Supplementary figure 1.

Estimation of AI in RPTC exposed to Stx2 in different concentrations. AI was determined by analyzing five to seven randomly selected areas with 100 to 200 cells in each area. Histograms represent means ± SEM. * - $p<0.05$, ** - $p<0.01$. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated eight times.

Supplementary figure 2.

Ouabain increases expression of Bcl-xL, decreases expression of Bax and cleaved caspase-3 in the absence of Stx2.

A. Expression of Bcl-xL, Bax, caspase-8 and caspase-3 in presence of absence of ouabain during 6 hours. Densitometric quantification of bands was done for the respective blots. The density of the band from control cells was set to 100%. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated four times.

B. Expression of Bcl-xL, Bax, caspase-8 and caspase-3 in presence of absence of ouabain during 16 hours. Densitometric quantification of bands was done for the respective blots. Histograms represent means ± SEM. * - $p<0.01$. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated four times.

Supplementary figure 3.

A. A cartoon illustrating the ouabain/Na,K-ATPase/IP3R signaling pathway.

Ouabain triggers the interaction between the N-terminus tail of the catalytic α subunit of Na,K-ATPase and the N-terminus of IP3R[15,16]. This activates the IP3 receptor and triggers slow intracellular calcium oscillations, which subsequently activate the NF-κB p65 subunit and leads to protection from apoptois[18,19]. The immune-precipitation (IP) study shows that ouabain will enhance the interaction between the catalytic α subunit of Na,K-ATPase and the IP3 receptor both in the absence (B) and presence of Stx2 (C).

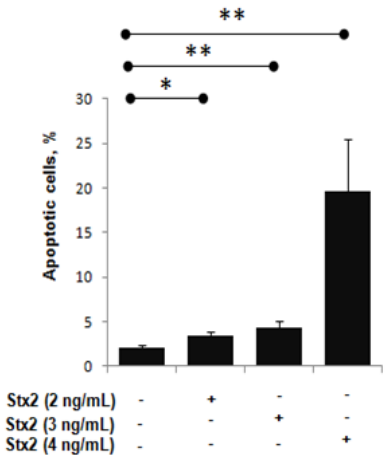
WB, Western blot. Input represents 5% of total cell lysate. The densities of immunoprecipitated IP3R band were adjusted based on the input densities; the density of the samples not exposed to ouabain was set to 100%. Histograms represent means ± SEM. *$p<0.05$ –

ouabain vs. control (left bar) and ouabain vs. control in presence of Stx2 (right bar). Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated three times.

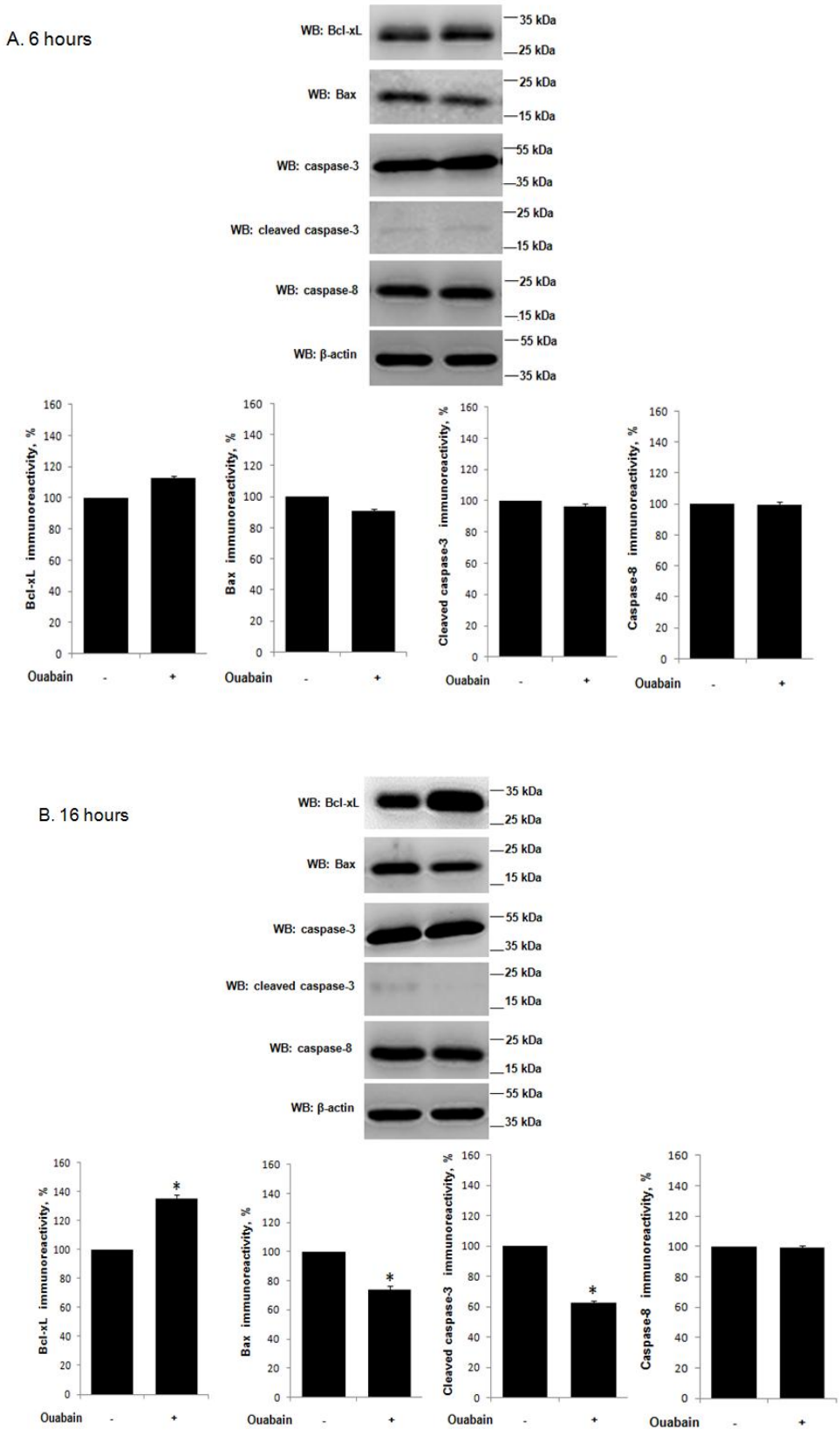
Supplementary figure 4.

Images obtained with confocal immunofluorescence microscopy shows Stx2 binding to the plasma membrane of RPTC (lower magnification, 25x/0,8NP water-immersion objective). Index of Stx2-positive cells was determined by analyzing five randomly selected areas with 25 to 45 cells in each area. Histograms represent means ± SEM. *$p<0.001$ – Stx2 vs. control. Statistical analysis was performed using the Mann-Whitney U test. Experiments were repeated three times.

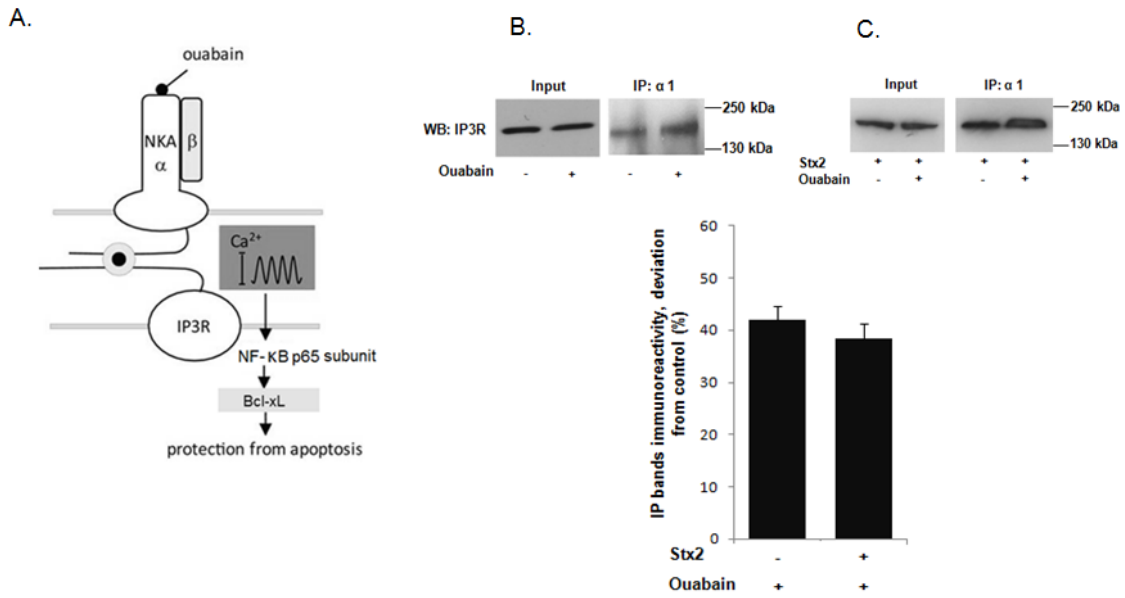
Supplementary figure 1.



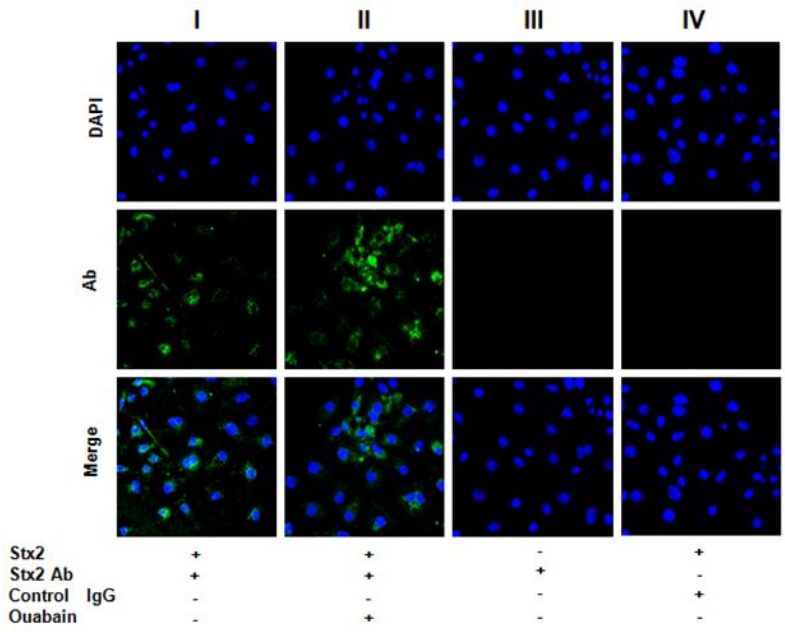
Supplementary figure 2.



Supplementary figure 3.



Supplementary figure 4.



Complete material and methods

Cells

RPTC were prepared from kidneys of 20 day-old male Sprague-Dawley rats as described previously.[40] The studies performed in Sweden followed the Karolinska Institutet regulations concerning care and use of laboratory animals, and were approved by the Stockholm North ethical evaluation board for animal research. The kidneys were removed and placed in 0.9% NaCl at room temperature. The cortical layers were dissected and placed in Hank's balance salt solution (Invitrogen, Grand Island, N.Y., USA) containing 10 nM Hepes, 2% BSA and collagenase 0.02 mg/ml for 15 min at 37°C and gently mixed using a fire-polished Pasteur pipette. The reaction was stopped by washing the cells twice in a solution containing 1% trypsin inhibitor. After washing, equal volumes of cell suspension were plated on 12-mm glass coverslips in 24-well Petri dishes. Cells were cultured for 3 days in supplemented DMEM (20 mM HEPES, 24 mM $NaHCO_3$, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 10% FBS) on glass coverslips for in 5% CO_2 at 37°C.

On day two in vitro, at which time they have been shown to maintain most of their characteristics as proximal tubule,[40,41] the cells were exposed to the indicated compounds (Stx2, obtained from C. Thorpe, Phoenix Lab, Tufts Medical Center, Boston, MA or from T.G. Obrig, Dept of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore), with or without and ouabain (Sigma-Aldrich, St.Louis, MO, USA) or vehicle (PBS) for 12 h. To obtain an adequate degree of Stx2-induced apoptosis in RPTC, cells were incubated with Stx2 at different concentrations (2-4 ng/mL). In pilot studies the Stx2 concentration of 4 ng/ml was found to give pronounced and reproducible apoptotic effects **(Supplementary Figure 1)**. This concentration was therefore used in all subsequent experiments.

Stx2 binding to rat proximal tubular cells

Cells were plated on 12-mm glass coverslips in 24-well cell culture plates. The cells were fixed in 4% paraformaldehyde, washed once with cold PBS and incubated with Stx2 (200 ng/ml) for 1h with gentle shaking. Cells were then incubated with mouse monoclonal anti-Stx2 IgG1 antibody (11E10, 200 ng/mL.a kind gift from T.G. Obrig, Dept of Microbiology and Immunology, University of Maryland, Baltimore, MD) or the isotype control antibody mouse IgG1 200 ng/ml (BD Biosciences, San Diego, CA) for 1h, washed twice with PBS, followed by

incubation with Alexa 488 fluorescence-conjugated goat anti-mouse IgG antibody (1:1000, Invitrogen, Grand Island, N.Y., USA) for 1h. After washing, the cells were mounted and observed with Zeiss LSM 510 laser scanning confocal microscope using 25X/0.8NA oil-immersion and 40X/1.2NA water objective. Alexa Fluor 488 stain was detected using 488 nm excitation and a 510-550 nm band-pass filter. In each preparation, eight to ten randomly selected areas were examined, and in each area, between 50 and 100 DAPI-stained cells were counted.

Detection of apoptotic cells

RPTC were plated on 12-mm glass coverslips in 24-well cell culture plates. On culture day two, when cells had achieved approximately 50% confluence, indicated stimulants were added to the medium. The ApopTag Red In Situ Apoptosis Detection kit (Chemicon International, CA) was used to determine the apoptotic index (AI). TUNEL assay was conducted according to the manufacturer's instructions. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1.5 µg/ml, Santa Cruz Biotechnology, CA).

Cells were mounted in Immu-Mount (Thermo Shandon, Midland, Canada) and images recorded with Zeiss LSM 510 laser scanning confocal microscope using 25X/0.8NP oil-immersion objective. TUNEL stain was detected using 488 nm excitation and a 510-550 nm band-pass filter. ApopTag was detected using 561nm excitation and a 575 nm long pass filter, DAPI was detected using 405 nm excitation and a 420-480 nm band-pass filter. Cells were considered apoptotic when they exhibited ApopTag Red staining and characteristic apoptotic morphology. The AI was calculated as the percentage of TUNEL-positive cells, the total number of cells was determined by DAPI stain. In each preparation, eight to ten randomly selected areas were examined, and in each area, between 100 and 200 DAPI-stained cells were counted.

FACS Staining

Approximately 1 million RPTC from each study group were harvested, washed, and labeled with fluorochrome-conjugated Annexin V (an indicator of early apoptosis) and 7-AAD (viability dye) in the dark at room temperature for 15 min, using "Annexin V-PE Apoptosis detection Kit I" (BD Biosciences Pharmingen, San Diego, CA, USA). This procedure was followed by FACSCalibur (Becton Dickinson Biosciences, USA) analysis. The quantification analysis was performed with CellQuest software version 3.3. Early apoptotic cells were indicated

as Annexin V-PE positive and 7-AAD negative, whereas late apoptotic and/or dead cells were indicated as Annexin V-PE and 7-AAD double positive and viable cells were double negative.[42]

NF-κB p65 subunit activity

NF-κB p65 subunit translocation to the nucleus was used as an index of NF-κB p65 subunit activation. NF-κB immunostaining was performed as described previously.[18] Briefly, cells were labeled with rabbit anti-human polyclonal NF-κB p65 antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA); secondary antibody was anti-rabbit IgG-Alexa 546 (1:3000). The immune-labeled cells were observed with Zeiss LSM 510 laser scanning confocal microscope using a 40X/1.2NA water-immersion objective. Alexa Fluor 546 stain was detected using 561 nm excitation and a 575 nm long pass filter. In each preparation, six to seven randomly selected images that contained approximately 100 cells each were observed and all cells in these images were analyzed. The ratio of the immunosignal between the nucleus and the cytosol was measured using ImageJ software (NIH Image, Baltimore, MD, USA). An area corresponding to 90% of nucleus and identical area of cytoplasm were used to measure intensity of the immunosignal. NF-κB p65 subunit DNA binding in nuclear protein extracts was assessed using a commercially available NF-κB p65 subunit transcription factor assay according to the manufacturer's instructions (Active motif, Carlsbad, CA, USA). Nuclear protein was extracted using The Nuclear Extract Kit (Active motif, Carlsbad, CA, USA). Briefly, cells are collected in ice-cold PBS in the presence of Phosphatase Inhibitors and then re-suspended in Hypotonic Buffer to swell the cell membrane and make it fragile. After separation from the cytoplasmic fraction, the nuclei were lysed and solubilized in the Lysis Buffer in the presence of the Protease Inhibitor Cocktail. Bio-Rad Benchmark Microplate Reader was used to read the sample absorbance, with data expressed as optical density at 450 nm.

Immunoprecipitation of NKA and IP3R

After incubation with Stx2 and ouabain for 24 h, RPTC were washed twice with PBS, lysed on ice in cold buffer (50 mM Tris/HCl (pH 7.4), 50mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 0.5% sodiumdeoxycholate, 1.5% Nonidet P-40 and protease inhibitors (Roche Diagnostic, Mannheim, Germany), sonicated and centrifuged at 14 000 rpm for 30 min at 4 °C. Supernatant protein concentration was measured by Bio-Rad DC protein assay (Bio-Rad, CA,

USA) using bovine serum albumin as the standard. Protein solutions were normalized to an equivalent amount of total protein. In order to study the interaction between NKA and IP3R, the protein solution (500 µg) was incubated with anti-Na,K-ATPase α_1 mouse mAb (Upstake Biotechnology Charlottesville, VA, USA) for 1 h at 4 °C. Immunocomplexes were then incubated with 30 µg/L of 50% slurry of protein A/GPLUS-agarose beads (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C with mixing. On the next day the beads were washed three times with PBS, proteins were eluted with 2 x Laemmli sample buffer and heated at 37 °C for 30 min and prosessed for SDS-PAGE and Western Blotting using anti-IP3R mAb (BD transduction Laboratories, Lexington Kentucky, USA). The protein bands were visualized by chemiluminescence using secondary antibodies labeled with horseradish peroxidase (GE Healthcare, Waukesha, WI USA). Quantitation of the protein content was done by densitometric analysis.

Immunoblotting for detection of Bax, Bcl-xL, caspase-3 and caspase-8

Proteins solubilized in Laemmli sample buffer were resolved in polyacrylamide gels by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked in 5% non-fat milk in TBS-T (136 mM NaCl, 10 mM Tris, 0.05% Tween 20) and immunoblotted using the Bax and Bcl-xL, caspase-3 Rabbit Ab (Cell Signaling Technology, Danvers, MA USA), rabbit caspase-8 Ab (Abcam, UK) and actin mouse mAb (BD, Lexington KY, USA) for 1 h at room temperature. The actin mouse mAb were used as a loading control. After three washes with TBS-T, the membranes were incubated with secondary anti-rabbit or anti-mouse antibodies labeled with horseradish peroxidase for 1 h at room temperature. Membranes were washed three times with TBS-T. The protein bands were visualized by chemiluminescent substrate ECL. Quantitation of the protein content was done by densitometric analysis.

Animal studies

The study was approved by the animal ethics committee of Lund University. Experiments were conducted on C57BL/6 male mice (8 weeks old) weighing 22 to 24 g. Mice received an intraperitoneal injection of Stx2 at 285 ng/kg. PBS injection was used for control mice. Ouabain was delivered in a dose corresponding to 15 µg/kg/day using subcutaneous neck mini-pumps

(ALZET, USA). Controls received PBS vehicle in the pump. All mice were sacrificed at day 3 after Stx2 injection. Mouse kidneys were removed and fixed in 4% paraformaldehyde in PBS (pH 7.4) immediately after sacrifice. Tissues were then embedded in paraffin and sectioned. Blood was collected by cardiac puncture of anesthetized animals. Citrated blood samples were centrifuged at 2000 x *g* for 15 min. The plasma was removed and stored at - 80°C until analysis.

TUNEL assay

Renal tissue (3-μm sections) used for TUNEL assay was deparaffinized and rehydrated prior to processing. Two sections from each mouse kidney were used for TUNEL assay. The ApopTag Red In Situ Apoptosis Detection kit (Chemicon International, CA, USA) was used to determine the apoptotic index. TUNEL assay was conducted according to the manufacturer's instructions. Nuclei were counterstained with DAPI. Sections were mounted in Immu-Mount (Thermo Shandon, Midland, Canada) and observed with Zeiss LSM 510 laser scanning confocal microscope using 25X/0.8NA oil-immersion objective. ApopTag was detected using 561nm excitation and a 575 nm long pass filter, DAPI was detected using 405 nm excitation and a 420-480 nm band-pass filter. DAPI-stained cells were viewed with an ultraviolet light source. Cells were considered apoptotic when they exhibited ApopTag Red staining and characteristic apoptotic morphology. In each slice, five to seven randomly selected areas were examined, and in each area, between 80 and 150 DAPI-stained cells were counted.

Immunohistochemistry

Renal tissue (3-μm sections) was deparaffinized and rehydrated prior to processing. Antigen retrieval was carried out by boiling in Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) for 20 min. Sections were treated with Triton X-100 0.3% (Sigma-Aldrich NV/SA, Bornem, Belgium) in PBS for 20 min. After three PBS washes, sections were incubated with blocking buffer (5% bovine serum albumin and Triton X-100 0.1% in PBS) for 1 hour. The rabbit polyclonal anti-Bcl-xL primary antibody (Cell Signaling Technology, Danvers, USA) and rabbit polyclonal anti-Bax (Santa Cruz, CA, USA) were applied at a dilution of 1:50 in 5% bovine serum albumin in PBS overnight at 4℃. Following three PBS washes, sections were incubated with a secondary Alexa Fluor 488 goat anti—rabbit IgG (1:500) for 1 h at room temperature.

Nuclei were counterstained with DAPI. All samples were stained for an identical length of time and under identical conditions. All recordings for each staining were done during one day using identical gain settings. Sections were mounted in Immu-Mount (Thermo Shandon, Midland, Canada) and observed with Leica TCS SP inverted confocal scanning laser microscope using 25X/0.8NA oil-immersion objective. Image analysis was performed using ImageJ software (NIH Image, Baltimore, MD, USA). Three areas in each section were analyzed.

For podocyte immunostaining kidney sections were prepared as above and incubated with rabbit polyclonal anti-WT1 primary antibody (Santa Cruz, CA, USA) at a dilution of 1:200 in 5% bovine serum albumin in PBS overnight at 4℃. Controls were performed in the same conditions, but the primary antibody was omitted. Following three PBS washes, sections were incubated with a secondary Alexa Fluor 488 goat anti-rabbit IgG for 1h at room temperature. The immune-labeled cells were observed with Zeiss LSM 510 laser scanning confocal microscope using a 63X/1.2NA oil-immersion objective. For quantitative determination of podocyte numbers, the WT-1–positive cells were counted in at least 12-15 randomly chosen glomeruli. Two sections from each kidney were analyzed.

Analyticals

Plasma creatinine was determined using a Mouse Creatinine (Cr) ELISA kit (CUSABIO, BIOTECH CO., LTD, China). The reaction was analyzed on an automated spectrophotometer (Wallac Victor$^{3}{}_{TM}$ 1420 Multitable Counter).

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Statistical analysis

Statistical analysis was performed with STATISTICA 6.0 software (Statsoft, Tulsa, OK USA) and SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA). Student t-test was used to compare quantitative variables between groups if the distribution was parametric, ANOVA followed by the *post hoc* test and nonparametric test (Mann-Whitney U test) were used to test significance of differences. Statistical significance was determined as $p<0.05$. Values are expressed as means ± SEM.