# Supplemental material

Complete Materials and Methods

**Antibodies and chemicals**

The following primary antibodies have been used trough out the study: mouse anti-PCNA, goat polyclonal anti-Ki-67, rabbit and mouse anti-WT1 (all from Santa Cruz Biotechnology, Santa Cruz, USA), mouse anti-Bax [6A7] and rabbit anti-SGLT-2 (both from Abcam, Cambridge, UK), rabbit anti-human polyclonal Bcl-xL, rabbit anti caspase-3, rabbit anti-TGF-beta (all from Cell Signaling Technology, Inc. Danvers, USA), rabbit anti-TGF-beta1 (Novus Biologicals, USA), rabbit anti-mouse collagen IV (Merk Millipore, Billerica, USA), goat anti-IL-1 beta, goat anti-IL-6 (both R&D Systems, Minneapolis, USA) and mouse anti-actin (BD Bioscience, Lexington, USA). The following fluorescent secondary antibodies have been used trough out the study: Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-mouse IgG, Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 546 rabbit anti-goat IgG (all from Life Technologies, Carlsbad, USA).
All chemicals and reagents were purchased from Sigma-Aldrich, St. Louis USA, and all cell culture materials were purchased from Life Technologies, Carlsbad USA, unless otherwise stated.

**Animals**

Forty days old Male Sprague-Dawley rats with initial body weights of 100 to 120 g were used for the passive Heymann nephritis, PHN, model. For cell preparation twenty days old male Sprague-Dawley rats were used. All animals were housed under controlled conditions of light and darkness (12:12h). Food, a standard diet containing 20 % protein by weight and tap water were available *ad libitum*. All experiments were performed according to Karolinska Institutet regulations concerning care and use of laboratory animals, and were approved by the Stockholm North ethical evaluation board for animal research.

**Rat Primary cultures**

Primary culture of rat proximal tubular cells (RPTC) was prepared as previously described by Khan et al. 2008. Animals were anesthetized by intraperitoneal injection of pentobarbital and the aorta cut, kidneys were excised and placed in 0.9 % NaCl (Sigma-Aldrich) at room temperature. The cortical layers were collected by using a microtome for 250µm slices. Slices were placed in 37°C basal solution (containing Hank's balanced salt (HBSS), 0.2% Bovine Serum Albumine (BSA, Sigma-Aldrich), 10mM HEPES (Sigma-Aldrich), 10 µg/mL penicillin and 10 µg/mL streptomycin supplemented with 0.035% collagenase I (Sigma-Aldrich), and incubated for 20 minutes with gentle mixing using a fire-polished Pasteur pipette. The reaction was stopped by washing the cells twice in basal solution with 0.01% trypsin inhibitor. After washing, cells were plated on 12 or 18-mm glass coverslips in 12 or 24-well Petri dishes and allowed to attach for 30 minutes before growth media was carefully added. Cells were cultured for 2 or 3 days in supplemented Dulbecco's modified Eagle's medium, 20 mM HEPES, 24 mM $NaHCO_3$ (Merck KGaA, Darmstat, Germany), penicillin 10 µg/mL, streptomycin 10 µg/mL, and 10 % Fetal Bovine Serum (FBS) in 37°C at an approximate humidity of 95 – 98 % with 5 % $CO_2$. Growth media was changed after 24 h. At day 3 in culture 99% of the cells are RPTC, expressing SGLT-2 a proximal tubular marker (FigS 1A).

Glomeruli isolation and podocyte culture were performed as described by Lal et al. 2015. The rats were anesthetized by intraperitoneal injection of pentobarbital and perfused trough the left ventricle first with HBSS to clear out blood and directly following with a solution of HBSS with 0.1% BSA and Dynabeads M-450 (Dynal Biotech ASA, Oslo, Norway). For each animal $8x10^7$ Dynabeads in 20 mL of solution was used. After perfusion, kidneys were removed and the medulla was discarded, the cortex was cut to small pieces and digested in 1mg/mL collagenase I and 10 U/mL DNAse in HBSS at 37°C for 30 min with gentle shaking. After digestion, the tissue was pressed gently through a 100-µm cell strainer (BD Falcon, Bedford, MA). Glomeruli containing Dynabeads were collected using a magnetic particle concentrator. The isolated glomeruli were washed three times with cold HBSS and used for subsequent studies. For primary culture approximately 500 glomeruli were plated on a 12 mm coverslip and cultured for 3 days in supplemented DMEM:F12 medium, 0.5% Insulin-Transferrin-

Selenium-Sodium Pyruvate and 5% FBS at an approximate humidity of 95–98% with 5% $CO_2$. Growth media was changed after 24 h.

Albumin treatment of primary cultures was started on day two or three in vitro for all experiments, cells were exposed to either vehicle (PBS) or fatty acid and endotoxin-free bovine albumin at concentrations of 0.2, 2.5, 5, 10, or 20 mg/mL (Sigma-Aldrich) in the growth media for 0 up to 18 hours. Some groups were also treated with ouabain 5 nM (Sigma-Aldrich) or with vehicle (PBS) in the growth media.

**Microscopy**

For all fluorescence imaging a Zeiss LSM 510 confocal microscope equipped with 25X/0.8NA oil, 63X/1.4NA oil and 40X/1.2NA water objectives was used. Immunofluorescence was detected as follows; DAPI excitation at 405 nm and detection 420-480 nm, Alexa Fluor 488 excitation at 488 nm and detection 510-550 nm, Alexa Fluor 546 excitation at 543 nm and detection 575 nm long pass. TUNEL labeling was detected by excitation at 543 nm and 575 nm long pass detection. JC-1 fluorescence ratios were recorded with excitation at 488nm and simultaneous 505-530 nm and long pass 560 nm detection.

**Detection of apoptosis in primary culture**

Terminal Deoxynucleotidyl (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) assay by use of ApopTag Red In Situ Apoptosis Detection kit (Merk Millipore, Billerica, USA) was used to determine the apoptotic index, AI, according to the manufacturer's instructions. The nuclei were counterstained 2 min with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Santa Cruz, USA). The samples were mounted in Immu-Mount (Thermo Shandon, Pittsburg, PA) and imaged using confocal microscopy. Cells were considered apoptotic when they exhibited ApopTag Red staining and characteristic apoptotic morphology with condensed nuclei. 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, each containing 100-200 cells. For determination of AI in cultured podocytes, podocytes were first identified by immunostaining using anti-WT1 antibody. Only podocytes outside of a glomerulus and positive for WT1 were included in AI calculations since the total number of podocytes located inside a glomerulus is not possible to determine in this preparation.

**Mitochondrial Membrane Potential Determination**

The integrity of the mitochondrial membrane potential was measured in RPTC using the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Life Technologies, Carlsbad, USA). Mitochondrial depolarization is detected by a shift in fluorescence emission from red (~590 nm) to green (~527 nm). After albumin treatment the cells were washed and incubated with 2.5 µg/mL JC-1 dye in cultured medium for 15 min at 37°C. Live cells were then analyzed using confocal microscopy. The mitochondrial membrane potential change was quantified by calculating the ratio of red to green fluorescence intensity using ImageJ software (NIH Image, Baltimore, USA). The control group red/green ratio was set to 100%.

**Immunocytochemisty**

RPTC and podocytes in primary culture were fixed in 4% Paraformaldehyde (PFA) for 10 minutes, washed once with cold PBS and treated with 0.3% Triton X-100 for 10 minutes. Following three washes with PBS, cells were incubated with blocking buffer with 5% BSA and 0.1% Triton X-100 for 1 hour. Primary antibodies were applied overnight at 4°C. Antibody controls were subjected to the same treatment, but the primary antibody was omitted. Following three washes with PBS, cells were incubated with secondary antibodies for 1 hour at room temperature. Cells were washed three times, and mounted with Immu-Mount for analysis.

**Bax, Bcl-xL translocation assessment**

RPTC on day two in vitro were labeled with the mitochondria-targeted green fluorescent protein CellLight® Mitochondria-GFP BacMam (Life Technologies, Carlsbad, USA) overnight at 37°C. On

day three in vitro, RPTC were incubated with albumin with or without ouabain or vehicle for up to 8 h. At the end of treatment cells were fixed with 4% PFA and immunostained for Bax and Bcl-xL, and mounted in Immu-Mount for analysis. Analysis of the Bax and Bcl-xL translocation to the mitochondria was performed with the Matlab image processing toolbox.
To assess co-localization between immune-labeled Bax/Bcl-xL and GFP expressing mitochondria, the method of Edlich et al. 2011 was used. In brief; two perpendicular line traces across the nucleus were drawn for each cell. Overlap of mitochondria and Bax signal peaks along the lines were analyzed, signal peaks were considered to overlap if spaced by no more than 140 nm. Results are shown as fraction of Bax/Bcl-xL – mitochondria overlap to total number of mitochondria along the line. The weakest peaks, representing background fluctuations, were ignored.

**Albumin internalization Protocol in vitro**
To assess albumin internalization RPTC were treated with vehicle, 2.5mg/mL or 10mg/mL delipidated and endotoxin-free albumin with a trace of Alexa 555 labeled albumin (Life Technologies, Carlsbad, USA) for 2-8 h. At the end of treatment cells were washed thoroughly with PBS and subjected to live cell imaging or fixed with 4% PFA and stained with DAPI. The amount of internalized albumin was assessed by calculating the intensity of Alexa 555 over the total number of cells in each field of view.

**Animal model, passive Heymann nephritis**
Animals were divided into control group and PHN group. PHN was induced in non-anesthetized rats by a single intravenous injection of 0.66 mg/100 g body weight of rabbit anti-Fx1A antibody (a kind gift from Professor David J Salant, Boston University Medical Center), controls were given vehicle, sterile PBS. On day 0 after PHN injection half the group was started on ouabain treatment (15 µg/mL/day) the other half was given vehicle (sterile PBS) delivered by mini-pumps implanted subcutaneous. All animals were followed for 4 months. Spot urine samples were collected every second week and albuminuria was measured. Blood pressure was measured every second week in vehicle and ouabain treated control groups, using tail cuff plethysmographyin in unanesthetized animals. Animals were trained to accept the tail cuff before beginning the study to avoid a stress response. During several days before initiating the study animals were placed in the chamber for increasing periods of time to become accustomed to the environment, the maximum time spent in the chamber is 1 hour. The blood pressure was not significantly different in vehicle and ouabain treated animals. At sacrifice animals were anesthetized, blood samples collected for serum creatinine determination and kidneys were removed from for histological and morphological studies.

**Analyticals**
Urine albumin concentrations were determined using Nephrat II (Exocell, Philadelphia, USA), immunoassay kit specific for determining rat urine albumin and analyzed with an automated spectrophotometer, according to the manufacturers instruction. The urine samples were collected in the morning at the same time intervals after induction of PHN throughout the experiment. Blood was collected from the left ventricle of anesthetized animals. Serum was obtained after whole blood clotting. Serum creatinine concentrations were determined using QuantiChrom™ Creatinine Assay Kit (DICT-500), a quantitative colorimetric creatinine determination assay and analyzed with an automated spectrophotometer, according to the manufacturers instruction.

**Renal Histology and Morphometric Analysis**
Dissected kidneys were fixed in Dubosq-Brazil for 6 hours, dehydrated in alcohol and embedded in paraffin. Kidney sections, 3-4 µm, were cut using an automated microtome (Leica), transferred to a warm water bath, picked up on glass coverslips and dried on a heating plate. Sections were stained with Periodic Acid-Schiff (PAS) Kit according to manufacturer's protocol. Sections including superficial and juxtamedullar glomeruli were evaluated. Tubulo-interstitial changes including fibrosis were evaluated. At least 10 randomly selected areas were examined for each slice.

To assess glomerular tubular disconnection serial sections (3-4 µm) from the thickest part of the kidney were PAS stained. On average 75 sections were examined for each animal. Each section covered the full width of the kidney and the total volume of the sections corresponded to at least 2% of

the full kidney volume. Approximately 700 glomeruli could be identified in each volume of serial sections. Only glomeruli located entirely within the serially sectioned tissue were included in the analysis. Identification of glomeruli started from the center section and each individual glomerulus was followed in the serial sections above and below the center section until they could be completely registered. Approximately 50% of all glomeruli fulfilled the criteria of being completely within the volume, resulting in 300-320 examined glomeruli. A full 3D morphological analysis was possible to perform by aligning the serial sections and manually outlining the 3D morphology in sequences of images. The glomeruli were classified as connected to a normal proximal tubule, connected to an atrophic proximal tubule, or without a tubular connection. The proximal tubule segments connected to glomeruli were considered atrophic when there was thinning of the tubular cells accompanied by loss of brush border and narrowing of the tubular lumen. The findings were expressed as percentage of the three classes of glomeruli over the total number of glomeruli examined.

**Immunohistochemical Staining**

Kidney sections, 3-4 µm, were deparaffinized and rehydrated: 20 minutes at 65°C followed by 4 x 5 minutes in xylene, 2 x 5 minutes in 99.5%, 5 minutes in 95% and finally 5 minutes in 70% ethanol (Solveco, Rosersberg, Sweden). Antigen retrieval was performed by boiling 20 min in citrate buffer (10mM citric acid, 0.05% Tween-20) pH 6.0 for 20 min. Sections were then treated 20 minutes with 0.3% Triton X-100. Following three washes with PBS, sections were incubated with blocking buffer, 5% BSA and 0.1% Triton X-100, for 1 hour. All primary antibodies were diluted in PBS containing 5% BSA and applied overnight at 4°C. Antibody controls were subjected to the same treatment, but the primary antibody was omitted. At the end of incubation sections were washed three times in PBS and the secondary antibody was applied. All secondary antibodies were diluted in PBS with 5% BSA and incubated for 1 hour at room temperature. Sections were washed three times and the nuclei counterstained 5 minutes with 1µg/mL DAPI followed by a wash for 5 minutes in PBS and mounted in Immu-Mount. Analysis of Bcl-xl and Bax was done in the outer cortex of 2 slices from each animal and in 6 randomly selected areas. For podocyte assessment WT1–positive cells were counted in at least 10 randomly chosen glomeruli for each rat. For semi-quantitative determination of PCNA, Ki-67 and TGF-beta 1 three randomly distributed areas were analyzed. All sections were stained under identical conditions, and analyzed using identical microscopy settings.

To assess matrix alterations, collagen IV immunostaining was performed. Sections were microwaved in 10mM sodium citrate (pH 6.0) 4 × 5 min, incubated with rabbit anti-mouse collagen IV (Merk Millipore, Billerica, USA) overnight at 4°C and subjected to immunoperoxidase staining using a Vectastain ABC kit (Vector Laboratories, Burlingame, USA), DAB was used as a chromogen. The results of the collagen IV staining were quantified by ImageJ software, assessing the area of positive staining in 20 consecutive glomeruli from each rat, deriving a collagen IV staining percent for each rat. The level of collagen IV staining in control animals was considered as base line and the level of collagen IV staining in the other groups was calculated as % increase from control.

**Detection of apoptosis in renal tissue**

To detect apoptotic proximal tubular cells, kidney sections were deparaffinized, rehydrated and subjected to antigen retrieval. The nuclei were stained with 1µg/mL DAPI for 5 min and mounted with Immu-Mount. All samples were stained under identical conditions and analyzed using identical microscopy settings. Analysis was done in the outer cortex of 2 slices from each animal and in 5 randomly selected areas containing 200-300 cells. Cells were classified as apoptotic if they had a condensed nucleus, where the nuclei was shrunk and had abnormal morphology with no visible interior. This method allows for analysis of a large number of cells, but may to some extent over-estimate the number of apoptotic cells since a nucleus could appear condensed if sectioned at a narrow angle. To lower the effect of this inherent over-estimation the control animals were set to 100% and PHN animals were normalized to control.

To detect apoptotic podocytes renal sections were first subject to immunohistochemistry using antibodies specific for WT1, a podocyte specific transcription factor, followed by ApopTag Red In Situ Apoptosis Detection kit to detect apoptotic podocytes. ApopTag Red In Situ was used according

to the manufacturer's instructions. Before mounting in Immu-Mount, nuclei were counterstained 5 min with 1 µg/mL DAPI. The staining was assessed on the same day using identical microscopy settings.

To evaluate the extent of apoptotic proximal tubular cells at the level of the glomerular-tubular junction two sections for each of the PHN animals at 4 months was used. Sections were deparaffinized and treated with proteinase K (20 µg/mL) for 20 minutes at 37°C. Apoptotic cells were detected by TUNEL staining using a Peroxidase In Situ Apoptosis Detection kit (Merk Millipore, Billerica, USA) according to the manufacturer's instructions. Sections were then counterstained with Harris hematoxylin (Richard Allan Scientific, USA). As a negative control, terminal deoxynucleotidyl transferase was omitted in TUNEL reaction mixture, and as a positive control, DNase was added for 15 minutes at 37°C. In each slice, 25 to 30 randomly selected glomeruli were examined and the number of apoptotic cells per glomerular-tubular junction was calculated.

**Protein detection by Western blotting**

Proteins solubilized in Laemmli sample buffer were resolved in polyacrylamide gels by SDS-PAGE electrophoresis and transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences). Membranes were blocked in 5% non-fat milk in TBS-T and immunoblotted using Bax, Bcl-xL, caspase-3, TGF-beta, IL-1, IL-6, or actin antibodies. Actin staining was used as a loading control. After three washes with TBS-T, the membranes were incubated with secondary anti-rabbit, anti-goat or anti-mouse horseradish peroxidase conjugated antibodies (GE Healthcare) for 1 h at room temperature. The membranes were washed three times with TBS-T, and immunoreactivity was detected using enhanced chemiluminescence by ECL plus Western blotting detection system (Amersham Biosiences). Molecular sizes were assessed using prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA). The protein content was analyzed by densitometric measurement using ImageJ software. The background corrected integrated pixel value was calculated for each band and normalized to the band of the loading control (actin). The protein content was then expressed in relation to control treatment (100%).

**Statistical Analysis**

Results are presented as the mean ± SEM. To determine differences among groups, two-way ANOVA followed by Fisher-LSD post-hoc test was used. If the distribution of the variables was not parametric, the data were analyzed using the non-parametric Mann-Whitney test. Comparisons between groups were made using Kruskal-Wallis one-way ANOVA on ranks with pair-wise multiple comparisons made by Dunn's method. The statistical significance level was defined as $p<0.05$.

## References

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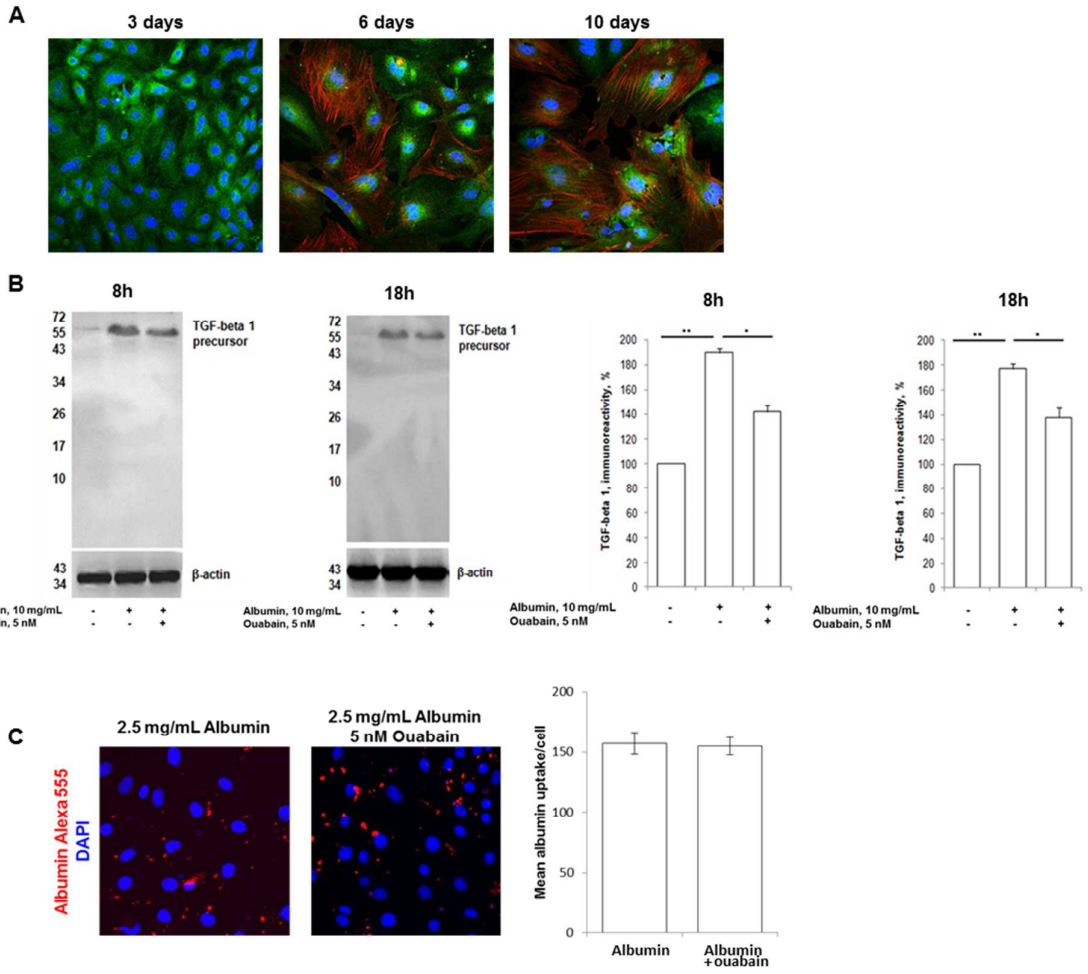


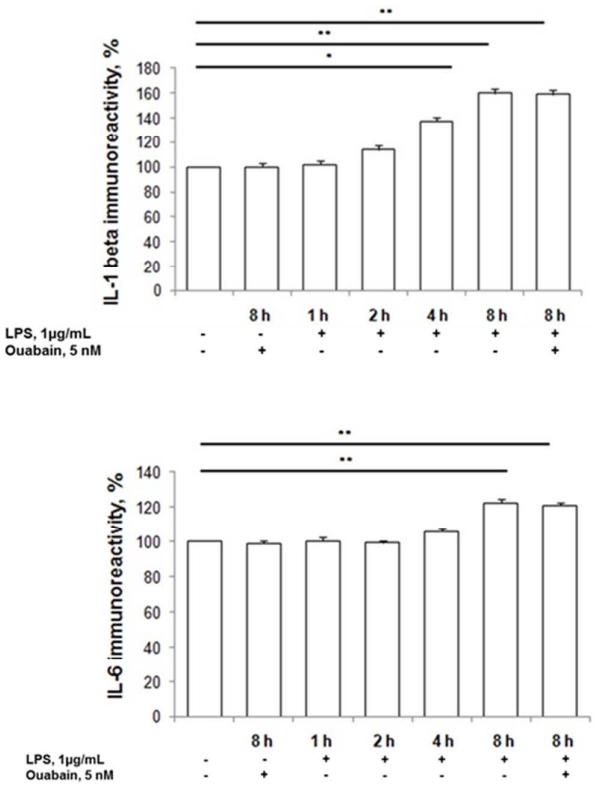

**Supplemental Figure 1**

**A.** Characterization of RPTC. RPTC on day 3, 6 and 10 in culture were immunostained for SGLT-2 (green), a marker of proximal tubule cells, and for alpha-smooth muscle actin (red), a marker of fibroblasts, and DAPI (blue) to count the total number of cells. At day three, 99% of the cells express SGLT-2, and 1% expressed alpha-smooth muscle actin. At day six 75% and at day ten 46% of the cells were SGLT-2 positive and alpha-smooth muscle actin negative. In addition at day six 25% and at day ten 53% express both SGLT-2 and alpha-smooth muscle actin.

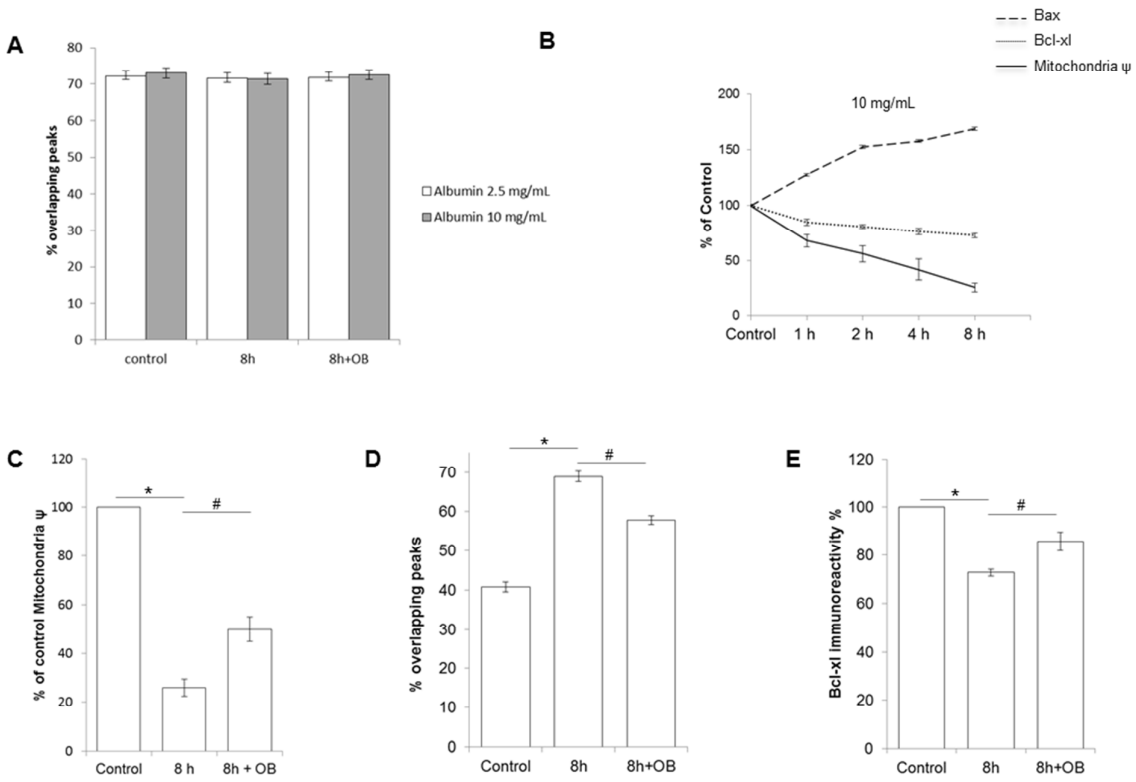
**B.** TGF-beta 1 expression detected by immunoblotting in RPTC cells incubated with or without, 10 mg/ml albumin and in combination with ouabain 5 nM for 8 or 18 hours.

Histograms show quantification of albumin-induced TGF-beta 1 expression data are shown as mean ± SEM, **p<0.001 *p<0.01 Experiments were repeated four times.

**C.** Ouabain does not influence RPTC albumin uptake. RPTC incubated with 2.5 mg/mL albumin and 10µl/mL Alexa 555 albumin with or without 5 nM ouabain for 2 hours. Histograms show quantification of Alexa 555 albumin signal per cell. Data are shown as mean ± SEM. Experiments were repeated six times.




**Supplemental Figure 2**
Quantification of expression of the inflammatory cytokines, IL-1β (top) and IL-6 (bottom), after incubation with LPS 1µg/mL for 0, 1, 2, 4 or 8 hours, and for 8 hours together with 5 nM ouabain. Experiments were repeated three times. Data are shown as mean ± SEM, **p<0.001 *p<0.01




**Supplemental Figure 3**

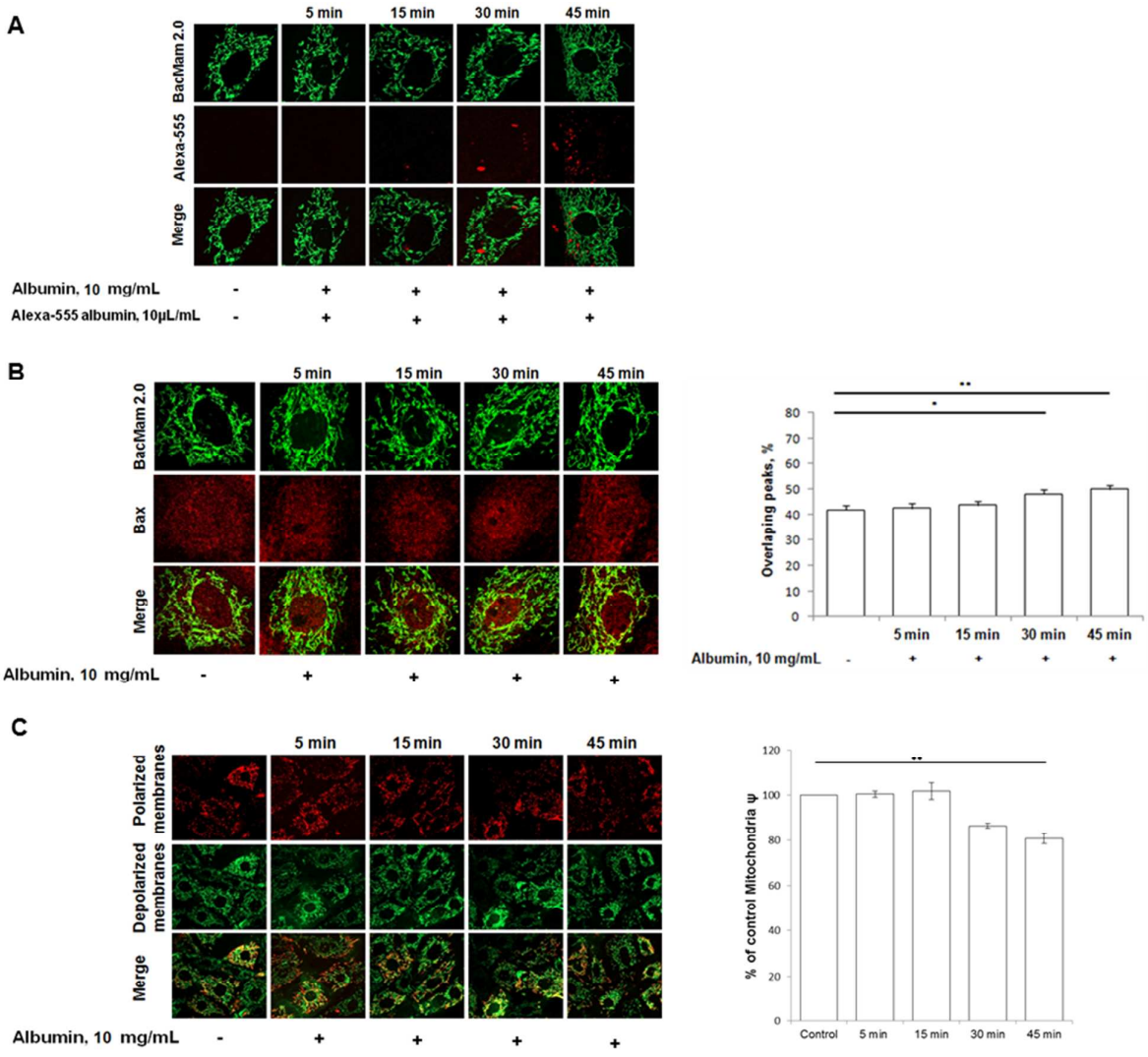
**A.** Quantification of Bcl-xl localization to mitochondria in RPTC incubated with 2.5 mg/ml (open bars) and 10 mg/ml (grey bars) albumin for 8 hours in presence and absence of 5 nM ouabain. All data are shown as % overlapping Bax/mitochondrial peaks, mean ± SEM. Experiments were repeated four times.

**B.** Plot show time dependent change as % of control for mitochondrial membrane potential (solid line), localization of Bax to mitochondria (broken line) and Bcl-xL expression (dotted line) in RPTC exposed to 10 mg/ml albumin.

**C.** Quantification of mitochondrial membrane potential change in RPTC incubated with vehicle (control) or 10 mg/ml albumin in presence and absence of 5nM of ouabain for 8h. *$p<0.001$ #$p<0.01$. Data are shown as % of control, mean ± SEM. Experiments were repeated three times.

**D.** Quantification of Bax localization to mitochondria in RPTC incubated with vehicle (control) or 10 mg/ml albumin in presence and absence of 5nM of ouabain for 8h. *$p<0.001$ #$p<0.01$. Data are shown as % overlapping Bax/mitochondrial peaks, mean ± SEM. Experiments were repeated four times.

**E.** Quantification of Bcl-xL expression in RPTC incubated with vehicle (control) or 10 mg/ml albumin in presence and absence of 5nM of ouabain for 8h. *$p<0.001$ #$p<0.01$. Data are shown as % Bcl-xL immune reactivity when control was set to 100%, mean ± SEM. Experiments were repeated four times.



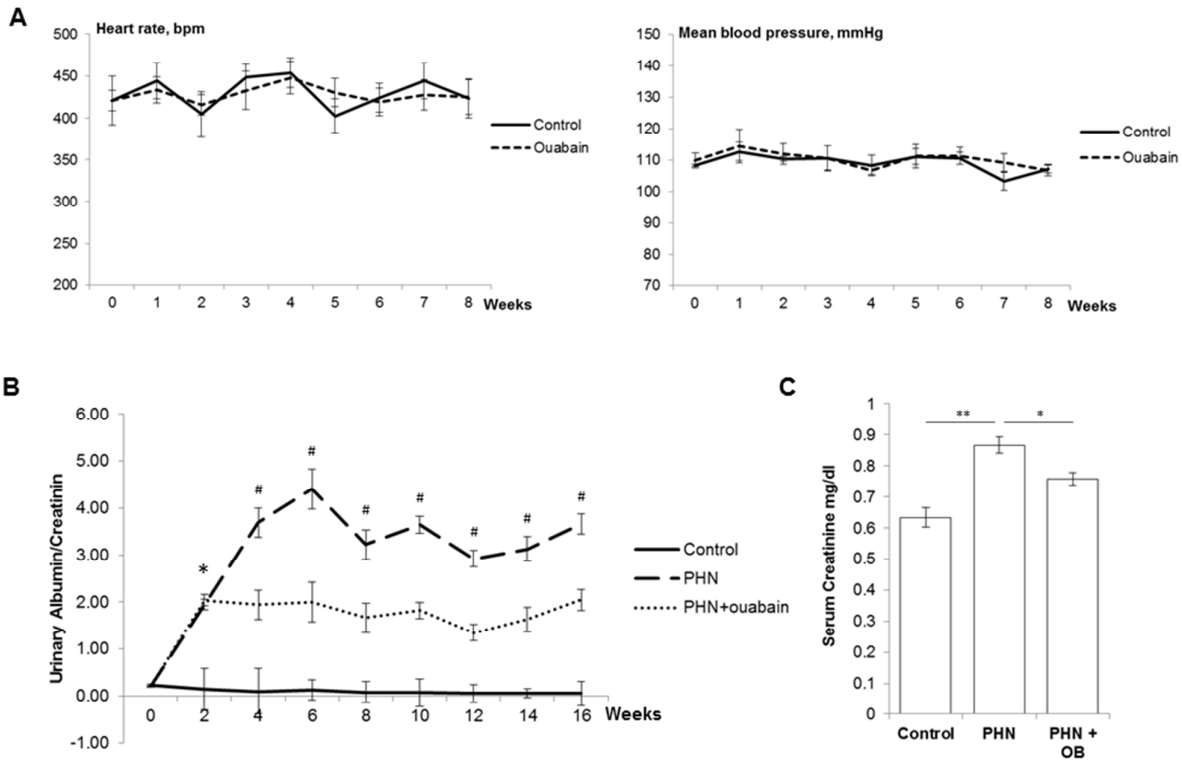

**Supplemental Figure 4**

Effects of short time exposure to Albumin 10mg/mL

**A.** Albumin uptake, RPTC were incubated with 10 mg/mL albumin and trace Alexa 555 coupled albumin for 5, 15, 30 and 45 minutes. All images represent a single section through the focal plane.

**B.** Localization of Bax to mitochondria in RPTC incubated with 10 mg/ml albumin for 5, 15, 30 and 45 minutes. Bax immunofluorescence staining is shown in red and mitochondria in green, by using the mitochondrial marker BacMam 2.0. Histogram show % overlapping Bax/mitochondrial peaks, mean ± SEM. Experiments were repeated three times. *p<0.05 and **p<0.01 vs control

**C.** Mitochondrial membrane potential change in RPTC incubated with albumin 10 mg/ml for 5, 15, 30 and 45 minutes. Mitochondrial membrane potential change was visualized using JC-1 dye; green shows depolarized membrane, red shows polarized membrane. Histogram show % of control, mean ± SEM. Experiments were repeated three times. **p<0.01 vs control



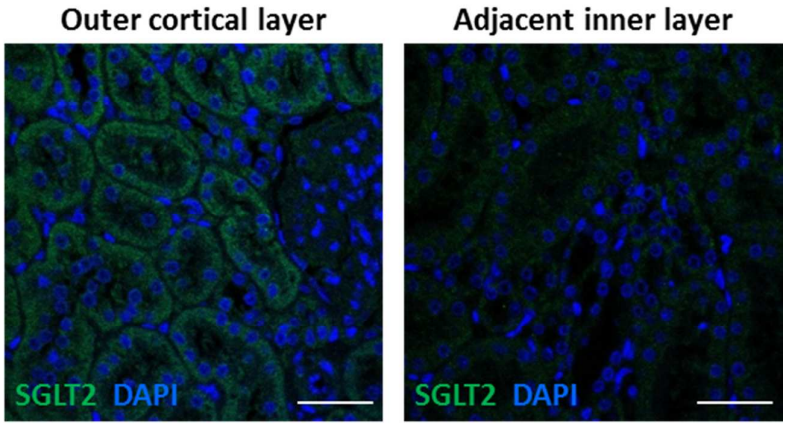

**Supplemental Figure 5**

**A.** Heart rate in mmHg (left), and blood pressure in bpm (right), for vehicle and ouabain (15 µg/kg/day) treated rats during 8 weeks. Measurements were performed on unanesthetized rats using tail cuff. Data are presented as mean ± SEM.

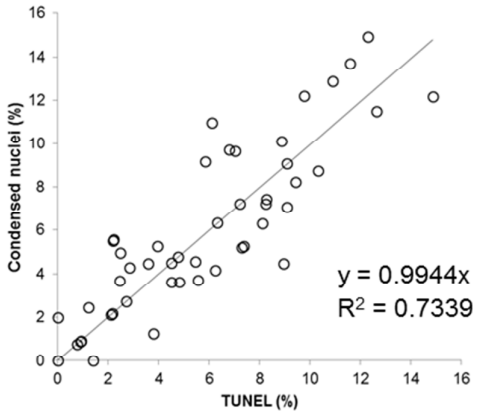
**B.** Urinary albumin/Creatinin levels in control, vehicle and ouabain (15 µg/kg/day) treated PHN rats during 16 weeks. Data are presented as mean ± SEM. *$p<0.05$ for Control vs. PHN and Control vs. PHN + ouabain at 2 weeks. $^{\#}p<0.05$ for Control vs. PHN, Control vs. PHN + ouabain and PHN vs. PHN + ouabain at the corresponding time points.

For all experiments statistical analysis was performed using two-way ANOVA.

**C.** Serum creatinine levels (mg/dl) in control, vehicle and ouabain (15 µg/kg/day) treated PHN rats at experiment end point, 16 weeks. Histograms show the mean ± SEM. The Mann-Whitney U test was used to determine if differences were statistically significant. **$p<0.001$ and *$p<0.01$.

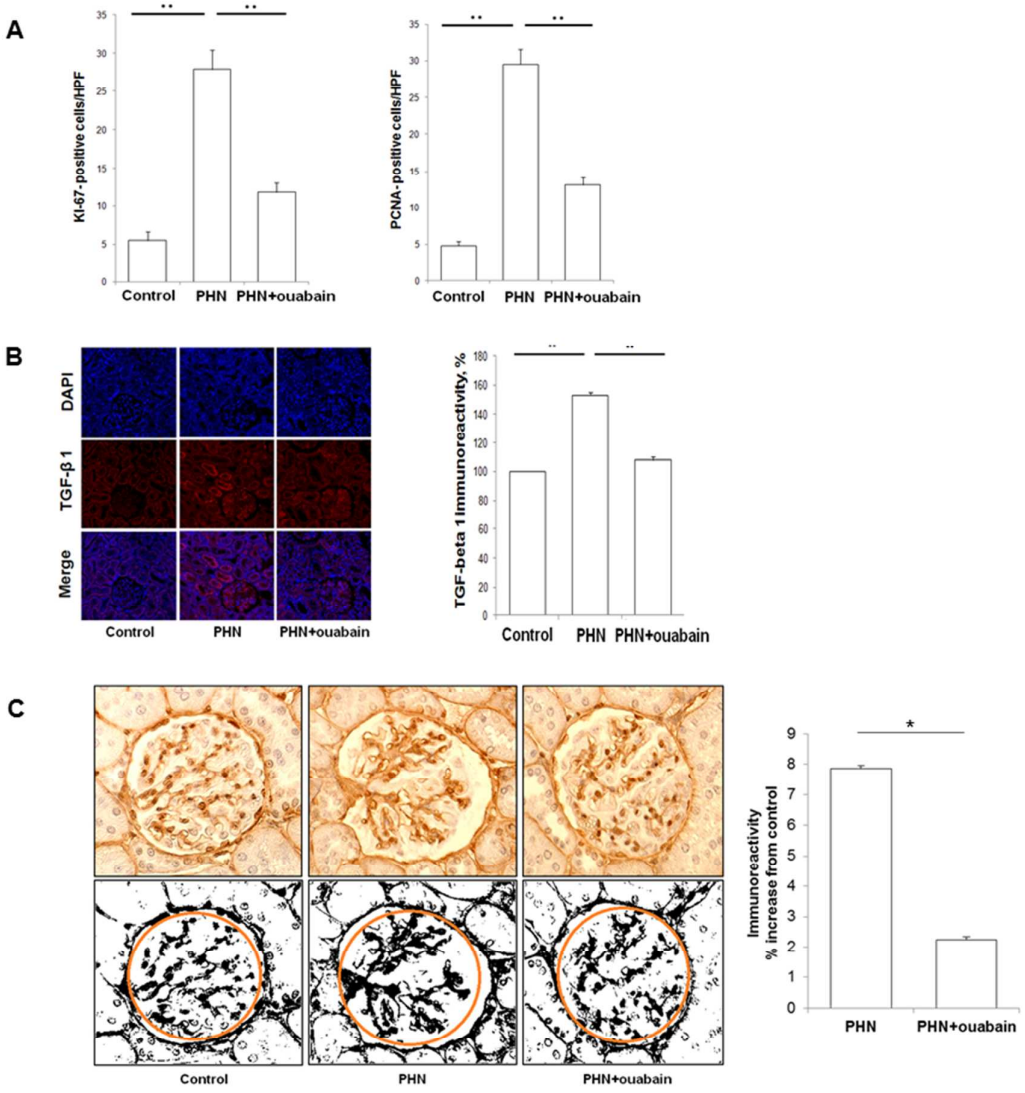



**Supplemental Figure 6**
Analysis of apoptosis by condensed nuclei, expression of Bax and Bcl-xl done in control rats, PHN rats and ouabain-treated PHN as presented in Figure 4 was performed exclusively in the outer cortical layer. The majority of tubules in the cortical area (left image) are proximal tubules as verified by immunostaining for SGLT-2 (green) used as a specific marker of proximal tubule cells. In comparison an area closer to the center of the kidney slice have few or no proximal tubule as shown by the absence of SGLT-2 staining (right image). DAPI staining of nuclei is shown in blue. Scale bars = 40µm




| Group | % Condensed nuclei | % TUNEL |
|---|---|---|
| Control | 2.29 | 2.00 |
| 2.5 mg/ml albumin | 4.53 | 4.67 |
| 5.0 mg/ml albumin | 6.69 | 6.70 |
| 10.0 mg/ml albumin | 10.42 | 9.78 |

**Supplemental figure 7**
Comparison of condensed nuclei and TUNEL methods for assessment of apoptosis. RPTC on the same coverslip was analyzed using both condensed nuclei and TUNEL. Studies were performed on control RPTC and RPTC exposed to 2.5 – 10 mg/ml albumin for 8 h. The plot (left) shows the % apoptosis expressed by condensed nuclei and by TUNEL for each coverslip analyzed. There is a linear correlation between the two methods, y=0.9944x and $R^2 = 0.7339$. The table (right) show the average % apoptosis for each treatment assessed with condensed nuclei and with TUNEL.

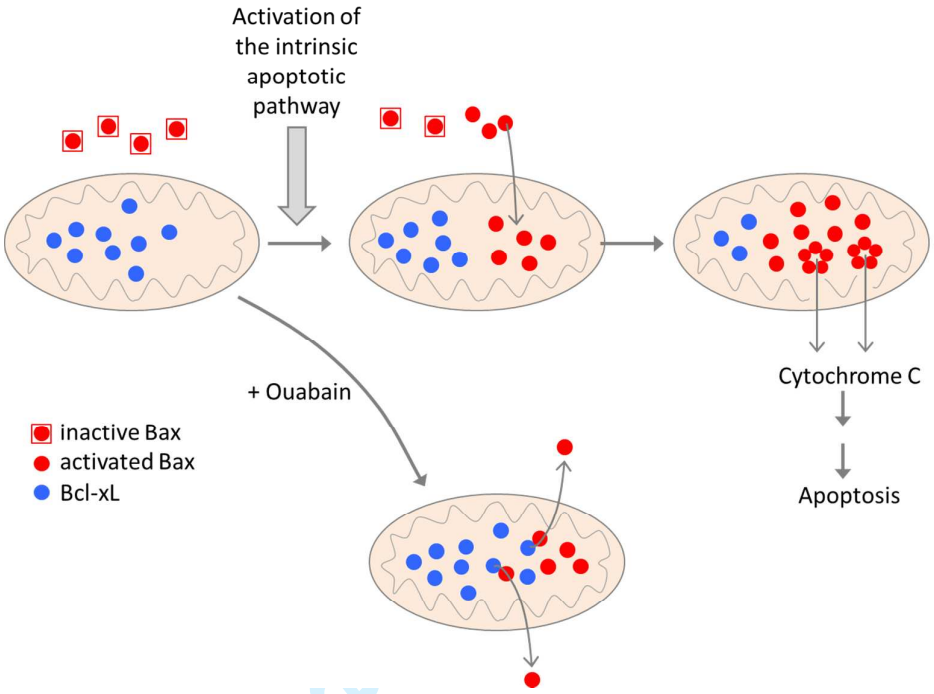



**Supplemental Figure 8**

**A.** Quantification of proliferative markers Ki-67 and PCNA from immunohistochemistry in kidney sections from control, PHN and ouabain-treated PHN rats. Histograms show mean ± SEM. Mann-Whitney U test was used to determine significant differences. **p<0.001

**B.** Representative expression of pro-fibrotic factor TGF-β1 (red) and nuclei (DAPI, blue) in controls, vehicle-treated PHN and ouabain-treated PHN rats. Semi-quantitative evaluation of the TGF-β1 signal was performed in section from controls, vehicle-treated PHN and ouabain-treated PHN animals, three areas corresponding to 75% of the cortex were analyzed. Immunoreactivity for TGF-β1 is expressed as % deviation from control. Histograms show mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. **p<0.001

**C.** Representative images of collage IV immunostaining in glomeruli of control, vehicle-treated and ouabain-treated PHN rats. The images are representative. Level of collagen IV in control animals was set to base line and data are expressed as % increase from control. Histograms show mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test. *p<0.05




**Supplemental Figure 9**
The intrinsic mitochondrial apoptotic program is controlled by the Bcl-2 family of apoptotic and anti-apoptotic proteins. Bcl-xl is a prominent member of the anti-apoptotic Bcl-2 proteins and Bax a prominent member of the apoptotic Bcl-2 proteins. Under control conditions Bax is mainly located in the cytosol, and Bcl-xl on the mitochondria. Apoptosis is initiated by the restructuring of Bax and its translocation to the mitochondria. Bcl-xl acts by re-translocating Bax to the cytosol. Accumulation of Bax on the mitochondria promotes its homo-oligomerisation as well as its hetero-oligomerisation with other apoptotic proteins (Chen et al. 2015, Große et al. 2016). This results in permeabilisation of the mitochondrial membrane and leakage of cytochrome c, which marks the point of no return in the apoptotic process. During the course of the apoptotic process the abundance of Bax increases and the abundance of Bcl-xL decreases.
Ouabain acts by protecting from the accumulation of Bax on the mitochondrial membrane as well as from up-regulation of Bax and down-regulation of Bcl-xL abundance.

| | Control | PHN | PHN + OB |
|---|---|---|---|
| **Characterization of glomerular tubular junction** (% of evaluated glomeruli) | | | |
| Normal | 96.02 | 67.08 | 80.49 |
| Atrophic and a-tubular | 3.98 | 32.91 | 19.51 |
| **Serum Creatinine** | | | |
| mg/dl | 0.634 ± 0.09 | 0.866 ± 0.08 | 0.758 ± 0.05 |
| Increase from control (%) | 0 | 36.68 | 19.69 |

**Supplemental Table 1.**

Correlation between atrophic/loss of glomerular tubular junction and Serum Creatinine in Control and PHN rats.