Role of Lysosomal Cathepsin Activities in Cell Hypertrophy Induced by NH4Cl in Cultured Renal Proximal Tubule Cells\textsuperscript{1,2}

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ABSTRACT

An increase of renal ammoniagenesis has been implicated in renal hypertrophy associated with various clinical disorders such as metabolic acidosis, diabetic nephropathy, and renal insufficiency. In vivo and in vitro studies have shown that ammonia promotes hypertrophy in tubulip epithelial cells. To elucidate its role on protein turnover, the effects of NH4Cl on the activities of cathepsins B, H, and L + B, as well as on protein synthesis and degradation in LLC-PK1 cells, were investigated. The results show that NH4Cl (20 mM) induced cell hypertrophy, as defined by an increase in both cell protein content and cell volume (+25.5 \pm 1.3 and +10.4 \pm 0.1\% after 48 h). This hypertrophy was associated with the suppression of the activities of cathepsins B and L + B (\textit{57.0} \pm 0.9 and \textit{-54.5} \pm 1.5\% after 48 h) and a reduction of protein degradation rate (\textit{-59.7} \pm 4.1\% after 48 h), but without enhanced protein synthesis. The findings were further supported with an additional experiment, showing that the protein synthesis inhibitor chloroquine (10 \mu M) did not blunt NH4Cl-induced cell hypertrophy. Moreover, NH4Cl (20 mM) resulted in a persistent elevation of the lysosomal pH, whereas the rise in the cytosolic pH was only transient. This alkalization in lysosomes may be causatively involved in the impairment of the activities of cathepsins B and L + B. In conclusion, the suppression of the activities of cathepsins B and L + B and the subsequent reduction of protein breakdown due to intralysosomal alkalinization contribute to NH4Cl-induced hypertrophy in LLC-PK1 cells.

Key Words: Cathepsin, ammonia, NH4Cl, protein turnover, lysosomal pH, LLC-PK1 cells

Nephron hypertrophy, characterized by an increase in cell protein content and cell size, is predominantly accounted for by an elevated renal tubule mass (1). This hypertrophy occurs in several disorders such as diabetic nephropathy, the remnant kidney, and renal insufficiency (1). In addition, chronic metabolic acidosis due to NH4Cl feeding can also lead to nephron hypertrophy in the rat (1). A growing body of evidence suggests that an increase in renal ammoniagenesis rather than metabolic acidosis itself may stimulate tubular hypertrophy (2). Accordingly, it could be demonstrated in vitro that NH4Cl induces hypertrophy in proximal tubule cells (3–5). Because the degree of hypertrophy in any cell depends on the balance between protein synthesis and protein degradation (6), NH4Cl-induced cell hypertrophy could be induced by enhanced protein synthesis, reduced protein degradation, or both. The mechanism involved in the ammonia-induced hypertrophy is not yet completely clear. In particular, the potential role of proteolytic enzymes in the induction of cell hypertrophy in LLC-PK1 cells remains to be clarified.

To elucidate the mechanism by which ammonia modulates protein turnover, we investigated the effects of NH4Cl on the activities of the lysosomal cysteine proteinases cathepsins B, H, and L + B, as well as on the protein breakdown and protein synthesis in LLC-PK1 cells. This cell line, originally derived from a Hampshire pig kidney, exhibits many biochemical and morphologic characteristics of the proximal tubule cells (7). Because lysosomal proteinases act optimally at acidic pH (8,9), the cytosolic and lysosomal pH values were determined to verify whether the potentially altered cathepsin activities were due to changes of the pH.

MATERIALS AND METHODS

Cell Culture

LLC-PK1 cells (2 \times 10^4/cm²) (American Type Culture Collection, Rockville, MD; Passages 220 to 230) were grown in Dulbecco’s modified Eagle medium (DMEM; GIBCO, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; GIBCO), 15 mM N-hydroxyethyipiperazine-N′-2-ethanesulfonic acid (HEPES) and 100 U/mL penicillin/100 ng/mL streptomycin at 37°C in a humidified 5% CO2/95% O2 atmosphere. Confluent monolayers with dome formation were observed between 4 and 6 days after seeding. Cytotox-
ticity and cell viability were monitored by phase contrast microscopy, trypsin blue exclusion, and lactate dehydrogenase test. NH₄Cl (0 to 20 mM; Sigma, Deisenhofen, Germany) did not induce cell damage or detaching after up to 72 h of incubation. In the experiments, the media were adjusted to 7.48 to 7.62 with HEPES. After incubation at 5% CO₂, the pH was in the range of 7.60 to 7.61 after 24 h and 7.52 to 7.54 after 48 h. There is little difference in the absence (pH 7.60) or presence (pH 7.61) of NH₄Cl (20 mM).

**Cell Protein Content and Cell Size**

Cells (10⁶/well) were plated in six-well plates. When the monolayers reached about 80% confluency, the 10% FCS incubation medium was replaced with DMEM for 24 h. NH₄Cl (20 mM) was applied to the cells in serum-free DMEM (with 0.5% FCS) with or without cycloheximide (10 μM; Sigma) and amiloride (1 mM; Sigma) for the time periods indicated in Results. For the experiments incubated longer than 24 h, the daily dose of 20mM NH₄Cl in fresh DMEM was given every 24 h (the same for other experiments). Cells not treated with NH₄Cl served as controls. At the end of the incubation period, the still subconfluent cells were harvested with 0.25% trypsin/0.02% EDTA to obtain a single cell suspension, which allows an optimal evaluation of the changes in cell protein content and cell size. After being washed with phosphate-buffered saline (PBS), cell mean diameter and cell volume were quantitatively evaluated by a CASY-1 Cell Counter and Analyser System (Schaefer System, Reutlingen, Germany) (10). This system applies a new measuring technology: the well-known principles of resistance measurements are combined with pulse area analysis methods. A total of 2,000 cells were gated in a volume of 200 μL with a cell concentration of 1.5 x 10⁵/mL for each measurement. To ascertain whether an increase in cell size is associated with hypertrophy, cell suspensions obtained by detachment from wells were centrifuged for 5 min at 100 g, and the cell pellets were lysed by sonification. Cell protein content was determined according to Smith et al. (11) with bovine serum albumin as the standard.

**Cell Count**

Cell culture was performed as mentioned above. The still subconfluent monolayers were quiesced by replacing the normal incubation medium with serum-free DMEM for 24 h, and they were allowed to grow in serum-free DMEM in the presence of NH₄Cl (20 mM) for another 24 h. The cells were then detached from wells by 0.25% trypsin/0.02% EDTA to obtain a single cell suspension. After the cells were washed twice in PBS, they were visually counted under a phase-contrast microscope.

**DNA Synthesis**

[¹³H]thymidine (Amersham, Frankfurt/Main, Germany) incorporation into DNA was measured as an indicator of DNA synthesis (12). Cells (10⁶/well) were plated in six-well plates in the incubation medium supplemented with 10% FCS. When the monolayers reached confluency, the incubation medium was replaced with DMEM for 24 h. The cells were then exposed to NH₄Cl (20 mM) in serum-free DMEM for another 24 h. During the last 2 h of culture, the cells were pulsed with [¹³H]thymidine by the addition of 1 μCi per well. At the end of the labeling period, the monolayers were rapidly rinsed three times with ice-cold PBS. Cells were solubilized in 2% sodium dodecyl sulfate and precipitated with 2 mL of 20% ice-cold trichloroacetic acid (TCA). The precipitates were collected in a GF Glassfiber filter (Schleicher & Schuell, Dassel, Germany) and were washed sequentially with 10 and 5% TCA and finally with absolute ethanol. The radioactivity was determined in a Tri-Carb 4000 liquid scintillation counter (Packard Instruments, Downers Grover, CT). [³H]thymidine incorporation was presented as disintegrations per minute per 10⁵ cell. Cell number was counted simultaneously in replicated wells.

**Protein Synthesis**

Cell protein synthesis was assessed by determining the incorporation of L-[¹⁴C]phenylalanine (Phe; Amersham) into acid-insoluble proteins (4). Confluent cells were incubated with serum-free DMEM supplemented with 0.6 mM unlabeled Phe, NH₄Cl (20 mM), or vehicle (as control) for 12, 24, and 48 h including 5 μCi/well of L-[¹⁴C]Phe for the last 4 h. The excess of unlabeled Phe (0.6 mM) was used to ensure the equilibration of intracellular and extracellular specific radioactivity. At the end of a 4-h labeling period, the cells were rapidly rinsed three times with ice-cold PBS. The radioactivity in the cells was determined as described in the DNA Synthesis section. The cell protein synthesis rate was expressed as disintegrations per minute per well.

**Protein Degradation**

To measure the protein degradation rate, the confluent cells were labeled by preincubating cells in serum-free DMEM containing 0.5 μCi/well of L-[¹⁴C]Phe for 48 h (4). In order to minimize the reincorporation of [¹⁴C]Phe, the experimental media contained an excess (2 mM) of unlabeled Phe. After a 2-h phase, the cells were washed with PBS to remove L-[¹⁴C]Phe released from the degradation of short-lived protein, and 4 mL of fresh experimental medium containing NH₄Cl (20 mM) or vehicle (as control) was added. At the indicated sampling periods up to 48 h, 0.3-mL aliquots of the media were taken and precipitated by the addition of ice-cold TCA (10% final concentration). The samples were centrifuged for 5 min, and the TCA-soluble supernatant was frozen at -20°C overnight for radioactivity measurement. At the end of an experiment, the cells were washed three times with ice-cold PBS. The radioactivity in the cells and in the media was measured as mentioned above. Total radioactivity released plus that remaining in the cells at the end of the experiments was expressed as the initial L-[¹⁴C]Phe incorporation into cell proteins. The percentage of radioactivity released versus the radioactivity remaining in the cells at the indicated time intervals was calculated as the protein degradation rate.

**Cathepsin Assay**

Confluent monolayers with dome formation were growth arrested with DMEM for 24 h. NH₄Cl (0 to 20 mM) with or without amiloride (1 mM) was then added to the cells in serum-free DMEM for the time periods indicated in Results (the same amount of identical vehicle PBS was added to the control cells). After the incubation period, the cell monolayers were washed twice with ice-cold 0.9% NaCl and scraped into 1.5 mL of ice-cold phosphate buffer (pH 5.8) with a rubber policeman. All subsequent steps were carried out at 4°C. The cell membranes were permeabilized by sonication (4 x 2-s bursts). The activities of cathepsins B and H and the combined activities of cathepsins B and L (cathepsin L + B) were assayed as described by Barrett and Kirschke (13), with fluorogenic peptidyl substrates (Bachem, Heidelberg, Germany): Z-Arg-Arg-AMC (AMC: 7-amino-4-methylcoumarin)
Measurement of Cytosolic and Lysosomal pH

The cells were grown to confluence on glass coverslips. Cytosolic pH in single cells was determined with the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein (BCECF; Sigma), incubated with cells at 3 μM for 15 min as described previously (14). Lysosomal pH in single cells was measured according to Okhuma and Poole (15) after 120 min of incubation in medium containing 5 mg/mL fluoresein isothiocyanate (FITC)-dextran and subsequent incubation in FITC-dextran free medium for 120 min. This indicator is concentrated within compartments that are acidic compared with the medium, with consequent quenching of fluorescence. The pH was calculated by the equation:

\[ pH = \log \left( \frac{[R-R_{\text{min}}]}{[R_{\text{max}}]-R} \right) \cdot K \]

where R is the actual ratio and K is the affinity constant determined in previous experiments. R_{\text{max}} and R_{\text{min}} were obtained for each individual cell by calibration with pH 8 and pH 4, respectively. After incubation with BCECF or FITC-dextran, the cells were rinsed 4 times with perfusion solution (NaCl, 122; KCl, 5.4; CaCl₂, 1.2; NaH₂PO₄/Na₂HPO₄, 1; MgCl₂, 0.8; d-glucose, 5.5; HEPES, 10; pH 7.4 at 37°C). Additional measurements were also performed in the same perfusion solution but with bicarbonate, to see its influence on lysosomal pH.

Incubation Experiments of Cell Lysates

Cell lysates were preincubated with or without NH₄Cl (20 mM) and phosphate buffer with pH 5.8 or 6.8, respectively, for 180 min. Then, the activities of cathepsins B, H, and L+B were assayed as described above.

Statistics

Results are presented as mean ± SE. Statistical analysis between two groups was performed by use of an unpaired t test, as appropriate. Differences among several groups were tested by the use of analysis of variance. All statistics were carried out by InStat software (GraphPad Inc., San Diego, CA). A value of P < 0.05 was considered to represent a significant difference.

RESULTS

Effects of NH₄Cl on Cell Protein Content and Cell Size

After exposure to NH₄Cl (20 mM) for up to 48 h, the cell monolayers were subjected to the measurements of protein content and cell size. In a parallel experiment, cells were also incubated with the protein synthesis inhibitor cycloheximide (10 μM) in the absence or presence of NH₄Cl (20 mM). As can be seen in Table 1, NH₄Cl induced an increase in cell protein content, as expressed per cell number, as well as an enlargement of cell mean diameter and cell volume, as determined by a Cell Analyzer System. The hypertrophic response was significant after an exposure for 48 h: protein content increased by 25.5%, cell mean diameter increased by 7.7%, and cell volume increased by 10.4%. Additionally, the NH₄Cl-induced rise in protein content or in cell size was not prevented by cycloheximide (Table 1). Coexposure with the Na⁺/H⁺ antiport antagonist amiloride (1 mM) was conducted for 48 h to clarify whether stimulation of the plasma membrane Na⁺/H⁺ antiport was required for NH₄Cl-induced hypertrophy in LLC-PK1 cells. As shown in Table 1, amiloride did not block the NH₄Cl-induced increase in protein content or enlargement of cell size.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein Content (μg/10⁶ cells)</th>
<th>Cell Diameter (μm)</th>
<th>Cell Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44.2 ± 6.3</td>
<td>17.1 ± 0.3</td>
<td>3,076.9 ± 125.4</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>48.7 ± 3.3</td>
<td>17.8 ± 0.2</td>
<td>3,391.0 ± 58.7⁰</td>
</tr>
<tr>
<td><strong>48 h</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>45.5 ± 1.5</td>
<td>16.9 ± 0.1</td>
<td>2,943.1 ± 58.9⁰</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>57.1 ± 2.8⁰</td>
<td>18.2 ± 0.1⁰</td>
<td>3,249.4 ± 29.8⁰</td>
</tr>
<tr>
<td>NH₄Cl + amiloride</td>
<td>55.7 ± 1.7²</td>
<td>18.1 ± 0.1²</td>
<td>3,146.7 ± 27.7⁰</td>
</tr>
<tr>
<td>NH₄Cl + cycloheximide</td>
<td>55.1 ± 0.3⁰</td>
<td>18.0 ± 0.3⁰</td>
<td>3,120.0 ± 42.6⁰</td>
</tr>
</tbody>
</table>

⁰ Data are given as the mean ± SE of nine determinations from three independent experiments. NH₄Cl, amiloride, and cycloheximide were used at the doses of 20 mM, 1 mM, and 10 μM, respectively.

ⁱ P < 0.05 versus controls.

² P < 0.01 versus controls.
Effects of NH₄Cl on Cell Number and DNA Synthesis

To distinguish the role of hypertrophy versus hyperplasia, cell count and [³H]thymidine incorporation were studied as indicators of cell proliferation. As summarized in Table 2, cell number and [³H]thymidine incorporation were reduced in the monolayers exposed to NH₄Cl (20 mM) after 24 h of incubation.

Effect of NH₄Cl on Cell Protein Turnover

Cell protein synthesis and breakdown were investigated in the cells exposed to NH₄Cl (20 mM) to elucidate the underlying mechanism of NH₄Cl-induced hypertrophy. As can be seen in Figure 1A, NH₄Cl did not stimulate protein synthesis up to an exposure for 48 h. In contrast, a significant decrease in protein degradation rate in a time-dependent fashion was observed starting after 3 h and continuing up to 48 h (Figure 1B).

Effects of NH₄Cl on Lysosomal Cathepsin Activities

Because more than 90% of the long-living proteins and a large fraction of the short-living proteins are degraded by lysosomal cathepsins (8), further experiments were performed to verify the potential involvement of cathepsin activities in the decline of protein breakdown induced by NH₄Cl. In addition, cellular neutral proteolytic activity was also measured by using collagen (Type I) as substrate to see whether nonlysosomal proteolytic enzymes also contribute to the reduction of protein degradation. As depicted in Figure 2A, cathepsin H activity was not significantly influenced in LLC-PK1 cells exposed to NH₄Cl (20 mM) for 48 h. Also, cellular neutral proteolytic activity remained unchanged (control, 32.4 ± 2.1; NH₄Cl, 33.5 ± 2.5 pmol/min per μg of DNA; P > 0.05). However, the activities of cathepsins B and L + B were markedly decreased: 12.5 and 14.5% after 3 h, 15.8 and 19.8% after 8 h, and 57.0 and 54.5% after 48 h, respectively (Figure 2A). As shown in Figure 2B, NH₄Cl caused a dose-dependent inhibition of the activities of cathepsins B and L + B. Moreover, this inhibition was not blunted by coinubcation with the Na+/H+ antiport antagonist amiloride (1 mM; Figure 3); also, amiloride did not modify cathepsin activities in the absence of NH₄Cl.

To exclude the possibility that NH₄Cl-altered cathepsin activities were due to elevated osmolarity in the incubation medium, the cells were incubated with mannitol (20 mM). There was no difference of cathepsin activities between the cells incubated with or without mannitol (control: B, 10.4 ± 1.2; L + B, 30.9 ± 3.4; the mannitol-treated cells: B, 11.6 ± 0.4; L + B, 31.8 ± 1.4 pmol/min per μg of DNA). In fact, NH₄Cl did not change osmolarity in the incubation medium (control, 265 to 287 mosmol/L; NH₄Cl-treated cells, 271 to 288 mosmol/L). Also, raising the pH in the incubation medium to 7.88 did not alter cathepsin activities (data not shown).

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Number (x 10⁶/well)</th>
<th>[³H]Thymidine Incorporation (x 10³ dpm/10⁵ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.59 ± 0.15</td>
<td>13.0 ± 1.3</td>
</tr>
<tr>
<td>NH₄Cl (20 mM)</td>
<td>1.96 ± 0.18b</td>
<td>9.5 ± 0.7b</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE from six determinations. p < 0.06 versus controls.

Figure 1. (A) Effect of NH₄Cl (20 mM) on protein synthesis. Values are given as the mean ± SE from two independent experiments (N = 6). (B) Effect of NH₄Cl (20 mM) on protein degradation. Values are given as the mean ± SE of the percentage of L-(¹⁴C)Phe released over controls from two independent experiments (N = 6). *P < 0.01 versus controls.
Figure 2. (A) Time-course effects of NH₄Cl (20 mM) on the activities of cathepsins B, H, and L + B. Data are presented as the mean ± SE of nine determinations from three independent experiments. *P < 0.05 versus controls. (B) Dose-dependent effects of NH₄Cl on the activities of cathepsins B and L + B after 24 h of incubation. Values are given as the mean ± SE from two independent experiments (N = 6). **P < 0.01 versus controls.

Effects of NH₄Cl on Cytosolic and Lysosomal pH

Because cysteine proteinases in lysosomes function optimally at an acidic pH (8,9) it would be reasonable to speculate that changes of intracellular pH were involved in the impairment of acidic cathepsin activities induced by NH₄Cl. In this study, the determination of cytosolic pH with BCECF showed that NH₄Cl (20 mM) induced a rapid, but transient rise, which returned to the baseline within 4 h, even though NH₄Cl was still present (Figure 4A). The lysosomal pH was also measured by the incubation of cells with FITC-dextran. Compared with the cytosol, the lysosomes responded to NH₄Cl with an immediate rise (6.33 to 7.36) that persisted during the entire incubation period of 3 h. After the removal of NH₄Cl, the lysosomal pH declined slightly (5.45 to 5.76) but did not reach the basal level (5.17 to 5.26) (Figure 4B). With bicarbonate-buffered perfusing solution, we observed identical changes of lysosomal pH, as in the bicarbonate-free medium.

Influences of NH₄Cl and the pH on Cathepsin Activities in Cell Lysates

As summarized in Table 3, the preincubation of cell lysates with NH₄Cl (20 mM) for 180 min did not influence cathepsin activities. On the other hand, the preincubation of cell lysates in phosphate buffer with pH 6.8 (instead of the pH 5.8 normally used in the measurement) resulted in the suppression of the activities of cathepsins B and L + B, whereas cathepsin H activity was increased.

DISCUSSION

Already in 1939, it was shown that metabolic acidosis induced by NH₄Cl feeding causes an increase in kidney weight, cell mass, and protein content (16). In contrast to this observation, acidification in vitro has failed to produce cell hypertrophy (2-4). Because metabolic acidosis stimulates renal ammonium production, it has become apparent only recently that the renal hypertrophic response to metabolic acidosis is an effect of increased ammonia production rather than of the acidosis per se (17). Thus, ammonia may play a pivotal role as a growth promoter (1-3).

In this study, in LLC-PK1 cells, NH₄Cl induced cell hypertrophy, as characterized by an increase in both protein content and cell size. The data are consistent with other studies in proximal tubule cells (3-5). This hypertrophy was not accompanied by cell hyperplasia, as demonstrated by the estimation of both thymidine incorporation and cell number. In addition, the turnover of total cell proteins was quantified by measuring the incorporation and release of L-¹⁴C Phe. The data showed no rise in cell protein synthesis; but a marked decline in protein breakdown after the application of NH₄Cl. In line with these observations, the suppres-
Figure 4. (A) NH₄Cl (20 mM) induced a rapid and transient rise in the cytosolic pH. The pH was recovered within 4 min in the presence of NH₄Cl. The determination was repeated five times at identical condition with consistent results. (B) NH₄Cl (20 mM) induced a sustained rise in the lysosomal pH (5.33 to 7.36) in the presence of NH₄Cl. After the removal of NH₄Cl, the pH declined slightly (5.45 to 5.76), but not to its basal value (5.17 to 5.26). The determination was repeated three times at identical condition with consistent results.

Ammonia and Tubular Cathepsin Activity

TABLE 3. Effects of NH₄Cl (20 mM) and different pH buffers on the enzyme activities in cell lysates.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cathepsin</th>
<th>B</th>
<th>H</th>
<th>L + B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (pH 5.8)</td>
<td>10.2 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>28.7 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>pH 6.8</td>
<td>7.8 ± 0.2b</td>
<td>6.5 ± 0.3b</td>
<td>23.4 ± 1.1b</td>
<td></td>
</tr>
<tr>
<td>NH₄Cl (20 mM)</td>
<td>9.5 ± 0.9</td>
<td>3.8 ± 0.2</td>
<td>27.2 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

*The preincubation of cell lysates in phosphate buffer with pH 5.8 and 6.8 as well as with NH₄Cl (20 mM) was performed at 37°C for 180 min, and then the activities of cathepsins B, H, and L + B were determined. Values were expressed as the mean ± SE of six measurements from two independent experiments. P < 0.01 versus control.

A change in protein synthesis with cycloheximide did not block the induction of cell hypertrophy, indicating that NH₄Cl-induced hypertrophy was caused by reduced protein degradation without enhanced protein synthesis. Impaired protein breakdown with un-

changed protein synthesis was observed not only in in vitro studies but also in in vivo experiments after ammonia feeding (4,18,19).

In the degradation of intracellular and extracellular proteins, various proteolytic enzymes are involved such as cysteine, serine, and metalloproteinases (9,20). In particular, lysosomal cysteine proteinase cathepsins B, L, and H play a major role in the breakdown of both intracellular and extracellular proteins (8,9,20). In our study, the neutral proteolytic activity measured by using collagen as substrate was not influenced by NH₄Cl. In contrast, the activities of cathepsins B and L + B declined in the presence of NH₄Cl in a time- and dose-dependent manner.

These investigations in cultured proximal tubule cells correspond well with previous in vitro investigations showing that decreased cathepsin activities in glomeruli and tubules are associated with the induction of renal hypertrophy in various rat models (21,22). In addition, the reduction of cathepsin activities induced by insulin-like growth factor-I resulted in hypertrophy in freshly isolated proximal tubule cells (23). This study, therefore, demonstrates that the suppression of the activities of cathepsins B and L + B and a subsequent decline of protein breakdown contribute to NH₄Cl-induced hypertrophy in LLC-PK1 cells. Furthermore, it provides additional pathogenic relevance for enhanced ammoniagenesis, which is currently thought to play an important role in progressive renal diseases (2,17). Accordingly, various growth promoters such as angiotensin II, insulin, and a high-protein diet stimulate ammonia production (24). Thus, the inhibitory role of ammonia on lysosomal cathepsins B and L + B may favor an accumulation of tubular proteins and subsequently tubular interstitial fibrosis. The latter may also be aggravated by the activation of an alternative complement pathway due to enhanced ammoniagenesis (2).

Concerning the signal transduction pathway of NH₄Cl-induced effects in LLC-PK1 cells, neither the suppression of the activities of cathepsins B and L + B nor the induction of cell hypertrophy was mediated through the activation of the Na⁺/H⁺ antiport, as shown by using amiloride. Hence, an enhanced
Na+/H+ antiport activity is not required for the decrease of protein breakdown as well as the cell hypertrophy induced by NH4Cl.

Previous studies in proximal tubule cells suggested that the NH4Cl-induced decline of protein degradation was independent of intracellular pH (3.4.18). Even exposure for up to 96 h did not induce significant changes in the intracellular pH (17). Also in this study, NH4Cl induced only a transient rise in the cytosolic pH. However, the determination of lysosomal pH with FITC-dextran revealed a rise within a minute by more than 1.1 pH units at a medium pH of 7.61. In contrast to the transient rise in the cytosolic pH, the lysosomal pH was elevated during the entire incubation period up to 3 h. On the other hand, the preincubation of cell lysates with NH4Cl did not modify the activities of cathepsins B, H, and L + B. Thus, these results indicate that the inhibition of the activities of cathepsins B and L + B was due to alkalization in lysosomes. These data correspond well with the fact that the activities of lysosomal enzymes are optimal at acidic pH (8,9). NH4Cl, like other weak bases, enters acidic pH (8.9). NH4Cl, like other weak bases, enters lysosomes by diffusion and becomes protonated and thus, the activities of lysosomal enzymes are consistent with the acidic pH optima of lysosomal hydrolytic enzymes. In contrast to cathepsins B and L + B, the activity of cathepsin H did not decline after the exposure of the cells to NH4Cl; in fact, the preincubation of cell lysates in different pH buffers revealed even a rise of cathepsin H activity at a relative high pH (pH 6.8). This finding is in agreement with earlier observations indicating that the optimal pH of cathepsin H is higher than those of other cathepsins in lysosomes (8,26). Thus, this study further emphasizes that an acidic pH of lysosomes is essential for the normal renal disposal of intracellular proteins (27).

The mechanism involved in the elevation of lysosomal pH after the exposure of NH4Cl is currently unknown. There is some evidence implicating the requirement of energy for pH maintenance. It was shown that ATP prevents the inhibition of lysosomal proteolysis by NH4Cl (25). According to the investigations in macrophages and liver cells, the time course of lysosomal accumulation of NH4Cl is biphasic with an initial rapid energy-independent phase followed by a slow ATP-dependent phase, which can be inhibited by metabolic inhibitors (25). Correspondingly, the existence of an ATP-dependent proton pump in lysosomes has been identified (28).

Taken together, this study demonstrates that NH4Cl induces hypertrophy but not hyperplasia in LLC-PK1 cells. The cell hypertrophy is caused by the reduction of protein degradation, mainly due to the depressed activities of cathepsins B and L + B, in the absence of enhanced protein synthesis. As a potential mechanism responsible for the impairment of protein breakdown, an intralysosomal alkalinization is emphasized.

REFERENCES