On the nature of proteinuria with acute renal injury in patients with chronic kidney disease

Rajiv Agarwal

Department of Medicine, Division of Nephrology, Indiana University School of Medicine, and Richard L. Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana

Agarwal, Rajiv. On the nature of proteinuria with acute renal injury in patients with chronic kidney disease. Am J Physiol Renal Physiol 288: F265–F271, 2005; doi:10.1152/ajprenal.00318.2004.—Albuminuria is an excellent marker of cardiovascular and renal prognosis. Commercially available tests of immunodetectable albumin in the urine may not identify posttranslationally modified albumin that makes it undetectable to antibodies. Also, it is unclear whether albumin is degraded to smaller fragments, such as through proteolysis, in the course of acute renal injury. In 20 men with chronic kidney disease, we measured excretion rates of urinary protein (pyragallol red), immundetectable urinary albumin (immunoturbidimetry), and urinary total intact albumin (HPLC) after a single dose of 100 mg intravenous iron sucrose administered over 5 min. Fragmentation of urinary albumin and carbonylation of urinary proteins were assessed by immunoblotting. Results showed that iron infusion increased carbonylation of plasma and urinary proteins in a time-dependent manner. A transient increase in urinary excretion rates of total protein, immundetectable urinary albumin, and total intact albumin was seen. Fragmentation and loss of immunoreactivity of albumin paralleled the changes in total protein excretion. In conclusion, fragmentation, loss of immunoreactivity, and oxidation of albumin in a time-dependent manner may underestimate the extent of injury with the immunoactive microalbumin assay. Measurement of total intact albumin may better quantify acute renal injury.

oxidative stress; albuminuria

PROTEINURIA IS A HALLMARK of chronic renal injury, the quantity and quality of which are excellent markers of both the cardiovascular and renal prognosis (13). Traditionally, the occurrence of albuminuria has been held as a marker of altered glomerular permselectivity (3), whereas the occurrence of small-molecular-weight proteinuria has been linked to renal tubular damage (2). While albuminuria has been well studied in models of chronic kidney disease (CKD), relatively little is known about the occurrence of albuminuria with acute renal injury in people with CKD (6). The processing of urinary albumin with acute renal injury is also poorly defined.

Data are emerging that antibody-based tests of albuminuria may not identify posttranslationally modified albumin that makes it immunonreactive (11). If immunoreactive domains on urinary albumin were lost in the setting of acute renal injury, it would make the use of urinary total intact albumin, a test not based on immunodetection but molecular size, more desirable over tests of urinary albumin based on immunodetection. Whether acute renal injury makes albumin immunoreactive is not known.

Acute renal failure is characterized by activation of intrarenal proteases, which can potentially degrade albumin to smaller fragments. Whether degradation of albumin occurs with acute renal injury is also unclear. If albumin were fragmented, then neither a total intact albumin test nor the conventional, commercially available, antibody-based urinary albumin assays may detect these fragments (10). Accordingly, studies are needed to evaluate the measurements of total urinary protein, urinary total intact albumin, and immunoreactive urinary albumin assays in the setting of acute renal injury.

In patients with stable CKD, parenteral iron increases plasma concentration and urinary excretion rate of malondialdehyde, a biomarker of lipid peroxidation, within 15–30 min of iron sucrose administration (1). This is accompanied by enzymuria and increase in proteinuria. In contrast, saturation of transferrin is not maximally seen until 3 h after the end of infusion. Oxidative stress, enzymuria, and proteinuria are transient and completely resolve in 24 h. Finally, N-acetyl-L-cysteine (NAC) reduces acute generation of systemic oxidative stress but fails to abrogate proteinuria or enzymuria. In this study, however, the nature of proteinuria was not characterized. Serial hourly observations in the same patient in an acute oxidative stress-induced human kidney injury model offered the opportunity to assess critically the performance of various protein assays, and albumin handling, in an evaluation of renal injury. In this model, I studied the time course of albumin fragmentation, loss of immunoreactivity, and oxidative damage to urinary albumin. These findings would have implications for the assessment of acute renal injury by measurement of urinary protein and identify potential shortcomings in our assessment of acute renal injury as reflected by immunodetection of albuminuria.

METHODS

Subjects. The study design and primary results of the study have previously been published (1). Briefly, 20 male veterans with an average (SD) age of 73 (7) yr, average weight of 96 (21.3) kg, and average estimated glomerular filtration rate of 26 (7.2) ml/min participated in the study after regulatory approval and written informed consent. Twelve patients had diabetes, six had hypertension, and two had uncertain causes of CKD. All patients completed the study except one, who died 2 days after the first injection of intravenous iron sucrose of causes unrelated to the intravenous iron or study procedures.

After baseline collection of blood and urine, subjects received an intravenous dose of iron sucrose (Venofer, American Regent Laboratories, Shirley, NY) at a dose of 100 mg infused over 5 min. Blood and urine collections were obtained at 0.25, 0.5, 1, 2, 3, and 24 h after iron dosing. Urine and plasma or serum were immediately frozen in aliquots and stored at −80°C until analysis. Subjects were assigned by a computer-generated permuted block randomization scheme to one of two open-label, 1-wk, parallel treatment arms: either no active

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intervention or treatment with the antioxidant NAC (Macomyst, Apothecon, Princeton, NJ) at a dose of 600 mg twice a day for 1 wk. After 1 wk, subjects had all study procedures repeated as on the initial study period.

**Urinary albumin and protein measurement.** Total urinary protein was determined using the dye-binding method using a complex of pyrogallol red and molybdenum acid (QuanTest Red Total Protein Assay System, Quantimetrix, Redondo Beach, CA). Immunodetectable urinary albumin was determined turbidimetrically by the clinical laboratory using the Roche Cobas Integra 400 system (Roche Diagnostics, Indianapolis, IN). Total intact albumin was determined by an HPLC-based assay (Accumin, AusAm Biotechnologies). This assay reduces the potential for false negative results that may arise with antibody-based assays for albumin that fail to detect all intact albumin in the urine (8). To ensure calibration between assays, we assayed Accumin albumin standards by the antibody-based turbidometric method. The slope was 1.013 ± 0.047 ($r^2 = 0.99$), confirming excellent calibration between Accumin and turbidometric assays. The reference standard for the pyragallol red assay supplied by the manufacturer contains protein containing 67% albumin and 33% globulin. Comparison of this reference standard to BSA Fraction V (96%, Sigma, St. Louis, MO), human apotransferrin (Sigma), and rabbit IgG (reagent grade, Sigma) yielded slopes of 1.07 ± 0.02, 0.96 ± 0.02, and 0.41 ± 0.01 (all with $r^2 = 0.99$), respectively. Thus albumin is overestimated by 7%, transferrin is underestimated by 4%, and IgG by 59% using the pyrogallol red colorimetric assay kit.

**Effect of albumin fragmentation on pyrogallol red assay.** Fragmentation of albumin, such as through proteolytic digestion, may lead to underestimation of albumin by the pyrogallol red assay. To test this notion, 20 μl of BSA in concentrations of 0, 50, 100, 250, 500, and 625 μg/ml were incubated with 2 μl of 0.25% trypsin, 1 mM EDTA (GIBCO BRL, Life Technologies) overnight at room temperature. A standard curve containing the same concentrations of BSA but with 2 μl of PBS was constructed. Next, 0, 50, 250, and 625 μg/ml BSA were incubated with 2 μl of 0.25% trypsin at 37°C for 1, 5, 15, 30, 60, 90, 120, and 180 min to test the notion that fragmentation of albumin is time dependent. A corresponding standard curve containing 0–625 μg/ml BSA but with 2 μl of PBS incubated at 37°C for 3 h was constructed as a time control. The concentrations of BSA were selected to mimic concentrations of urinary albumin that may be seen in people with microalbuminuria and overt albuminuria.

**Immunoblotting of urinary albumin and carbonyl.** Oxidation of urine proteins was detected by the carbonyl assay by immunoblotting using an OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA). Urine samples of 10 μl were added to an equal volume of 12% SDS solution, vortexed, and then derivatized with 20 μl of dinitrophenylhydrazine solution for 10 min at room temperature. Subsequently, 15 μl of neutralization solution were added to the above mixture, vortexed, and then 5 μl of 2-mercaptoethanol were added. Derivatized urine samples were loaded on 4–20% gradient SDS-PAGE gels (Bio-Rad, Hercules, CA) using Tris·HCl buffer in volumes calculated to give 5 μg protein/sample to a maximum volume of 19 μl. Bio-Rad Precision molecular weight standards were run with each gel. Electrophoresis was performed according to the method of Laemmli for 240 volt-hours. Electroboblotting was then carried out on 0.2-μm nitrocellulose paper for 60 volt-hours, and the membrane was blocked overnight at 4°C using 0.5% casein, 0.1% Tween in PBS (pH 7.4) buffer. Three washes of the protein blot were carried out using Tris-saline-Tween buffer for 6 min each, one wash with a higher salt-containing buffer, and finally two additional Tris-saline-Tween washes. All of the above procedures were carried out in duplicate.

In one set of protein blots, detection of carbonyl bands was performed using rabbit anti-dinitrophenylhydrazine antibody in casein buffer for 2 h at room temperature. Washes were repeated as above. The secondary antibody was goat anti-rabbit IgG horseradish peroxidase conjugate in casein buffer for 1 h at room temperature. Washes were again repeated as stated above. In the second set of protein blots, detection of albumin was performed using mouse anti-human serum albumin monoclonal antibody (Zymed Laboratories, South San Francisco, CA) diluted 1:1,000 in casein-containing buffer. The washing steps were identical to those above. The secondary antibody used was 1:10,000 diluted goat anti-mouse IgG horseradish peroxidase conjugate (Zymed Laboratories) in casein-containing buffer.

Bands in both the immunoblots were visualized with chemiluminescence using Pierce West Pico Super signal reagents (Pierce Bio-
technology, Rockford, IL) and captured on film. Bands were analyzed densitometrically with dedicated software using the GelLogic 100 System (Kodak, Rochester, NY).

To ensure the same analytic conditions within patients, samples for individual patients were analyzed on the same blot. Following visualization of the immunoblots, the nitrocellulose paper was stained for protein with amido black (Amido Black Staining Solution, Sigma), and the band density for each was calculated by an epi-illumination imaging technique using the equipment as noted above.

The ratio of the density of the carbonyl band vs. the corresponding amido black band was used to calculate the percent carbonylation. In situations where urine albumin concentration was <20 mg/l, we concentrated the urine specimen 10-fold using 10,000 molecular weight-cutoff Amicon filters (Millipore, Billerica, MA) to obtain enough protein to analyze carbonylation of urinary protein.

**Densitometry.** To calculate the extent of carbonylation, the ratio of total carbonyl protein density was divided by the total protein density as measured by amido black staining. Similarly, to calculate the extent of albuminuria, the ratio of total albumin monoclonal antibody-detectable bands was divided by total protein density on amido black staining of the corresponding blot.

**Statistical analysis.** Test performance was compared by regression and agreement analysis (4). Because multiple measurements were carried out within the same subject, subjects were treated as an independent variable in one-way ANOVA when differences between two tests were compared, e.g., immunodetectable urinary albumin and urinary total intact albumin. Urinary protein excretion rates were log2 transformed before repeated-measures ANOVA. The presence of nonintact albumin protein was tested by analyzing the difference between the total intact albumin and total protein assay at each time point.

### Table 1. Concentration-dependent underestimation of albumin after trypsin digestion with pyragallol red assay for urinary protein

<table>
<thead>
<tr>
<th>Nominal Albumin Concentration, µg/ml</th>
<th>Measured Protein Concentration in Undigested Sample, µg/ml</th>
<th>Measured Protein Concentration in Digested Sample, µg/ml</th>
<th>Percent Underestimation in Digested vs. Undigested Sample*</th>
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<tbody>
<tr>
<td>625</td>
<td>642</td>
<td>522</td>
<td>19%</td>
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<tr>
<td>500</td>
<td>474</td>
<td>345</td>
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<td>36</td>
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<td>0</td>
<td>6</td>
<td>10</td>
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BSA was incubated overnight with trypsin, and total protein was analyzed in time controls and trypsinized specimens. Digestion of albumin leads to underestimation of total protein by the pyragallol red assay. The percent error at higher concentrations was less than at lower concentrations, suggesting that fragmentation of albumin in macroalbuminuria range may cause less underestimation of urinary protein compared with fragmentation in the microalbuminuria range. *Adjusted for the slight protein overestimation due to presence of trypsin in the sample.

### Table 2. Time-dependent underestimation of albumin after trypsin digestion with pyragallol red assay for urinary protein

<table>
<thead>
<tr>
<th>Incubation Time, min</th>
<th>Percent Underestimation in Digested Sample</th>
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<tbody>
<tr>
<td></td>
<td>Nominal BSA Concentration (625 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>1%</td>
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<tr>
<td></td>
<td>5%</td>
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<td>48%</td>
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<td>52%</td>
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BSA was incubated at 37°C with trypsin and total protein was analyzed in time controls and trypsinized specimens at 3 different BSA concentrations. Digestion of albumin leads to underestimation of total protein by the pyragallol red assay in a time-dependent manner. The percent error at higher concentrations was less than at lower concentrations, suggesting that fragmentation of albumin in macroalbuminuria range may cause less underestimation of urinary protein compared with fragmentation in the microalbuminuria range.

### Fig. 2. Differences between assays are plotted against the average analyte concentration. Each data point with SE difference reflects data from 1 patient.
point in a repeated-measures design. Similarly, the presence of immunodetectable albumin fragments was tested by analyzing the difference between the total intact albumin and immunodetectable urinary albumin assay in a repeated-measures design. All $P$ values are two sided, and significance was set at $<0.05$. All statistical analyses were performed using Statistica for Windows, version 5.5 (StatSoft, Tulsa, OK).

RESULTS

The test performance of the assays of total protein, total intact albumin, and immunodetectable urinary albumin is shown in Fig. 1. Immunodetectable urinary albumin concentration was 25% lower than that estimated by urinary total protein assay, with a coefficient of determination of 0.99 (Fig. 1A). Urinary total intact albumin was, on average, 29% higher compared with immunodetectable urinary albumin (Fig. 1B). Finally, there was no difference in the relationship between total intact albumin concentration and total urinary protein concentration (Fig. 1C).

Table 1 shows the relationship between digestion of albumin and estimation of protein by the pyragallol red method. At lower concentrations of albumin, the underestimation of albumin was between 35 and 45%, whereas at higher concentrations it was ~20–30%. Thus the pyragallol red method is likely to underestimate albumin fragments especially in the microalbuminuria range, whereas in conditions of overt albuminuria, underestimation is less likely.

To characterize further the phenomenon of albumin fragmentation and detection of protein by the pyragallol red method, samples of BSA simulating microalbuminuria (50 $\mu$g/ml), borderline albuminuria (250 $\mu$g/ml), or overt albuminuria (625 $\mu$g/ml) were incubated with trypsin for varying time periods. Total protein was assayed at the end of the experiment. Table 2 shows a time-dependent underestimation of albumin irrespective of the concentration of the albumin studied. After 3 h of incubation with trypsin, total protein was underestimated by 87% in 50 $\mu$g/ml albumin, but only underestimated 32% in 625 $\mu$g/ml albumin. Therefore, at lower concentrations of albumin, the magnitude of underestimation is greater compared with that at higher concentrations.

The difference between total urinary protein and immunodetectable urinary albumin was related to the average concentration of the analyte (Fig. 2A). Thus at very high concentrations of urinary protein, there was substantially more urinary protein compared with immunodetectable albumin. After between-subject variation was accounted for, within-subject variation in the error estimate was small (Fig. 2A). Similar results were obtained for the difference between total urinary intact albumin and immunodetectable urinary albumin. This difference, a measure of immunounreactive albumin, was greater at higher concentrations of the analyte (Fig. 2B). Finally, there was no significant between-subject variation in the difference between total protein and intact albumin except at very high concentrations.
concentrations when urinary total protein was higher than intact albumin (Fig. 2C).

There was a time-dependent increase in proteinuria as measured by the dye-binding assay and in albuminuria as measured by total intact albumin and immunodetectable urinary albumin assays (all \( P < 0.0001 \)) (Fig. 3A). The majority of excreted protein was intact albumin. The difference between total urinary protein and intact albumin reflects albumin that is fragmented and nonalbumin urinary proteins. There was a time-dependent increase in such nonintact albumin proteins (Fig. 3B). Furthermore, there was a time-dependent increase in immunounreactive albumin excretion as measured by the difference in total urinary intact albumin and immunodetectable urinary albumin (Fig. 3C).

Concentrating the urine specimens did not yield high-quality protein bands on immunoblotting for analytic purposes. Thus seven subjects in whom protein bands were clearly visible without concentrating the specimen for the analysis of fragmentation of urinary albumin. Figure 4 shows the relationship of the total albumin density to total protein density increasing over 3 h, indicating that albumin excretion as a fraction of total protein increased over time (\( P = 0.001 \)). This could be due to increased excretion of intact albumin, increasing fragmentation of albumin, or both. That fragmentation of protein of 67-kDa molecular mass over 180 min occurs in vivo after intravenous iron administration is demonstrated by amido black staining of the protein bands on nitrocellulose paper in Fig. 5A. These fragments were identified as albumin by the monoclonal albumin antibody (Fig. 5B). These data demonstrate that albumin is increasingly fragmented over time with intravenous iron-induced renal injury.

There was a significant increase in plasma carbonyls, which peaked at 3 h (\( P = 0.034 \)) (Fig. 6A) and were not reduced with NAC treatment (\( P = 0.20 \) for visit \( \times \) treatment interaction, Fig. 6B). Urinary carbonyls increased from 0.93 ± 0.84 at baseline to 1.42 ± 1.07 at 15 min, 1.38 ± 1.14 at 3 h, and returned to 1.10 ± 0.87 at 24 h (\( P = 0.053 \)). There was no effect of NAC treatment on urinary protein carbonylation (\( P > 0.2 \)). Carbonylation of individual urinary protein fragments is shown by total protein staining (Fig. 5C) and by immunoblotting (Fig. 5D).

**DISCUSSION**

As expected, immunodetectable urinary albumin was lower than total protein in the urine sample, suggesting the presence of nonalbumin proteins and immunoundetectable albumin in the urine. The coefficient of determination of 0.99 may tempt us to the conclusion that this may be an excellent estimation of immunodetectable albumin with an inexpensive, convenient, dye-binding assay (Fig. 1A). However, further analysis showed that the error in estimation between total protein and immunodetectable urinary albumin was related to the level of proteinuria (Fig. 2A). Thus there was an increase in error at higher levels of proteinuria.

Conventional detection of albumin that is the basis for most of the commercially available “microalbuminuria” assays is dependent on immunological methods. Loss of antibody-recognizable epitopes on albumin has been postulated as the cause of “ghost albumin” that is immunoundetectable (11). Total intact albumin was 29% higher than the conventional immunodetectable urinary albumin assay, supporting the reports of others (8). Furthermore, immunounreactivity, defined as the difference in total urinary intact albumin and immunodetectable urinary albumin (Fig. 2A). Thus there was an increase in error at higher levels of proteinuria.

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immunoreactivity (Fig. 3C) appears to be the reason for these discrepant observations.

The concentration of total intact albumin in the urine was no different from the pyragallol red assay for urine protein, implying that the dye-binding pyragallol red assay can serve as a reasonable approximation of total intact albumin assay. Unlike the previous two analyses, there was no significant difference between subjects with respect to the difference between total intact albumin and total protein (Fig. 2C). Although there was no bias in the pyragallol red assay for predicting intact albumin, agreement between the pyragallol red assay and the total intact albumin assay was poor. Thus the confidence limits for the prediction interval would be wide if the pyragallol red assay were used to predict urinary total intact albumin. The pyragallol red assay overestimates albumin by 7%, underestimates transferrin by 4%, and IgG by 59%. Albumin fragments lead to underestimation of urinary proteins by a variety of commercially available assays (9). Slight overestimation of albumin, underestimation of albumin fragments, and gross underestimation of IgG by this method are likely the explanation for finding an excellent correlation between total intact albumin and total urinary protein. Urinary globulins and other proteins, which were not studied, can also undergo postglomerular processing and impact the pyragallol red assay. Stated differently, the errors in assessment of urine protein by the pyragallol red assay mathematically summate to produce an excellent correlation. Thus the pyragallol red assay cannot be substituted for the total intact albumin assay as it does not have a biological basis.

Albuminuria was the major constituent of urinary proteins with injury induced by intravenous iron. This is consistent with oxidative stress-induced impairment in podocyte selectivity seen in animals (12). The occurrence of renal injury was associated with transient loss of immunoreactivity of albumin that may be due to loss of antibody-recognizing sites on the albumin molecule. Furthermore, fragmentation of albumin was confirmed by immunoblot analysis (7). Although it is possible that the reducing conditions of electrophoresis may be enough to fragment albumin (14), this would not be a sufficient explanation for increasing time-dependent in vivo fragmentation. Albumin fragmentation does not occur in the plasma, but fragmentation is seen in urine in animal models of diabetes (5).

The pathophysiology of albumin fragmentation was studied further with carbonylation assays. I reasoned that albumin carbonylation would make it susceptible to lysosomal degradation. Indeed, I found that fragmentation of albumin and carbonylation of proteins were correlated in both their time course and their location on gel electrophoresis. I hypothesized that if urinary fragmentation was seen despite blocked protein carbonylation, then the conclusion that carbonylation occurs following urine albumin fragmentation would be reasonable. The dose and duration of the antioxidant were insufficient to block urinary albumin carbonylation. Therefore, whether the carbonylation of albumin led to its fragmentation and loss of immunoreactivity, or the reverse argument holds true, cannot be answered by the present data.

It is also important to note that whereas the quantitative abnormalities in urinary protein excretion were reduced at 180 min compared with 15 min, the qualitative abnormalities, such as urine carbonylation and fragmentation (Figs. 4 and 5), continued to increase at 180 min. This raises the possibility that qualitative abnormalities in urine albumin processing may be more sensitive in detecting injury and may also take longer to repair.

These results have important implications for the interpretation of urinary protein electrophoresis. The sizing of proteins by PAGE is typically used for classifying proteinuria as tubular or glomerular. However, the sizing of proteinuria by PAGE has some drawbacks. Filtered albumin can undergo fragmentation by lysosomal processing in the renal tubules (14); this phenomenon can misclassify patients as having small-molecular-weight proteinuria. My results show that urinary albumin was fragmented in patients with CKD. More importantly, the occurrence of albumin fragmentation in the setting of acute intravenous iron exposure suggests that albumin may be damaged with acute renal injury. Therefore, sizing proteinuria may not be sufficient to ascertain the origin of proteinuria, glomerular or tubular, in the setting of acute renal injury.

In conclusion, our data show that iron-induced oxidative stress increased protein oxidation in a time-dependent manner in plasma and urine and led to an increase in intact albumin, immunoreactive albumin, immunoreactive albumin, and albumin fragmentation. We found that many small-molecular-weight proteins are actually albumin fragments, not tubular proteins, implying that PAGE is not sufficient to characterize the origin of urinary proteins. Fragmentation, loss of immunoreactivity, and oxidation of albumin in a time-dependent manner may underestimate the extent of injury with the commercial immunodetectable urinary albumin assay. The measurement of total intact albumin would better characterize renal injury in the setting of acute renal failure.

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