Renal Tubular Cell Protein Breakdown in Uninephrectomized and Ammonium Chloride–Loaded Rats

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ABSTRACT
Kidney enlargement after unilateral nephrectomy or the induction of a systemic acidosis with ammonium chloride is associated with an increase in kidney protein content. This reflects an imbalance between protein breakdown and protein synthesis. Because it has been shown in diabetic nephromegaly that depressed protein breakdown contributes to the increase in kidney protein content, this study examined whether altered protein breakdown is common to all forms of renal hypertrophy. Accordingly, protein turnover was measured in isolated proximal tubules from kidney in rats undergoing renal enlargement after uninephrectomy or chronic ammonium chloride–induced acidosis. In both conditions, kidney protein content and protein synthesis ([14C]valine incorporation) increased significantly. Fractional protein degradation was depressed in renal tubules isolated from the acidicotic rats and was accompanied by a decrease in proximal tubule cathepsin B and combined B and L activities. These changes are comparable to earlier observations with the diabetic kidney. In contrast, after unilateral nephrectomy, protein breakdown is not reduced, and it can reasonably be concluded that, in this condition, protein gain reflects increased protein synthesis alone. It was concluded that the pattern of protein turnover leading to protein accretion in renal hypertrophy varies according to the initial stimulus for renal growth.

Key Words: Compensatory renal growth, proteolysis, acidosis, protein turnover, kidney proximal tubules

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rats were pair-fed with UNX rats and euthanized at 96 h. These rats served as paired controls. For the measurement of proximal tubule protein degradation, control and UNX rats were euthanized 5, 24, and 96 h after surgery.

**Ammonium Chloride–Induced Renal Hypertrophy**

Female Sprague-Dawley rats weighing 191 to 201 g were studied. Treated rats received ammonium chloride dissolved in water at a dose of 40 mmol/kg body wt per day by gavage and were allowed free access to food and water. Controls were pair-fed and received water by gavage. Rats were euthanized after 4 or 7 days of treatment. At the time of euthanasia, arterial blood was taken from the aorta for pH and bicarbonate measurements.

**Isolation of Rat Kidney Proximal Tubules**

Proximal tubules were isolated from rat kidneys as originally described by Gesek et al. (8). In short, rats were anesthetized with pentobarbital (50 mg/kg ip). The kidneys were then perfused through the abdominal aorta with an oxygenated Krebs-Ringer-Henseleit buffer containing collagenase (1 mg/mL) and hyaluronidase (0.33 mg/mL) at 37°C. After 10 min, the kidneys were excised and the cortex was minced. Cortical tissue was then incubated with the oxygenated enzyme–containing buffer at 37°C for another 10 min. Proximal tubules were then separated from the resulting cortical tubule suspension by filtration through a 150-μm-pore-size nylon mesh and then centrifugation in a 45% iso-osmotic percoll gradient and prepared for study. Tubules from a control rat and an experimental rat were isolated on the same day and studied in parallel in order to reduce experimental variability.

**Measurement of Protein Degradation in Isolated Tubules**

This was determined as previously described from this laboratory (1.9). In brief, long-lived tubule cell proteins were radiolabeled by the iv injection of 25 μCi of [14C]valine into sodium methohexital (50 mg/kg)-anesthetized rats 24 h before study. Isolated proximal tubules were then suspended in incubation medium at a concentration of 4 to 6 mg of tubule protein/mL, and 5-mL aliquots were placed in sealed 37°C incubation medium/Fl2 with 5 mM lactate, 5 mM butyrate, 1 mM glycine, 10 mM valine, 1% bovine serum albumin and was gassed with 95% O2–5% CO2. Aliquots of the incubation medium were taken at the beginning and then after 75 min of incubation. Trichloroacetic acid (TCA), final concentration 10%, containing 5 mM valine and cooled to 4°C was then added to the samples, which were then centrifuged for 15 min. The precipitate was washed with 10% TCA followed by centrifugation, and the supernates were combined. The pellet from the initial sample was solubilized in 0.1% sodium dodecyl sulfate with 0.1% NaOH. Radioactivity in the supernate and solubilized pellet was counted in a liquid scintillation counter (Searle Analytic Inc., Elk Grove Village, IL). The fractional rate of long-lived protein degradation was calculated as previously described (9) from the release over 75 min of radioactivity soluble in TCA. This was expressed as a percentage of total radioactivity present in long-lived proteins at the beginning of the experimental period. The early 37°C digestion period and the washes involved in isolating the tubules allowed for the release of unincorporated [14C]valine and [14C]valine produced from the degradation of radiolabeled short-lived proteins. In all of these experiments, 5 mM unlabeled valine was added to the incubation medium to expand the free valine pool so as to minimize the reincorporation of released [14C]valine into protein and to minimize the breakdown of the radiolabeled amino acid.

**[14C]Valine Incorporation in Isolated Tubules**

The incorporation of [14C]valine into TCA-insoluble material was taken to represent protein synthesis and was determined as previously described (10). In brief, isolated proximal tubules were incubated for 75 min under the same conditions as described for the protein degradation studies above except that, in addition to the 5 mM cold valine, [14C]valine (1 μCi/mL) was included in the incubation medium. Rats did not receive [14C]valine in vivo. Aliquots of the tubule suspension were then placed on ice and washed five times with cold (4°C) 2% TCA plus 5 mM valine and then once with 10% TCA plus 5 mM valine to remove free [14C]valine. The TCA precipitate was solubilized in 1 N NaOH and counted. The radioactivity incorporated was expressed as disintegrations per minute per 60 μg of tubule protein. Unlabeled valine (5 mM, 25 times the normal plasma level) was added to the incubation medium so as to maintain optimal precursor specific activity, to avoid problems of compartmentalization, and to minimize the dilution and degradation of the tracer over the period of the study (11). To test that these conditions were being met, we carried out preliminary studies (N = 3) in which we examined the effect of raising the concentration of cold valine on [14C]valine incorporation. Increasing the concentration of valine from 5 to 10 mM was associated with similar rates of incorporation, 1.74 ± 0.50 versus 2 ± 0.70 nM/75 min, suggesting that the desired conditions were being met.

**Cathepsin Assay**

Cathepsin B activity and the combined activities of cathepsins B and L were determined in assays that specifically measured these enzyme activities in kidney. These are described in detail by Olbricht et al. (12). The combined activity of cathepsins B and L was determined by measuring fluorescence generated by cathepsins B and L activities on the substrate Z-Phe-Arg-7-amino-4-methyl-coumarin (7-Phe-Arg-NMec), which releases the highly fluorescent product NMec. Cathepsin B activity was determined from fluorescence generated when it cleaves Z-Arg-Arg-NMec to release fluorescence and NMec. Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and NMec were purchased from Enzyme System Products (Dublin, CA). The substrate was diluted to its final concentration of 0.5 mM with 0.1% Brij (Sigma Chemical Co., St. Louis, MO). And the standard curve was created by using NMec. On the basis of protein concentration, aliquots of proximal tubules were incubated at 37°C for 10 min with the substrate. The reaction was stopped by the addition of 100 nM iodoacetate in sodium acetate buffer on ice. Fluorometric measurements were made on a Perkin Elmer LS 50 luminence spectrometer (Elmer Perkin Corporation, Norwalk, CT) with FLDM software on an IBM PS/2 computer. Samples were excited at 365 nm, and emission was recorded at 450 nm with a slit width of 2.5 mm. The assay was linear with respect to incubation time and protein concentration. The specific activity of the enzymes is expressed as picomoles of NMec per 5 mg of protein/min.
Results are expressed as the mean ± SE. Differences between the two groups were analyzed by t test for paired and unpaired data as appropriate. Multiple comparisons were evaluated by analysis of variance, and if the F test was significant, then Scheffe's test was used to make comparisons between the groups. A P value of < 0.5 was taken as significant.

RESULTS

Compensatory Renal Growth

Kidney Size and Protein Content. Over the 4 days after left UNX, the wet weight and protein content of the remaining (right) kidney increased significantly when compared with the right kidney of control rats euthanized at Day 0 (Table 1). As early as 24 h after UNX, there was a significant gain in wet weight (11%) and protein content (8%). By the fourth day, the protein content of the remaining kidney was 29% greater than that of the right kidney of Day 0 control rats (P < 0.05). When the 4-day remnant kidneys were compared with kidneys from sham-operated, 4-day, pair-fed rats, it was evident that they were significantly enlarged (P < 0.05). Kidney wet weight (1.198 ± 19 versus 968 ± 23 mg) and protein content (123 ± 3 versus 105 ± 5 mg) were on average 24 and 17% higher after UNX.

Protein Degradation. The results of the protein degradation measurements made in isolated proximal tubules from kidneys undergoing CRG are depicted in Figure 1. Five hours after UNX, fractional long-lived protein degradation in kidney tubules from the contralateral kidney was similar to that observed in control tubules (1.80 ± 0.18 versus 1.71 ± 0.15%/h). However, 24 h after UNX, there was a significant increase in the fractional rate of protein degradation compared with controls (2.67 ± 0.25 versus 1.75 ± 0.53%/h respectively). This represents a 53% increase in protein degradation. By 96 h, fractional protein degradation in the experimental group had returned to baseline and did not differ significantly from the 96-h controls (2.02 ± 0.20 versus 1.74 ± 0.12%/h).

[14C]Valine Incorporation. The results of the measurement of [14C]valine incorporation into tubule cell protein, taken to represent protein synthesis, are given in Figure 2. [14C]Valine incorporation by renal tubules isolated from UNX rats was significantly greater than in tubules from sham-operated control rats. Five hours after surgery, [14C]valine incorporation by tubules from the experimental rats exceeded that of controls by 63%, after 24 h by 82%, and after 96 h by 39%.

Ammonium Chloride–Induced Renal Growth

Kidney Size and Protein Content. Ammonium chloride loading induced a severe metabolic acidosis and a significant (P < 0.05) increase in kidney size over the periods of study (Table 2). After 4 days of ammonium chloride loading, arterial pH had dropped to 6.88 ± 0.15. Kidney wet weight was 24% (985 ± 4 versus 795 ± 20 mg) and kidney protein content was 18% (131 ± 3 versus 111 ± 2 mg) greater than in pair-fed control rats. After 7 days, kidney wet weight and protein content were 13% greater than in the controls.

TABLE 1. Changes in kidney wet weight and protein content after left UNX

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>No.</th>
<th>Body Wt (g)</th>
<th>Wet Weight (mg)</th>
<th>Protein Content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>7</td>
<td>179 ± 6</td>
<td>793 ± 34</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>UNX</td>
<td>1</td>
<td>7</td>
<td>173 ± 16</td>
<td>884 ± 39b</td>
<td>103 ± 3b</td>
</tr>
<tr>
<td>UNX</td>
<td>2</td>
<td>7</td>
<td>185 ± 21</td>
<td>1,013 ± 25b</td>
<td>116 ± 3b</td>
</tr>
<tr>
<td>UNX</td>
<td>4</td>
<td>6</td>
<td>186 ± 6</td>
<td>1,198 ± 19bc</td>
<td>123 ± 3bc</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>6</td>
<td>187 ± 6</td>
<td>968 ± 23b</td>
<td>105 ± 5b</td>
</tr>
</tbody>
</table>

*Measurements were made on the day of euthanasia. 

b P < 0.05 versus Day 0 control.

bc P < 0.05, Day 4 UNX versus Day 4 control.
Because UNX is followed by an early increase in one kidney, the protein content of the remaining kidney decreased by 50%.

On Day 4, degradation in the treated group exceeded the control value by 53%. Because net kidney protein degradation exceeded the control value by 53% at this time, cathepsin B activity was reduced by 38%, and combined B and L activities were reduced by 50%.

**Protein Degradation.** As depicted in Figure 2, fractional protein degradation in renal proximal tubules, isolated from rats after 4 and 7 days of ammonium chloride treatment, was significantly depressed compared with that in their respective controls (P < 0.05). On Day 4, degradation in the treated group was on average 27% lower than in controls (1.32 ± 0.08 versus 1.80 ± 0.18% h⁻¹), and on Day 7, it was 33% lower (1.42 ± 0.22 versus 2.11 ± 0.16% h⁻¹).

**[¹⁴C]Valine Incorporation.** Loading rats with ammonium chloride induced a significant increase in proximal tubule [¹⁴C]valine incorporation (Figure 4). After 4 days of ammonium chloride, [¹⁴C]valine incorporation in the treated group exceeded the control values by 46% and after 7 days by 41%.

**Cathepsin Activity.** To determine whether proximal tubular lysosomal protease activity was also affected by the ammonium chloride treatment, cathepsins B and L activity was measured in tubules isolated from 7-day control and treated rats (Figure 5). Cathepsin B activity in control and tubules averaged 429 ± 48 and 264 ± 77 pmol/5 mg of protein per minute, respectively (P < 0.01). Total (B plus L) cathepsin activity averaged 892 ± 159 and 448 ± 107 pmol/5 mg of protein in the control and treated tubules, respectively (P < 0.01). Thus, on average, cathepsin B activity was reduced by 38% and combined B and L activities were reduced by 50%.

**DISCUSSION**

As is well established, the surgical removal of one kidney or chronic ammonium chloride-induced metabolic acidosis was followed by an increase in kidney size and protein content. Four days after the loss of one kidney, the protein content of the remaining kidneys increased by 17% over that of the ipsilateral kidneys of sham-operated, pair-fed control rats. A similar increment, 18%, in protein content was observed after 4 days of ammonium chloride loading. Because UNX is followed by an early increase in kidney protein content (Table 1; 4), we made our earliest measurements 5 h after UNX. At this time, fractional protein degradation in proximal tubules isolated from UNX rats did not differ significantly from that in sham-operated controls (1.71% h⁻¹). When measured at 24 h, however, fractional protein degradation in the UNX group exceeded that of the time controls by 53%. By the fourth day of study, fractional protein degradation had returned to values that did not differ from controls (Figure 1). [¹⁴C]Valine incorporation, taken to reflect protein synthesis, was increased significantly at all of these times. The largest increase was noted at 24 h, when [¹⁴C]valine incorporation in proximal tubules from the UNX rats was near double that of controls. Thus, the net gain in kidney protein content after UNX appears to be solely due to an increase in protein synthesis. Because the increase in kidney protein content occurs later in ammonium chloride acidosis (6), we first studied protein degradation after 4 days of ammonium chloride loading. In contrast to the UNX experiments, fractional protein degradation measured at this time was depressed significantly and on average was 28% lower than in the controls. Depressed fractional protein degradation was still evident after 7 days of ammonium chloride treatment (Figure 2). Proximal tubule lysosomal cathepsin B and combined B and L activities measured at this time interval were depressed significantly. Cathepsin B activity was depressed by 38%, and combined B and L activities were depressed by 50%. This suggests that reduced lysosomal protease activity may contribute to the depression of proximal tubule long-lived protein degradation in ammonium chloride–induced metabolic acidosis. In contrast to depressed protein degradation, [¹⁴C]valine incorporation was increased in tubules from ammonium chloride–treated rats.

Because organ protein content reflects the balance between cellular protein synthesis and degradation (5), it appears that both depressed fractional protein degradation and increased protein synthesis contribute to the increase in kidney protein content and, thus, kidney mass, seen in ammonium chloride–induced acidosis. In contrast, depressed fractional protein degradation does not seem to play any role in the increase in kidney protein content after UNX, which appears to be solely a consequence of increased protein synthesis. In this respect, the response of kidney differs from liver; in the regenerating liver, after partial hepatectomy, depressed protein degradation is a major factor determining the increase in organ protein content (13). It is noteworthy that in the kidney undergoing CRG, the fractional protein degradation did not differ from controls when measured 5 or 96 h post-UNX. However, at 24 h, degradation exceeded the control value by 53%. Because net kidney protein content had increased over this 24-h period, it appears that protein synthesis must have increased to an even greater extent. Indeed, [¹⁴C]valine incorporation increased by 82% at this time. An increase in protein degradation has also been described with rapidly growing heart and skeletal muscle. It has been
TABLE 2. Changes in kidney wet weight and protein content after ammonium chloride loading

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>No.</th>
<th>Body Wt (gm)</th>
<th>Wet Weight</th>
<th>Protein Content</th>
<th>Arterial pH</th>
<th>HCO3 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>5</td>
<td>190 ± 2</td>
<td>795 ± 20</td>
<td>111 ± 2</td>
<td>7.42 ± 0.07</td>
<td>32.0 ± 2.3</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>5</td>
<td>189 ± 3</td>
<td>784 ± 19</td>
<td>115 ± 3</td>
<td>7.35 ± 0.02</td>
<td>30.1 ± 0.9</td>
</tr>
<tr>
<td>Experimental</td>
<td>4</td>
<td>5</td>
<td>190 ± 2</td>
<td>985 ± 04b</td>
<td>131 ± 3b</td>
<td>6.82 ± 0.15b</td>
<td>6.7 ± 5.3b</td>
</tr>
<tr>
<td>Experimental</td>
<td>7</td>
<td>6</td>
<td>192 ± 4</td>
<td>889 ± 20b</td>
<td>130 ± 3b</td>
<td>7.09 ± 0.01c</td>
<td>17.5 ± 0.4b</td>
</tr>
</tbody>
</table>

- Measurements were obtained on the day of euthanasia.
- p < 0.01.
- p < 0.05.

Fractional long-lived protein degradation in proximal tubules isolated from kidneys removed from rats after 4 or 7 days of NH4Cl loading. Rats received NH4Cl (40 mM/kg body wt per day) in water or water alone (controls) by gavage. N = 6 rats per group.

Figure 3. Fractional long-lived protein degradation in proximal tubules isolated from kidneys removed from rats after 4 or 7 days of NH4Cl loading. Rats received NH4Cl (40 mM/kg body wt per day) in water or water alone (controls) by gavage. N = 6 rats per group.

suggested that the accelerated degradation may play a role in tissue remodeling during rapid growth (14). In contrast to our finding of unchanged or increased fractional protein degradation in isolated proximal tubules, Hill and Malamud (15) have reported that, in mice after UNX, whole-kidney protein degradation is depressed. However, this disparity may reflect a technical problem. Their results could reflect increased reincorporation of the tracer amino acid released during proteolysis by the kidneys undergoing CRG rather than depressed degradation. In this study, an excess of unlabeled valine was added to the in vitro assay to avoid this artifact.

With regard to the role of protein synthesis in CRG, there are several studies describing an increase in protein synthesis as measured by [14C]leucine incorporation into protein (4, 16, 17). However, the methods used had major shortcomings and these studies must be treated with circumspect. In particular, the specific activity of [14C]leucine in the immediate protein precursor pool, that is, the [14C]leucine bound to tRNA, was not measured. Thus, rather than representing an increase in protein synthesis, the increase in [14C]leucine incorporation may have reflected an increase in the size of the precursor pool due to heightened leucine transport into the cell or decreased intracellular leucine degradation (10, 18, 19). In this
study, we used \(^{14}\)C-valine as the tracer, and to minimize problems of compartmentalization, dilution, or degradation of the tracer, incorporation was measured in the presence of an excess of unlabeled valine (5 mM) (11). Nevertheless, the increase in incorporation might still not reflect protein synthesis. On the other hand, because protein degradation was not depressed, the increase in kidney protein content must be due to an increase in protein synthesis, a conclusion consistent with increased \(^{14}\)C-valine incorporation.

The situation after ammonium chloride-induced acidosis is more complex because, whereas depressed protein degradation appears to contribute to the accretion of protein, an increase in protein synthesis may well play an important role, as suggested by our finding of an increase in \(^{14}\)C-valine incorporation. A review of the literature reveals conflicting reports with respect to the effects of ammonium chloride on protein synthesis. We observed that cultured primary rabbit proximal tubular cells respond to ammonium chloride by increasing cell size and protein content, but protein synthesis was not increased (20). Jurkowitz et al. (19), studying cultured LLC-PK cells, experienced similar results. Bignall et al. (17), studying \(^{14}\)C-leucine incorporation into kidney slices of rats treated with ammonium chloride for up to 36 h, failed to detect an increase in \(^{14}\)C-leucine incorporation. It should be noted, however, that the increase in kidney protein content occurred after 48 h; also, kidney slices were studied and no precautions were taken to minimize the degradation or compartmentalization of the tracer. On the other hand, Golchini et al. (21), in their study with JTC kidney cells, noted an increase in \(^{14}\)C-leucine incorporation after 24 h of exposure to ammonium chloride, although that study has been questioned on methodologic grounds. There are also studies with cultured astrocytes (22) and sea urchin eggs (23) that suggest that ammonium chloride stimulates protein synthesis. Our study, in which \(^{14}\)C-valine incorporation was increased in tubules isolated from rats after 4 days or more of ammonium chloride loading, is consistent with an increase in protein synthesis contributing to renal hypertrophy.

The observation that fractional protein degradation is depressed in proximal tubules from rats with ammonium chloride acidosis is in agreement with previous cell culture studies. Using cultured kidney cells originating from the proximal tubule, we (10,20), Golchini et al. (21), and Jurkowitz et al. (19) showed that ammonium chloride depressed long-lived cell protein degradation and increased cell protein content. This response was unrelated to intracellular or extracellular pH (19,20) and could be reproduced when ammonia was present in the form of ammonium acetate or sulfate (20), indicating a direct effect of ammonia on fractional protein degradation. From studies with a variety of cultured cells, it has been established that ammonia acts by inhibiting the autophagic-lysosomal pathway of long-lived protein degradation. These proteins account for up to 99% of the cell protein content, and the autophagic-lysosomal apparatus plays an important role in their disposal (24). Long-lived cellular proteins are taken up into autophagic vacuoles, which deliver their contents to lysosomes, where degradation occurs. Apparently, ammonium chloride elevates the pH of acidic organelles, and this impairs the fusion of autophagic vacuoles with lysosomes, inhibits lysosomal enzyme activity, and lowers intralysosomal enzyme content (21,25,26). Taken together, it is likely that the increased ammonia produced by the kidney in response to ammonium chloride loading inhibits the autophagic-lysosomal system. Consequently, long-lived protein degradation decreases and kidney protein content increases. Our finding of lowered cathepsin B and L activities in proximal tubules is consistent with this proposed mechanism of action. Depressed proximal tubule protein degradation with reduced lysosomal cathepsin B and combined B and L activity is not unique to ammonium chloride-induced acidosis. We (1) and also Olbricht et al. (27) have reported similar findings in diabetic nephromegaly. Interestingly, Olbricht et al. (27) found that, although cathepsin B and combined B and L activities were reduced in diabetic rat kidneys, the activity was not reduced in kidneys undergoing CRG after UNX.

Increased renal ammoniagenesis is associated with renal enlargement in a number of diverse conditions (28). These include sustained metabolic acidosis (5), chronic hypokalemia (29), dietary antioxidants (30), and after the loss of renal tissue (31). Because multiple and different processes are altered in these disorders, it would be simplistic to attribute renal hypertrophy to a common mechanism, namely, increased tissue ammonia content. Thus, although our findings with ammonium chloride acidosis are compatible with a direct role for ammonia in the hypertrophic process through the inhibition of proteolysis and the stimulation of protein synthesis, CRG growth was not associated with depressed protein breakdown. Different factors appear to be involved in these diverse conditions.

In summary, it appears that, as previously observed in diabetes, depressed protein degradation contributes to the increase in kidney protein content seen in chronic ammonium chloride-induced acidosis, a condition in which kidney protein synthesis appears to be increased. On the other hand, in CRG after the loss of renal tissue, protein degradation is not depressed and the increase in kidney protein content appears to be entirely due to increased protein synthesis. Taken together, we have shown that, in renal hypertrophy, changes in protein turnover leading to protein gain are not uniform and vary according to the cause of enlargement.

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