circulation [14–16]. This means that the appearance of fragments in the urine is a direct result of the renal passage of albumin. This indicates that the changes seen in the ratio of intact versus fragmented albumin are not the result of a change in the permselectivity of the glomerular capillary wall but an alteration in a post-glomerular site that does not allow backflux into the circulation. In any case, recent studies have demonstrated that these changes would not be associated with biophysical alterations in glomerular transport. The influence of glomerular charge on trans-glomerular transport of albumin has now been shown to be far smaller than originally thought [16,25–28]. The fact that glomerular charge selectivity is essentially negligible invalidates the concept of large pores or ‘shunts’ that are thought to allow non-selective passage of albumin across the capillary wall [29,30]. Albumin is size selected as a 36-Å radius molecule which would mean that its fractional clearance would be unchanged or reduced in diabetic states [31,32]. We have demonstrated that size selectivity is essentially unchanged for the 4-week STZ-diabetic rat [27]. Overall, our results suggest that the change in the ratio of intact versus degraded excreted albumin that accompanies the increase in albumin excretion rate in early diabetes is due to the inhibition of degradation of albumin at a post-glomerular site; that is, after the albumin has passed the glomerular filtration barrier. It is not yet clear whether the glomerular epithelial cells or tubular proximal cells are the site of the degradation process.

The inhibition of albumin degradation is likely to reflect alterations synonymous with decreased activities of enzymes associated with lysosomal degradation in diabetes [27,33–35]. This alteration in enzyme activity is directly demonstrated by the change in size of the albumin fragments that are excreted in diabetic urine (Figure 1b). We cannot eliminate indirect change in lysosomal activity associated with the internalization and intracellular trafficking of albumin to and from the lysosome. Furthermore, a hydrodynamic effect of higher flow in the primary ultrafiltrate cannot be eliminated as a factor in modulating overall albumin degradation, but its effect is likely to be small.

Could the increased excretion of intact albumin in the diabetic rat be considered as simply a saturation phenomenon associated with uptake by the proximal tubules? Since there is considerable evidence that the glomerular sieving of albumin is not altered in early diabetes [27,31,32] then the question of saturation of sites on, say, the proximal tubular cells is relevant when one considers the increase in glomerular filtration rate (Table 1). The flux of intact albumin across the glomerular capillary wall in the control rat is 7.98 mg/24 h and this would be increased to 10.37 mg/24 h in the 4-week STZ-diabetic rat. Yet we know that of the 17.47 mg/24 h total albumin excreted in the diabetic rat there must be 12.93 mg (i.e. fragmented albumin) being processed by the tubules which means that the saturating level for albumin processing must be equal to or greater than this value. Clearly this is higher than the flux increase associated with the change in glomerular filtration rate, which
means that the saturation phenomenon is not a significant factor.

Fragments of albumin (> 15 kDa) have been previously detected in urine as measured by electrophoretic-immunochemical methods [36,37], but these fragments have only been detected in minor amounts. It is quite clear that the low-molecular-mass peptides are quantitatively detected through tritium labelling of albumin. Previous studies on the renal handling of albumin using 125I-albumin suffer from methodological deficiencies in detecting degradation products as they precipitate the urine fraction with trichloroacetic acid [38] which will remove a significant quantity of these low-molecular-mass degradation products.

The presence of both intact and degraded albumin implies that only partial inhibition of the degradation process occurred in the 4-week STZ-diabetic rat (Figure 1b). In contrast, in our previous study of experimental albuminuria induced by puromycin aminonucleoside, complete inhibition of degradation was observed [14] and thus we would anticipate that further inhibition of albumin degradation would occur with more advanced stages of diabetic nephropathy.

In conclusion, this study raises the possibility that (1) variation in the rate of albumin degradation contributes to the high variability of albumin excretion rate in normal and diabetic subjects [39], (2) early increases in albumin excretion rate in incipient diabetic nephropathy may be related to changes in renal albumin degradation and this will need further investigation in human subjects, and (3) reversibility of albuminuria may relate to the albumin degradation process rather than being exclusively related to the properties of the filtration barrier.

ACKNOWLEDGMENTS

We gratefully acknowledge Mr Steve Sastra and Mr Matthew Waldron for their technical assistance in performing the radioimmunoassays. This work was supported by grants from the Diabetes Australia Research Trust and the Austin Hospital Medical Research Foundation.

REFERENCES


Received 9 January 1998/25 February 1998; accepted 10 March 1998

© 1998 The Biochemical Society and the Medical Research Society