MATERIAL AND METHOD

Experimental animals. All experiments were approved by the Animal Care and Use committee at Thammasat University. To avoid the possible effects of the female reproductive cycle, (e.g. estrogen and progesterone triggering Na^+ reabsorption at collecting ducts), the study was performed in male Sprague Dawley rats weighing 180-200 g. The rats had free access to water and to a standard rodent diet. They were kept in cages with a 12:12-h artificial light cycle at 21°C and humidity of 55%. Rats were randomly assigned into two groups: 4 hours of bilateral ureteral ligation (4 h BUO, n=4) and sham-operated controls (C, n=4) for a non-targeted proteomic study. Three independent experiments were performed for the non-targeted proteomic study. The rats were anesthetized with isoflurane and were placed on a heating pad to maintain body temperature at 37°C during surgery. Through a mid-abdominal incision, both upper parts of ureters were ligated using 3-0 silk in 4 rats. Four Sham-operated controls were prepared in parallel with 4 h BUO group. Rats were allowed to recover following surgery in metabolic cages. We collected urine from sham controls for osmolality measurement. Four hours after the operation, the rats were decapitated using a guillotine while conscious to avoid stress-induced AVP secretion that might alter IMCD protein abundances. Trunk blood was collected for serum urea, creatinine, sodium and potassium analyses. We collected urine dripping from the urethra of sham controls for osmolality measurement. The kidneys were harvested followed by IMCD isolation.

Protocol 1. Eight rats were allocated to 4 hours of BUO (4 h BUO, n=4) and sham (n=4). Inner medullas (IM) were isolated and IMCD was prepared for a non-targeted proteomic study. Three independent experiments were performed.

Protocol 2. Eight rats were allocated to 4 h BUO (n=4) and sham (n=4). IM were isolated followed by IMCD isolation for a targeted proteomic study. Three independent experiments were performed.

Protocol 3. Twelve rats were allocated to 4 h BUO (n=6) and sham (n=6). IM were isolated for immunoblotting. Trunk blood was collected for serum urea, creatinine, sodium and potassium analyses. Rats stayed in metabolic cages after surgery to collect urine for urine volume until euthanization. Urine dripping from the urethra of sham controls was collected for osmolality measurement. For 4 h BUO rats, urine was aspirated from their pelvis for osmolality measurement.

Protocol 4. Six rats were allocated to 4 h BUO (n=3) and sham (n=3). The left kidneys were harvested for immunofluorescence (IF) and the right kidneys for electron microscopy (EM). Two independent experiments were performed.

Protocol 5. Six rats were allocated to 4 h BUO (n=3) and sham (n=3). The IM were dissected for immunogold electron microscopy. Two independent experiments were performed.

Protocol 6. Twenty-eight rats were allocated to BUO for 10 hours (10 h BUO, n=7) *vs.* sham for 10 hours (n=7) and BUO for 24 hours (24 h BUO, n=7) *vs.* sham for 24 hours (n=7). IM from right kidney were dissected for immunoblotting and the left kidneys were harvested for electron microscopy (EM). Rats were put in metabolic cages after surgery until euthanization to collect urine for urine volume. Urine dripping from the urethra of sham controls was collected for osmolality measurement. Urine was aspirated from pelvis of BUO rats for osmolality measurement.

IMCD and peptide preparation. IMCD was prepared from inner medullas according to Stroke (14) with modifications (11). In briefly, kidney inner medulla was digested by incubating at 37 °C for 70-90 min in digestion solution (14). The resulting suspension was centrifuged at 70 *g* for 30 s to harvest the IMCD-enriched fraction. The pellets from rats in each group were pooled and lysed in 8 M urea/50 mM TrisHCl/75 mM NaCl containing protease inhibitors (Roche, Mannheim, Germany). Protein samples were sonicated, centrifuged at 14,000 *g* for 10 min at 4°C and supernatants were corrected. Protein concentration was determined using the BCA protein assay (Pierce, Thermo Fisher Scientific, IL). A total of 200 µg of protein from each group was reduced with 10 mM DTT, alkylated using 40 mM iodoacetamide at room temperature for 30 min in dark, and quenched using 40 mM DTT/50 mM NH_4HCO_3 followed by trypsin digestion at 37°C overnight as previously described (13). After desalting by using Oasis HLB cartridge (Waters, Milford, MA), peptides were quantified by Quantitative Fluorometric Peptide Assay (Pierce).

Dimethyl labeling and peptide fractionation. A total of 100 ug of peptides from each group were dried in *vacuo*. followed by reconstituted with 100 nM TEAB at pH between 5-8.5. In-solution stable isotope dimethyl labeling was performed as previously described (2). Briefly, peptides from control group were labeled with regular formaldehyde (CH_2O) (Sigma-aldrich, MO, USA) and sodium cyanoborohydride ($NaBH_3CN$) (Sigma-aldrich, MO, USA) which generated a mass increase of 28.0313 Da per primary amine on a peptide (light labeling). While heavy labeling peptides from BUO group were generated by combining deuterated 13C labeled-formaldehyde ($^{13}CD_2O$) with sodium cyanoborodeuteride ($NaBD_3CN$) (Cambridge Isotope Laboratories, MA, USA) increasing a mass of 36.0757 Da per primary amine (2). After incubation for 1 hour, samples were quenched with 1% ammonia solution and acidified with formic acid. The two differentially labeled samples were mixed and dried in *vacuo*. The mixed sample was fractionated into 5

fractions using high pH reversed-phase peptide fractionation kit as manufacture's protocol (Pierce, Thermo FisherScientific, IL).

LC-MS/MS analysis and database searching. The fractionated samples were submitted to nano-liquid chromatography EASY-nLC 1000 (Thermo Fisher Scientific) coupled with Q-exactive plus mass spectrometer (Q Exactive Plus Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific) through an EASY-Spray nanoelectrospray ion source (Thermo Fisher Scientific). The gradient was supplied using an EASY-nLC 1000 UHPLC system and consisted of 5%-40% acetonitrile in 0.1% formic acid for 50 minutes, 40-60% acetrontrile in 0.1% formic acid for 10 minutes, and 60-90% acetonitrile in 0.1% formic acid for 10 minutes and at 300 nl/ml for 70 minutes. The MS methods included a full MS scan at a resolution of 70,000 followed by 10 data-dependent MS2 scans at a resolution of 17,500. The full MS scan range of 300 to 1600 m/z was selected, and precursor ions with the charge states of +1, +2 or greater than +8 were excluded. Fragmentation of precursor ions was performed using higher-energy collisional dissociation (HCD). MS2 spectra were searched and analyzed *by* using Maxquant software (version 1.6.0.13) based on database from Uniprot Rattus norvegicus (canonical and isoform sequence data, containing 7994 proteins). Precursor ion tolerance was 2 ppm, while minimum peptide length was 6, and maximum missed cleavage was 2. Static modification included carbamidometylation of cysteine (+57.021 Da). Variable modifications included methionine oxidation (+15.995 Da), protein N-acetylation (+42.01056 Da) and monoisotopic mass increment of 28.0313 Da for light and 36.0757 Da for heavy labeling, respectively. All datasets were filtered to include < 1% false positive, estimated based on target-decoy analysis. Labeled peptide analysis and quantification were performed using Maxquant software (3) and Perseus software (15). *The median peptide abundance of all peptides identified for a given protein was used to infer the protein abundance change.* Values were presented as *the*

mean ± S.E.M of log_2 of BUO/Sham ratios for each protein across all three biological replicates. All proteins that passed criteria two-tailed *t*-tests against $log_2(1)$ ($P< 0.05$) were considered significantly changed. The DAVID bioinformatics tool (http://david.abcc.ncifcrf.gov/) (6) was used to identify clusters of proteins based on GO terms. A modified Fischer's exact test was used to assess the level of enrichment.

Parallel Reaction Monitoring. We performed three new independent experiments. Each experiment contains 8 rats which randomly assigned into two groups: bilateral ureteral ligation for 4 hours (4 h BUO, n=4) and sham-operated controls (n=4). IMCDs from rats in each group were prepared and pooled followed by trypsinization and peptide quantification as described above. Ten micrograms of peptides from each group were analyzed using a Q-Exactive orbitrap MS (Thermo Scientific). The target peptides were selected based on prior non-targeted proteomic data and the following parameters (1) polarity; positive, (2) charge; 2, (3) resolution; 70,000, (4) AGC target; 1e5, (5) maximun IT; 150 ms, (6) Isolation window; 2.0 m/z and (7) NCE; 27. The raw file without any conversion was performed to quantitative analysis of protein by using skyline 4.2. The peptide setting on skyline was set to trypsin as cleavage specificity, using 2 missed cleavages, modification selected dimethyl: 2H (6) 13C (2) at N terminal and lysine. The transition sitting was set on the following parameters; two and three charge states for precursors, and the three most intense "b" and/or "y" fragments were selected for the MS/MS. The MS/MS spectra filtering was set up at a resolving power of 60,000 (at 200 m/z) and Orbitrap as mass analyzer. Peak areas for each peptide were extracted using Skyline software (8). Values were presented as *the mean of log_2 of BUO/Sham ratios for each protein across all three biological replicates*. The quantification was based on the sum of the area under the curves (AUC) of the selected peptides and the ratio between light isotope

peptide fragments and the heavy isotope peptide fragments. All proteins that passed criteria two-tailed *t*-tests against $\log_2(1)$ ($P< 0.05$) were considered significantly changed.

We performed a new independent experiment for immunoblotting, immunofluorescence (IF), and electron microscopy (EM). Twelve were allocated to 4 h BUO (n=6) and sham (n=6). The left kidneys were harvested for IF and EM. Whole inner medullas (IM) of the right kidneys were homogenized in Laemmli sample buffer containing a protease inhibitor. The total protein concentration of the homogenate was measured using the BCA protein assay. Loading gels with Coomassie blue stain were performed to adjust the final protein levels in each sample prior to immunoblotting. Anti-AQP2 (5) and anti-phospho-Ser256-AQP2 (10) were kindly provided by Dr. Mark Knepper (NIH, USA). Primary antibodies used were against RhoA (67B9, Cell Signaling Technology), Cttn (PA5-17730, Pierce), cleaved PARP, cleaved caspase 6, cleaved caspase 9 (9929, Cell Signaling Technology). Electrophoresis and immunoblotting were carried out as previously described (12). The images were developed and quantified using near-infrared fluorescence (Li-Cor Odyssey, Nebraska).

Immunofluorescence microscopy. Renal inner medullas from 3 Sham and 3 BUO rats were prepared for immunofluorescence labelling of kidney sections as previously described (7). Two independent experiments were carried out. Primary antibodies used were against AQP2 (LL265)(1); Lamp1 (17768) from Santa Cruz (Dallas, TX); RhoA (67B9), Itgb1 (4706), Vcl (4650), ERM (3142), LC3A/B (4108), ATG12 (4180) from Cell Signaling Technology (Danvers, MA); Ctnnb1 (PA5-17784) and Cttn (PA5-17730) from Pierce (Rockford, IL); ATP5a (14748) from Abcam (Cambridge, MA); and UT-A1 (L179) anti-UT-A1(9). Secondary antibodies were conjugated with Alexa Fluor 488 (anti-rabbit: green channel), 555 (anti-chicken: red channel), 633 (anti-mouse: pink channel) from Invitrogen (CA). Nuclei were stained with 4', 6-diamidino-

2-phenylindole (DAPI, D3571, Invitrogen). Fluorescence images were acquired using an Axio Observer Z1 microscope in conjunction with ApoTome2 (Carl Zeiss, Jena, Germany). The fluorescence colocalization was analyzed by Pearson correlation coefficient and $P< 0.05$ was considered significant.

Electron microscopy. Fresh renal inner medullas from control and BUO rats were minced into 1 mm^3 cubes, and fixed in 4% paraformaldehyde with 1% glutaraldehyde fixative. Post-fixation was performed in 1% osmium tetroxide in Millonig buffer for 1 h at 4 °C followed by dehydration in a graded ethanol series and propylene oxide. The tissues were embedded in epoxy resin and polymerized at 60 °C for 48 hours. The ultrathin sections were mounted on naked copper grids before staining with 2% uranyl acetate and lead citrate solutions then examined under a transmission electron microscope (FEI Tecnai G2 TWIN 200 kV).

Immunogold electron microscopy. We performed a new experiment using 6 rats for this study. Fresh renal inner medullas from 3 Sham and 3 BUO rats were fixed in 4% paraformaldehyde solution in 0.1 M Na^+ cacodylate, pH 7.2, for 3 h, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin (70 nm) cryosections were cut on a Reichert Ultracut S cryo-ultramicrotome (Leica) and mounted. Sections were blocked by incubation in PBS containing 0.05 M glycine and 1% BSA before incubation overnight at 4°C with various antibodies diluted in PBS containing 0.1% skim milk powder. Primary antibodies used were a rabbit polyclonal antibody against AQP2 (5), and goat polyclonal antibodies against Cathepsin D (Research Diagnostics), MAP LC3β (clone G-2, Santa Cruz) or LAMP1 (C20, Santa Cruz). Primary antibodies were visualized using various combinations (see figure legends) and sequential application of donkey anti-rabbit IgG, goat anti-rabbit IgG or donkey anti-goat IgG conjugated to 5nm, 6nm or 10nm colloidal gold particles (BioCell Research Laboratories) diluted 1:50 in PBS with 0.1% skim milk

powder and polyethylene glycol (5 mg/ml). After counterstaining with 0.3% uranyl acetate in 1.8% methylcellulose for 10 min, sections were examined in a FEI Morgagni electron microscope. Immuno-labeling controls included incubation without primary antibodies, use of only one secondary antibody (in double labeling experiments) and use of alternatively sized colloidal gold conjugated secondary antibodies.

Immunogold electron microscopy. All procedures have been described in detail previously (4). In brief, after anaesthesia, rat kidneys were removed, inner medullas dissected out, fixed in 4% paraformaldehyde solution in 0.1 M Na^+ cacodylate, pH 7.2, for 3 h, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin (70 nm) cryosections were cut on a Reichert Ultracut S cryo-ultramicrotome (Leica) and mounted. Sections were blocked by incubation in PBS containing 0.05 M glycine and 1% BSA before incubation overnight at 4°C with various antibodies diluted in PBS containing 0.1% skim milk powder. Primary antibodies used were a rabbit polyclonal antibody against AQP2(5), and goat polyclonal antibodies against Cathepsin D (Research Diagnostics), MAP LC3β (clone G-2, Santa Cruz) or LAMP1 (C20, Santa Cruz). Primary antibodies were visualized using various combinations (see figure legends) and sequential application of donkey anti-rabbit IgG, goat anti-rabbit IgG or donkey anti-goat IgG conjugated to 5nm, 6nm or 10nm colloidal gold particles (BioCell Research Laboratories) diluted 1:50 in PBS with 0.1% skim milk powder and polyethylene glycol (5 mg/ml). After counterstaining with 0.3% uranyl acetate in 1.8% methylcellulose for 10 min, sections were examined in a FEI Morgagni electron microscope. Immuno-labeling controls included incubation without primary antibodies, use of only one secondary antibody (in double labeling experiments) and use of alternatively sized colloidal gold conjugated secondary antibodies.

Statistics. Value are presented as mean ± S.E.M. Individual sample size (n) is shown in figure legends. The statistic comparisons between two groups were made by a Student's unpaired *t*-test. *P* values < 0.05 was considered significant.

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