Urinary proteins induce lysosomal membrane permeabilization and lysosomal dysfunction in renal tubular epithelial cells

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Liu WJ, Xu B, Ye L, Liang D, Wu H, Zheng Y, Deng JK, Li B, Liu H. Urinary proteins induce lysosomal membrane permeabilization and lysosomal dysfunction in renal tubular epithelial cells. *Am J Physiol Renal Physiol* 308: F639–F649, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00383.2014.—Lysosomal membrane permeabilization (LMP) has been shown to cause the release of cathepsins and other hydrolases from the lysosomal lumen to the cytosol and initiate a cell death pathway. Whether proteinuria triggers LMP in renal tubular epithelial cells (TECs) to accelerate the progression of renal tubulointerstitial injury remains unclear. In the present study, we evaluated TEC injury as well as changes in lysosomal number, volume, activity, and membrane integrity after urinary protein overload in vivo and in vitro. Our results revealed that neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 levels were significantly increased in the urine of patients with minimal change nephrotic syndrome (MCNS) and the culture supernant of HK-2 cells treated by urinary proteins extracted from MCNS patients. Urinary protein overload also induced apoptotic cell death in HK-2 cells. Importantly, we found that lysosomal volume and number were markedly increased in TECs of patients with MCNS and HK-2 cells overloaded with urinary proteins. However, lysosome function, as assessed by proteolytic degradation of DQ-ovalbumin and cathepsin-B and cathepsin-L activities, was decreased in HK-2 cells overloaded with urinary proteins. Furthermore, urinary protein overload led to a diffuse cytoplasmic immunostaining pattern of cathepsin-B and irregular immunostaining of lysosome-associated membrane protein-1, accompanying a reduction in intracellular acidic components, which could be improved by pretreatment with antioxidant. Taken together, our results indicate that overloading of urinary proteins caused LMP and lysosomal dysfunction at least partly via oxidative stress in TECs.

Urinary proteins; tubular epithelial cells; lysosomal membrane permeabilization; lysosomal dysfunction

The lysosome is known as a cytoplasmic membrane-enclosed organelle, which contains various hydrolytic enzymes and finishes the degradation process of cellular components and macromolecules (20). Several degradation pathways, including endocytosis, phagocytosis, and autophagy, end up in lysosomes to be degraded (3). Normal degradation function is crucial to maintain cellular homeostasis and enable cell survival in the physiological state. However, given its high content of hydrolytic enzymes, lysosomes are potentially harmful to the cell when damage occurs to the lysosomal membrane under pathological conditions, so-called lysosomal membrane permeabilization (LMP) (4, 9). LMP might result in indiscriminate degradation of cellular organelles since lysosomes release hydrolytic enzymes into the cytoplasm. In addition, lysosomal destabilization has a close relationship with various types of cell death in association with oxidative stress and inflammation (11). In certain conditions, the extent of lysosomal membrane damage is linked with different outcomes of cell injury. For example, partial lysosomal rupture induces apoptosis, whereas massive lysosomal leakage leads to necrosis (4).

Lysosomal degradation and transport for reuse are important in dealing with reabsorbed urinary proteins (16, 23). However, whether LMP occurs in TECs and whether LMP leads to TEC apoptosis after urinary protein overload remain unclear. It has been implicated that in the case of glomerular injury, the increased filtration of urinary proteins may overload lysosomal pathways and cause lysosomal rupture in tubular cells, leading to renal tubular injury (15, 25). This idea is attractive but lacks direct evidences. In the present study, we assessed morphological and functional changes of lysosomes after urinary protein overload in TECs in vivo and in vitro.

**MATERIALS AND METHODS**

**Patients.** The present study was approved by the Institutional Review Board of the Affiliated Hospital of Guangdong Medical College. Kidney specimens were obtained from biopsy-proven untreated minimal change nephrotic syndrome (MCNS, n = 11), focal segmental glomerulosclerosis (FSGS, n = 11), or membranous nephropathy (MN, n = 11) patients. Patients with urinary protein excretion of >3.5 g/24 h and age range from 16 to 40 yr were enrolled. Control kidney specimens (n = 4) were obtained from patients with hematuria only and biopsy-proven minimal change disease.

**Cell culture and treatments.** Human proximal tubular HK-2 cells (American Type Culture Collection) were maintained in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FBS (GIBCO) under standard conditions. HK-2 cells were exposed to 1, 2, 4, and 8 mg/ml urinary proteins for 0, 2, 4, 8, and 16 h before analysis. To suppress oxidative stress, HK-2 cells were pretreated with 2,000 U/ml catalase (Millipore, Billerica, MA) or 1 mM N-acetylcysteine (NAC; Sigma, St. Louis, MO) before exposure to 8 mg/ml urinary proteins for 16 h. Urinary proteins were extracted from urine of patients with un-

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treated, biopsy-proven, and uncomplicated idiopathic MCNS using an ammonium sulfate precipitation method, as previously described (22).

**Immunofluorescence experiments.** Immunostaining analysis for tissues or cells was conducted as previously described (14). Antibodies of rabbit anti-lysosome-associated membrane protein-1 (LAMP1), mouse anti-cathepsin-D (Abcam, Cambridge, MA), and mouse anti-cathepsin-B (Santa Cruz Biotechnology, Santa Cruz, CA) were used in the staining. Immunoreactivity was visualized with Alexa fluor 488 donkey anti-rabbit IgG or Alexa fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). Microscopic images were taken using a TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany) or an Olympus BX43 microscope (Olympus, Tokyo, Japan). The average fluorescence intensity value was calculated from at least 50 LAMP1-positive cells for each test. LMP in HK-2 cells was graded as the percentage of cells displaying diffuse staining of cathepsin-B immunostaining from six fields in each experiment.

**Transmission electron microscopy.** Kidney tissue specimens from patients were fixed, embedded, and stained as previously described (14). Ultrathin sections were examined using a Philips CM100 electron microscope (Tokyo, Japan) following the manufacturer’s protocol. This was done in these patients compared with control subjects (Fig. 3A). In addition, two renal tubular injury markers, urinary NGAL and KIM-1, were significantly increased in these patients compared with control subjects (Fig. 1B). In agreement with in vivo experiments, we also found that exposure of HK-2 cells to urinary proteins for 16 h enhanced NGAL and KIM-1 secretion in a dose-dependent manner. In addition, exposure to 8 mg/ml urinary proteins significantly elevated NGAL and KIM-1 levels in the culture supernatant from 2 to 16 h (Fig. 1, C and D).

It is well known that FITC annexin V can detect the exposure of phosphatidylserine on the outer leaflet of the plasma membrane, which occurs in the early stages of apoptosis, whereas only the membranes of late apoptotic cells are permeable to propidium iodide (PI) (7). Thus, early and late apoptosis were subsequently evaluated after coupled staining with FITC annexin V and PI. When studying the relationship between urinary protein dose and cell injury, we found that both early (annexin V+/PI−) and late (annexin V+/PI+) apoptosis were enhanced at 8 mg/ml in HK-2 cells. Obvious early and late apoptosis were observed at the prolonged treatment time of 16 h (Fig. 2A). In addition, urinary protein-induced apoptosis was confirmed by immunoblot analysis of cleaved caspases-3 at 8 mg/ml and 16 h (Fig. 2B).

**Urinary proteins increased the number and enlarged the volume of lysosomes in TECs.** To assess the impact of urinary proteins on lysosomes in vivo, we examined changes of lysosomal number and volume in renal TECs from MCNS patients. The lysosomal number per cell was counted on transmission electron microscopy images. The number of lysosomes was significantly higher in renal proximal TECs of patients with MCNS than in control subjects. Morphologically, some TECs exhibited swelling and vacuolar and granular degeneration in patients with MCNS (Fig. 1A). In addition, two renal tubular injury markers, urinary NGAL and KIM-1, were significantly increased in these patients compared with control subjects (Fig. 1B). In agreement with in vivo experiments, we also found that exposure of HK-2 cells to urinary proteins for 16 h enhanced NGAL and KIM-1 secretion in a dose-dependent manner. In addition, exposure to 8 mg/ml urinary proteins significantly elevated NGAL and KIM-1 levels in the culture supernatant from 2 to 16 h (Fig. 1, C and D).

**RESULTS**

**Urinary proteins induced TEC injury.** To explore the action of urinary protein overload in vivo, we first evaluated renal TEC lesions in patients with nephrotic syndrome. Considering that other mechanisms (e.g., leakage of the glomerular ultrafiltrate into the peritubular interstitial space, autoantibodies, and so on) in addition to urinary protein overload are involved in TEC lesions in FSGS or MN patients (10, 12), we predominantly focused on MCNS patients in this study. Clinical characteristics of MCNS and control patients are shown in Table 1. There were no statistically significant differences in age, serum creatinine, blood urea nitrogen, or serum uric acid between the two groups. Twenty-four-hour urinary proteins, total cholesterol, and triglyceride increased, whereas plasma albumin decreased, in MCNS patients compared with control subjects.

<table>
<thead>
<tr>
<th>Number</th>
<th>Control Subjects</th>
<th>Patients With Minimal Change Nephrotic Syndrome</th>
<th>P Value</th>
</tr>
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<tr>
<td>Men/women</td>
<td>3/1</td>
<td>9/2</td>
<td>0.05</td>
</tr>
<tr>
<td>Age, yr</td>
<td>27.1 ± 6.1</td>
<td>23.3 ± 3.2</td>
<td>0.56</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>4.4 ± 0.5</td>
<td>10.5 ± 1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>1.3 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma albumin, g/l</td>
<td>46.7 ± 3.6</td>
<td>18.0 ± 2.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>83.8 ± 7.9</td>
<td>80.3 ± 7.3</td>
<td>0.79</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/l</td>
<td>3.5 ± 0.5</td>
<td>7.7 ± 0.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Serum uric acid, μmol/l</td>
<td>324.0 ± 20.8</td>
<td>376.6 ± 22.7</td>
<td>0.21</td>
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Data are expressed as counts or means ± SE.

**Table 1. Clinical characteristics of the enrolled patients**
phenomenon was also seen in HK-2 cells after exposure to urinary proteins, of which exposure to 8 mg/ml urinary proteins significantly increased lysosomal number and volume, as assessed by lysosome-specific fluorescence intensity and LAMP1-positive granules (Fig. 3B).

Urinary proteins suppressed the degradation of DQ-ovalbumin. To evaluate the efficiency of lysosome-mediated proteolytic degradation, a self-quenched substrate for proteases, DQ-ovalbumin, was used. Bright dots of DQ-ovalbumin were seen in HK-2 cells treated with the solvent as the control. However, these dots were markedly reduced in HK-2 cells loaded with 8 mg/ml urinary proteins (Fig. 4). These results suggested that degradability of the lysosome was compromised despite the fact that the lysosomal volume was larger and the amount was higher after exposure to urinary proteins.

Urinary proteins increased the protein level but decreased the enzymatic activity of cathepsins. To investigate whether the suppressed proteolytic degradation could be attributed to a variation of lysosomal enzymatic activity, we examined the activity and protein levels of lysosomal proteolytic enzymes...
with fluorescence-based enzymatic assays in HK-2 cells. Exposure to urinary proteins induced a significant decrease in cathepsin-B and cathepsin-L activities (Fig. 5, A and B) but not in cathepsin-D activity (Fig. 5C). Interestingly, the protein level of cathepsin-B was increased, whereas cathepsin-D was not changed, after exposure to urinary proteins compared with the control (Fig. 5, D and E).

Urinary proteins triggered defective lysosomal acidification and LMP in TECs. Since enzymatic activity is closely related to the acid milieu of lysosomes (3), we next tested whether urinary proteins attenuated the acidification of lysosomes in renal TECs. We used Lyso-Tracker red to label acidic intracellular compartments (lysosomes) in living cells. Punctuated red fluorescence (lysosomes) was clearly seen under control condition. However, exposure to urinary proteins abolished Lyso-Tracker red labeling in a dose-dependent manner (Fig. 6, A and C). In parallel, the number of pale cells, as examined by flow cytometry, was also significantly increased after exposure to urinary proteins (Fig. 6, B and D). These data suggest that acidification of lysosomes was attenuated, resulting from lysosomal destabilization, namely, LMP (4). To test if LMP actually occurred, we examined if exposure to urinary proteins elicited lysosomal rupture. In the control, cathepsin-B immunoreactivity displayed a punctate pattern and colocalized to a large extent with LAMP1-positive dots in renal TECs. In contrast, in TECs from patients with MCNS, both cathepsin-B and LAMP1 exerted as a diffuse immunostaining pattern, suggesting a release from the lysosome to the cytosol. Patients with FSGS and MN, two common pathological types in nephrotic syndrome, were also studied in this experiment. Similar patterns of cathepsin-B and LAMP1 staining were obtained in TECs of patients with heavy proteinuria (Fig. 7A). In HK-2 cells, cathepsin-B release was also observed after exposure to 8 mg/ml urinary proteins extracted from MCNS patients, accompanied by larger and irregular LAMP1 immunostaining at 16 h (Fig. 7, B and C).

Fig. 2. Effect of urinary proteins on HK-2 cell apoptosis. A: early and late apoptosis of HK-2 cells were detected by annexin V/propidium iodide (PI) flow cytometry after exposure to urinary proteins at different concentrations for 16 h or at 8 mg/ml for different times. B: Western blot analysis of cleaved caspase-3 after exposure of HK-2 cells to urinary proteins at different concentrations for 16 h or at 8 mg/ml for different times. *P < 0.05.
Oxidative stress was involved in urinary protein-triggered LMP. We recently reported that some signaling pathways are activated by oxidative stress after exposure of TECs to urinary proteins (14). Thus, we subsequently tested whether oxidative stress was also an important mechanism underlying LMP. In our study, we found that the production of ROS was significantly enhanced after exposure of HK-2 cells to urinary proteins, which was suppressed by pretreatment with antioxidant

**Fig. 3.** Effects of urinary proteins on lysosomal amount and volume. A: lysosomal numbers and diameters with transmission electron microscopy in TECs from control and MCNS patients. Scale bars = 2 μm. B: immunofluorescence intensity of lysosome-associated membrane protein-1 (LAMP1; green) after exposure to different concentrations of urinary proteins for 16 h. The nucleus was counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars = 10 μm. *P < 0.05.

**Fig. 4.** Effect of urinary proteins on the degradation of DQ-ovalbumin. A: HK-2 cells were treated with different concentrations of urinary proteins or vehicle for 16 h and then incubated with DQ-ovalbumin (10 μg/ml) for 1 h. Scale bars = 10 μm. B: degraded products presented as green puncta were quantified. ***P < 0.001.
catalase or NAC (data not shown). Similar to the results shown in Fig. 6, exposure to urinary proteins abolished Lyso-Tracker red labeling but elevated the proportion of pale cells. However, pretreatment with catalase or NAC markedly increased the mean fluorescence intensity of Lyso-Tracker red (Fig. 8, A and C) and reduced the proportion of pale cells (Fig. 8, B and D), indicating that oxidative stress was involved in the lysosomal dysfunction and LMP induced by urinary proteins.

DISCUSSION

The aim of the present study was to investigate the effect of urinary proteins on renal tubules with respect to TEC injury and lysosomal dysfunction. It is well known that, in addition to urinary proteins, tubulointerstitial injury has a relationship with some other factors, such as the clinical stages, pathological types of nephrotic syndrome, and ages of patients. There-
fore, to minimize the influence of these factors, untreated young patients with MCNS and normal serum creatinine were enrolled in this study. We found that exposure to urinary proteins induced renal proximal tubule injury and apoptotic death of TECs. It has been widely accepted that TEC apoptotic death, which could be triggered by LMP, is the driver for the development of fibrotic renal lesions, so it is important to explore whether urinary protein overload induces lysosome destabilization and LMP. In the present study, our results revealed that cathepsin-B was accumulated in parallel with increased amount and volume of lysosomes both in vivo and in vitro after urinary protein overload. These data indicate an increased lysosomal mass in response to increased amounts of internalized proteins. However, it is not clear whether an increase in lysosomal content is sufficient to complete the clearance of overloaded proteins due to endocytosis. Notably, a previous study (6) suggested that larger lysosomes were a result of cellular injury due to an increased requirement for hydrolysis. In addition, other studies (17, 18, 29) have demonstrated that larger lysosomes were more susceptible to breakage and that an increase in lysosomal volume was a common event in cell death induced by various factors. In our study, significant increases in lysosome size and volume were observed in TECs from proteinuria patients and HK-2 cells after urinary protein overload, indicating potential damage of lysosomes.

Next, we investigated if the proteolytic power of the lysosomes changed after urinary protein overloading. Our data revealed that the lysosomal proteolysis capacity of cathepsin-B and cathepsin-L, but not cathepsin-D, decreased, as characterized by the DQ-ovalbumin assay. Most enzymes of lysosomes exert maximum activity at low pH, and the elevation of

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**Fig. 6. Effects of urinary proteins on Lyso-Tracker red (Lyso-Red) fluorescence and proportion of “pale” cells.** A and C: HK-2 cells were treated with urinary proteins at different concentrations for 16 h. After incubation with Lyso-Tracker red for 1 h, the intensity of cell fluorescence was analyzed. Scale bars = 10 μm. B and D: alternatively, cells were trypsinized, centrifuged, and resuspended for flow cytometry analysis. Cells with decreased red fluorescence (pale cells) were gated, and their percentages are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
lysosomal pH could impair the activity of hydrolytic enzymes and elicit declined degradability of lysosomes (1, 3, 21). In addition, our results showed the defective acidification of lysosomes after exposure to urinary proteins. These data suggest that the attenuated lysosomal milieu resulted in the reduction in enzymatic activity of cathepsin-B/cathepsin-L but not cathepsin-D, which was reported to remain active at neutral pH (4). This is consistent with a previous report (13) showing that after BSA overloading, cathepsin-L was accumulated in parallel to a decreased capacity of lysosomal degradation. It indicates that the increased lysosomal contents might not be sufficient to maintain normal clearance of overloaded proteins.

Fig. 7. Effect of urinary proteins on the distribution of CB and LAMP1. A: immunofluorescent staining of LAMP1 and CB in TECs from control, MCNS, focal segmental glomerulosclerosis (FSGS), or membranous nephropathy (MN) patients. The red immunofluorescence in the bottom image illustrates the leakage of CB from lysosomes into the cytoplasm. Scale bar = 10 μm. B: immunofluorescent staining of LAMP1 and CB in HK-2 cells after exposure to urinary proteins at different concentrations for 16 h. The percentage of cells displaying diffuse staining of CB was counted in at least 10 random fields. Scale bar = 10 μm. ***P < 0.001.
Fig. 8. Effects of antioxidant on Lyso-Tracker red fluorescence and proportion of pale cells in HK-2 cells overloaded by urinary proteins. A and C: HK-2 cells were pretreated with vehicle (CON), catalase (CAT; 2,000 U/ml), or N-acetylcysteine (NAC; 1 mM) before exposure to 8 mg/ml urinary proteins (UP) for 16 h. After an incubation with Lyso-Tracker red for 1 h, the intensity of cell fluorescence was analyzed. Scale bar = 10 μm. B and D: alternatively, cells were trypsinized, centrifuged, and resuspended for flow cytometry analysis. Cells with decreased red fluorescence (pale cells) were gated, and their percentages are shown. *P < 0.05; **P < 0.01; ***P < 0.001.
The alteration of intralysosomal pH and release of lysosomal enzymes were used as an indicator of LMP (4, 9, 28). Therefore, the decreased labeling of Lyso-Tracker red and redistribution of cathepsins from lysosomes to the cytosol might indicate LMP after urinary protein overload in TECs. We have also shown that oxidative stress is one of the crucial factors to trigger LMP, which agrees with a previous study (27) in neurons. Our recent study (14) revealed that exposure to urinary proteins for 8 h activates autophagy at least partly via oxidative stress, which mounts an adaptive response in TECs. This suggests that short-term treatment with urinary proteins (e.g., 8 h) does not trigger LMP, since the lysosome-mediated degradation system plays a key role in autophagy activation. With long-term exposure (e.g., 16 h), LMP occurs, and the reprotective role of autophagy cannot continue. It is well known that LMP has emerged as a trigger of cell death (3, 11). Therefore, the translocation of cathepsin-D to the cytoplasm observed in our study might play an important role in triggering apoptosis, as previously reported (2, 19).

In summary, urinary protein overload results in TEC injury and apoptosis, accompanied by an increase in the amount and mass of lysosomes and decreases in lysosomal enzymatic activity and degradation ability. LMP triggered by oxidative stress is likely an important mechanism underlying TEC injury and apoptosis after urinary protein overload (Fig. 9). Stabilization of the lysosomal membrane might be an intriguing strategy to alleviate renal tubular damage induced by urinary proteins.

REFERENCES