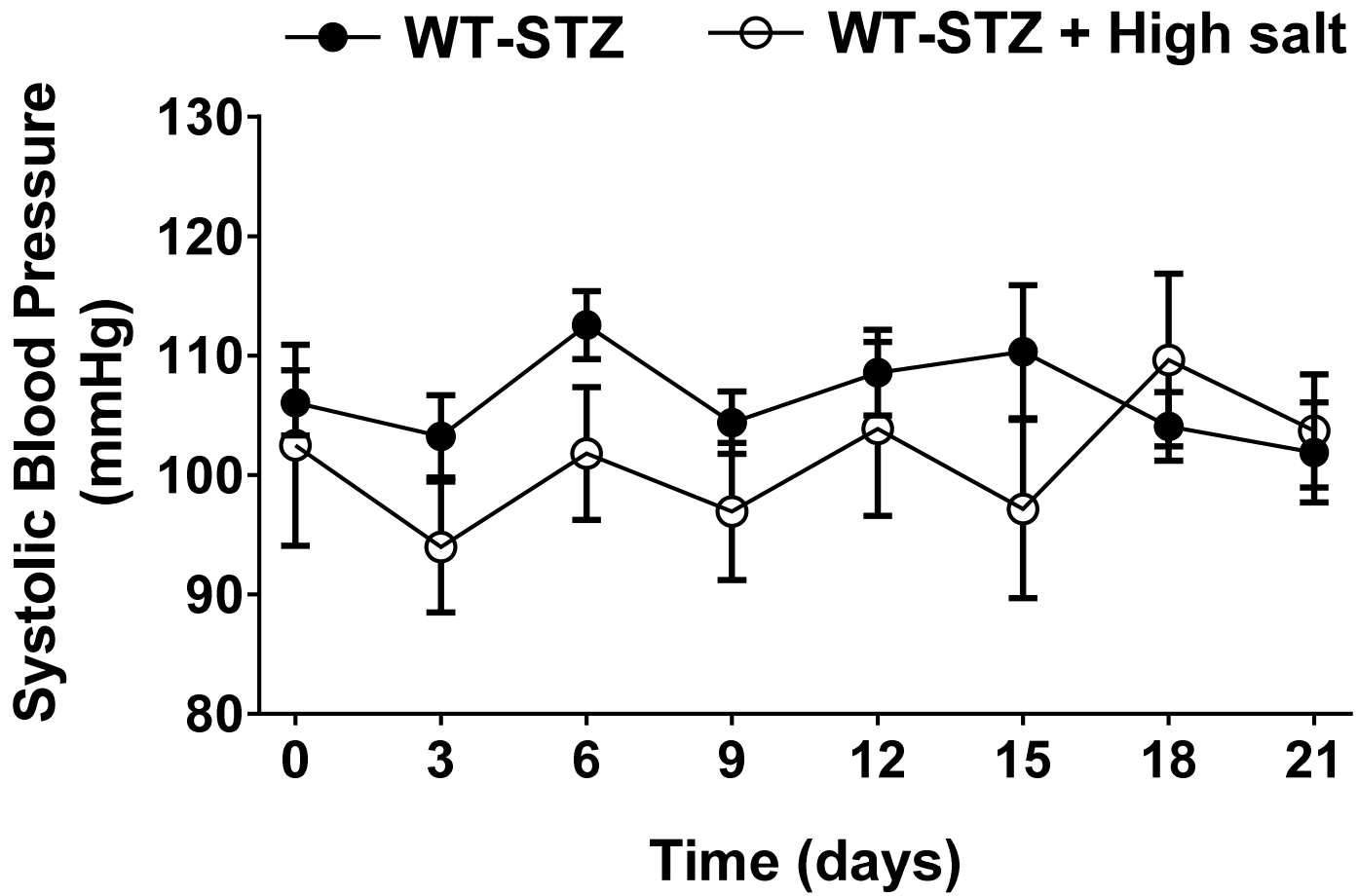
**Manuscript:** JASN-2018-03-0323

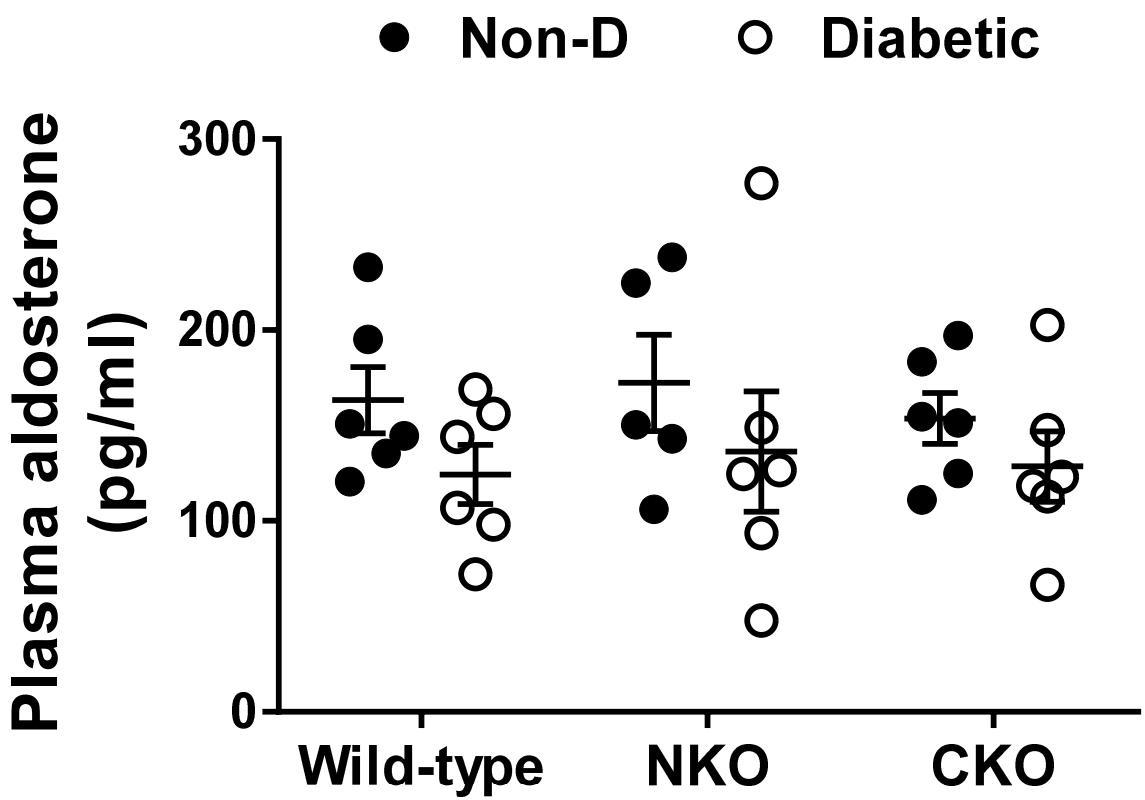
**Title:** The absence of the ACE N-domain decreases renal inflammation and facilitates sodium excretion during diabetic kidney disease

**Supplemental Material – Table of Contents**

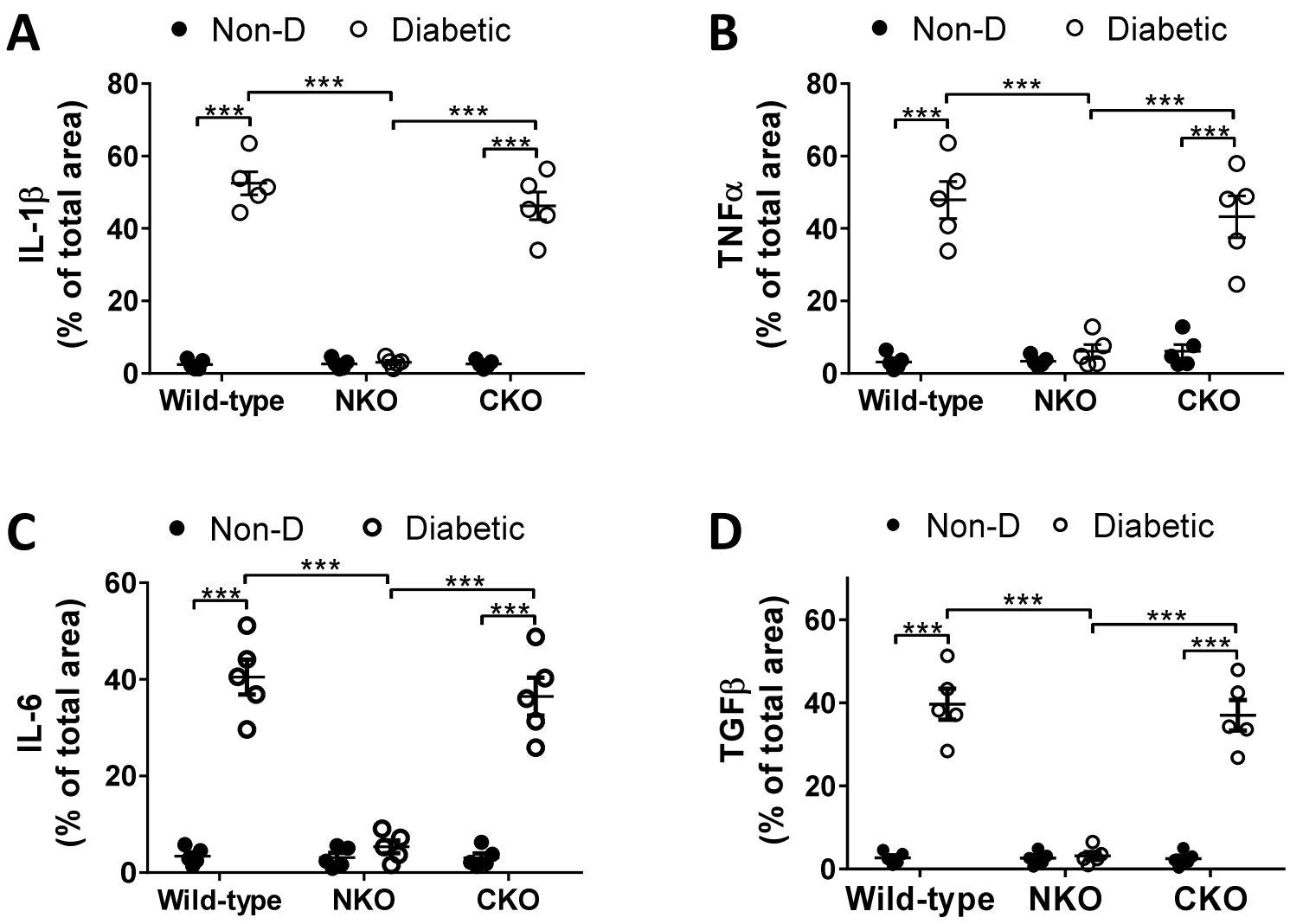

- Supplemental Figure 1
- Supplemental Figure 2
- Supplemental Figure 3
- Supplemental Figure 4
- Supplemental Figure 5
- Supplemental Figure 6
- Supplemental Figure 7
- Supplemental Figure 8
- Supplemental Figure 9
- Supplemental Figure 10
- Supplemental Table 1
- Supplemental Table 2



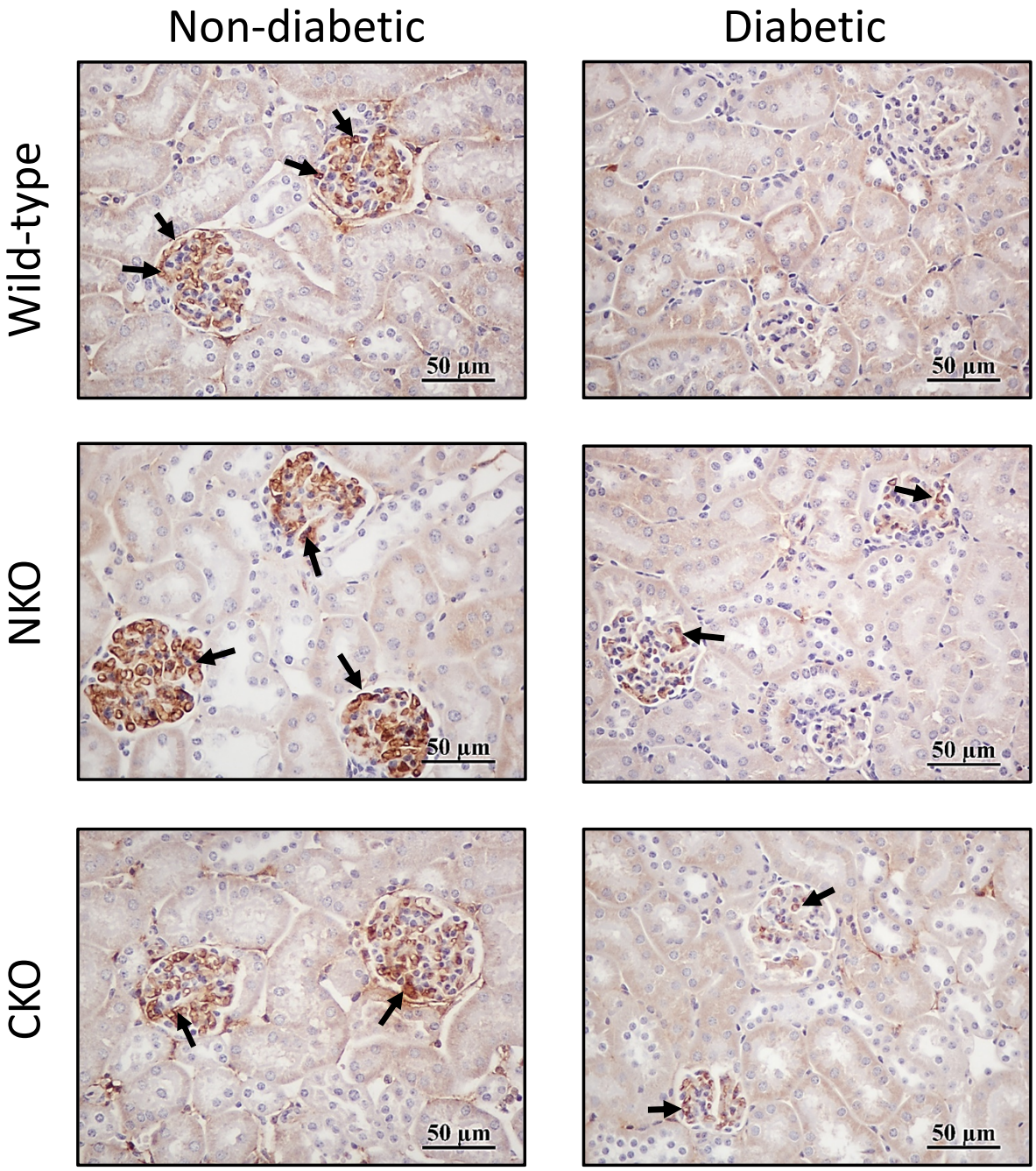

**Supplemental Figure 1** – Systolic blood pressure was measured in diabetic wild-type exposed to a high salt diet (4% w/w NaCl in the food; WT-STZ + High salt) for 3 weeks. Diabetic WT mice receiving normal diet (0.4% w/w NaCl in the food; WT-STZ) were used as controls. The treatment started 5 months after the onset of diabetes. Blood pressure was assessed by tail-cuff plethysmography. Data represent mean ± SEM.

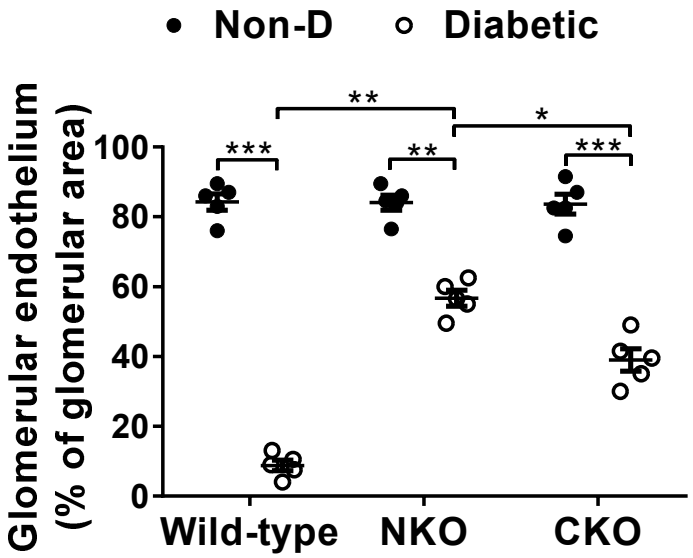



**Supplemental Figure 2** – Plasma aldosterone was measured in diabetic wild-type, NKO and CKO mice after 6 months of diabetes by a commercial ELISA. Mice not treated with streptozotocin were used as controls (Non-D). Data are represented as individual values for each mouse (dots). Horizontal bars represent the mean ± SEM.

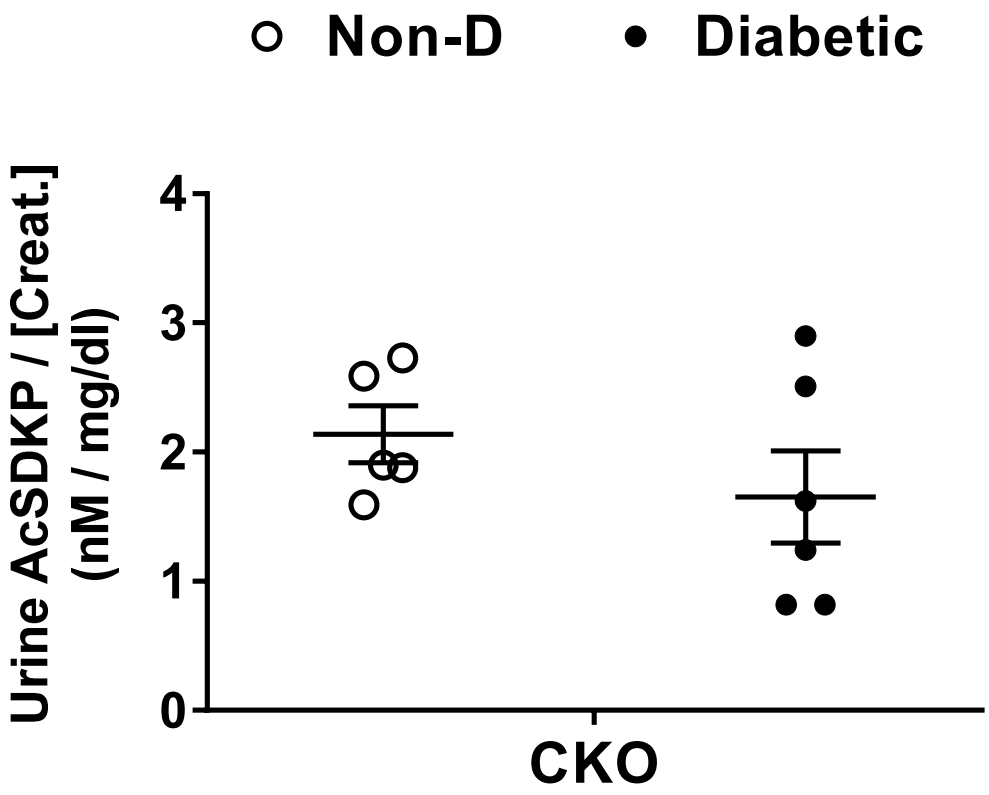



**Supplemental Figure 3** – Quantification of immunohistochemical analysis of (A) IL-1β, (B) TNFα, (C) IL-6, and (D) TGFβ was assessed in each animal on 20 consecutive microscopic fields at x40 magnification. n=5 per group, ***$P$<0.001. Data are expressed as percentage of positive staining per total area and represented as individual values for each mouse (dots). Horizontal bars represent the mean ± SEM.

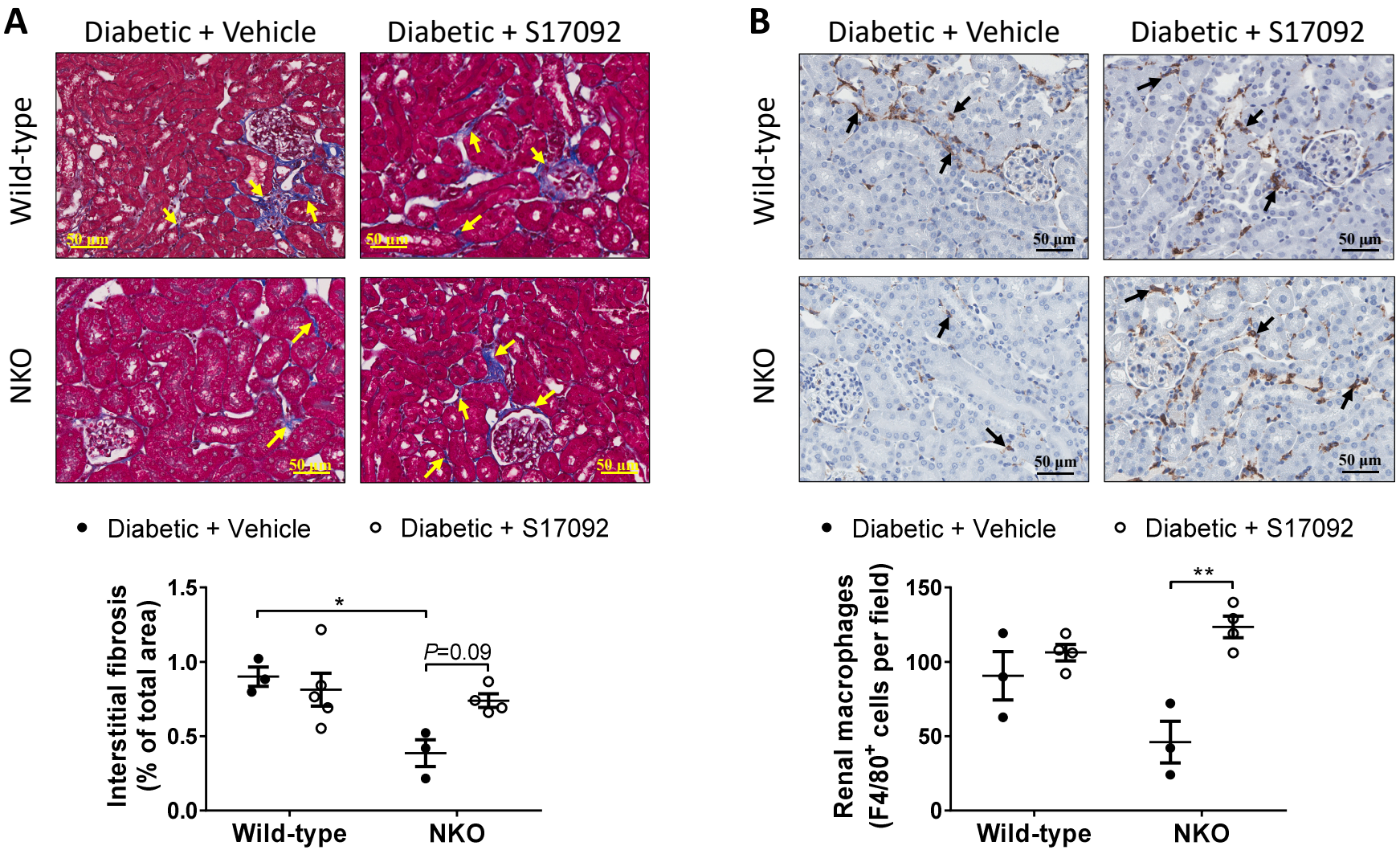


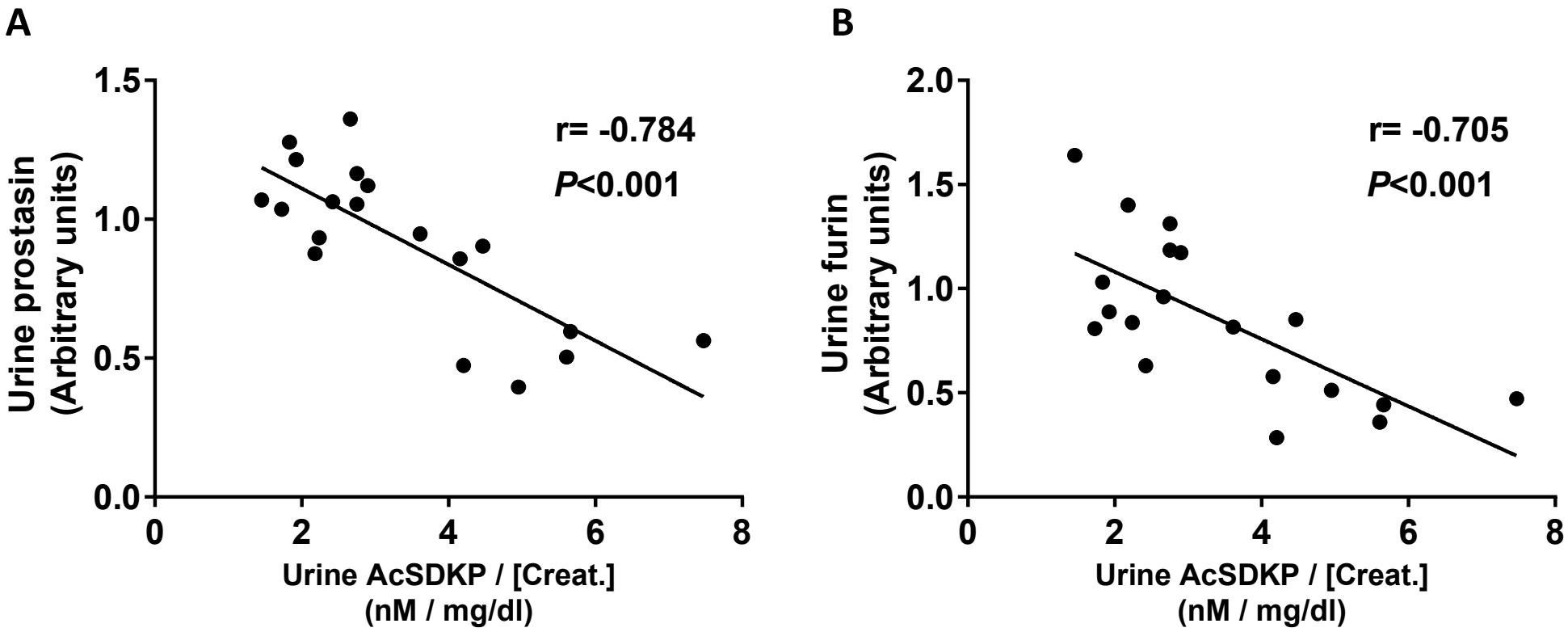

**Supplemental Figure 4** – Glomerular endothelial cells were measured using CD31 immunostaining and expressed as percentage of positive staining per glomerular area. Twenty fields per sample were analyzed. Original magnification x40. n=5 per group. **P*<0.05, ***P*<0.01, ****P*<0.001. Data are represented as individual values for each mouse (dots). Horizontal bars represent the mean ± SEM.



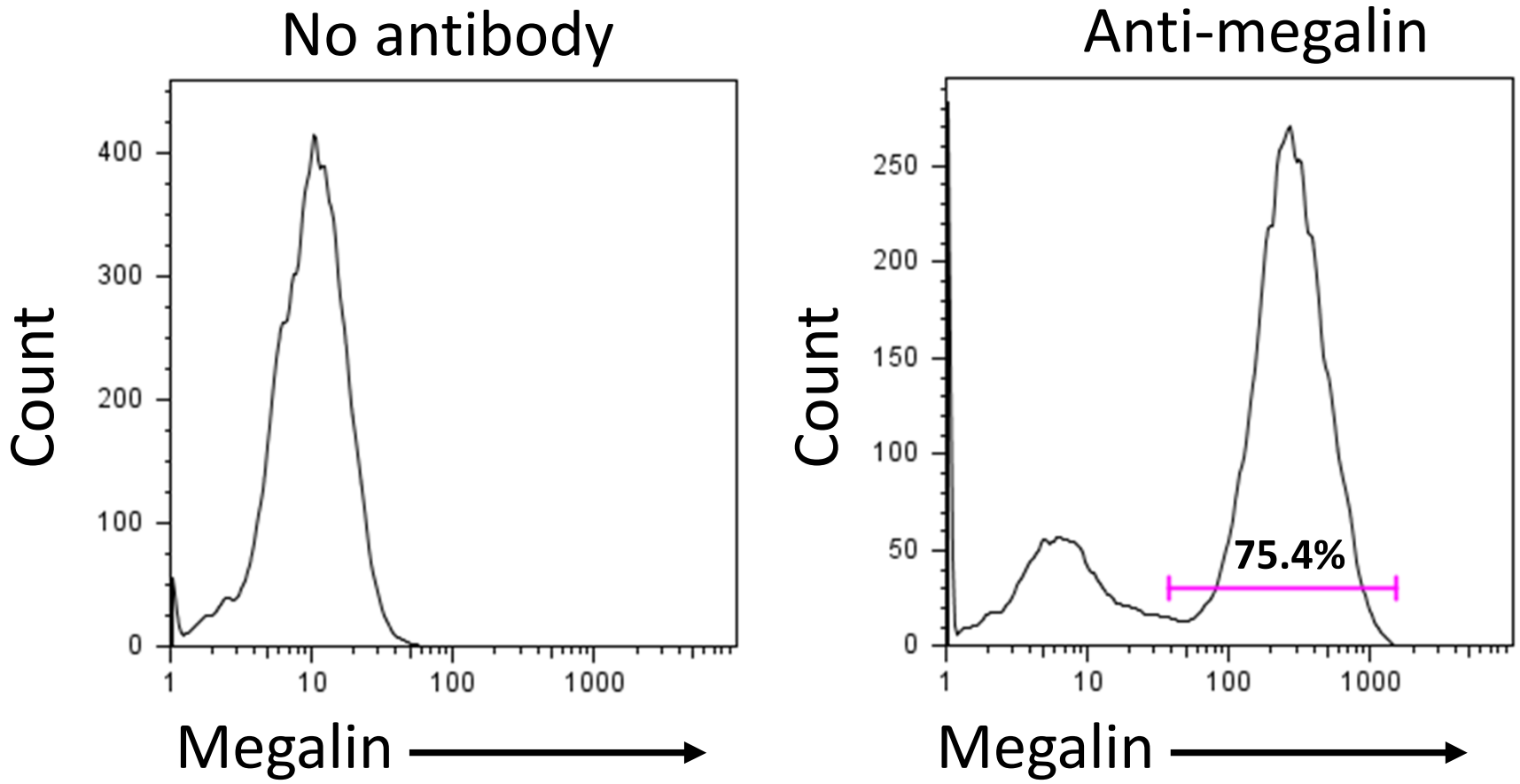

**Supplemental Figure 5** – AcSDKP was assessed by ELISA in urine samples of CKO diabetic and non-diabetic (Non-D) controls. n=5-6 per group. Data are represented as individual values for each mouse (dots). Horizontal bars represent the mean ± SEM.



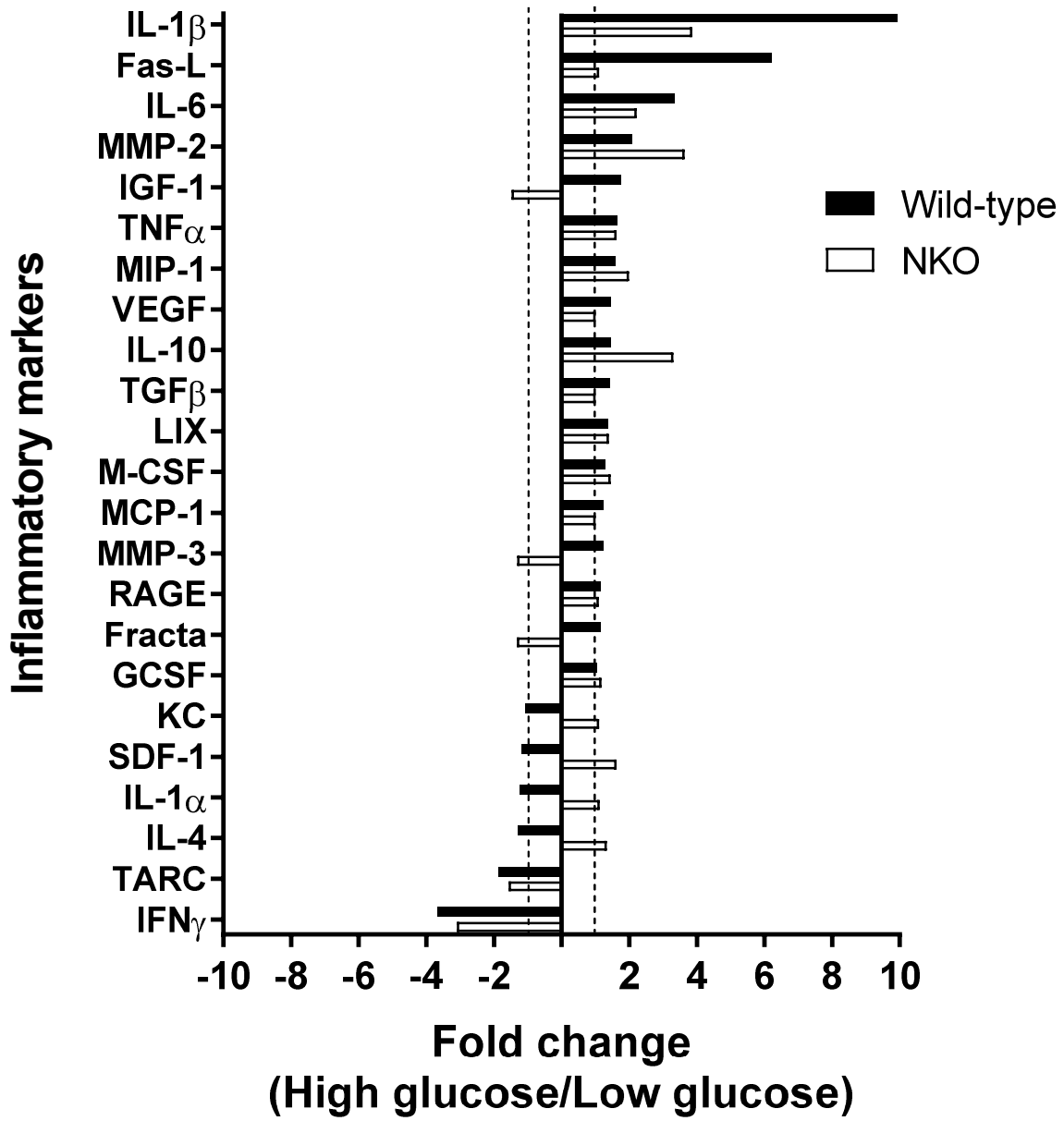

**Supplemental Figure 6 – Inhibition of AcSDKP synthesis with S17092 increases interstitial fibrosis and macrophage infiltration in diabetic NKO mice.** (A) Interstitial fibrosis in renal cortex was evaluated using Masson's trichrome staining and expressed as the percentage of fibrotic area of total cortical area from a whole-slide image. (B) Renal macrophage infiltration was assessed using F4/80 immunostaining and expressed as F4/80 positive cells per field. Fifteen fields per sample were analyzed. Original magnification x40. n=3-5 per group. *$P$<0.05, **$P$<0.01. Data are represented as individual values for each mouse (dots). Horizontal bars represent the mean ± SEM.



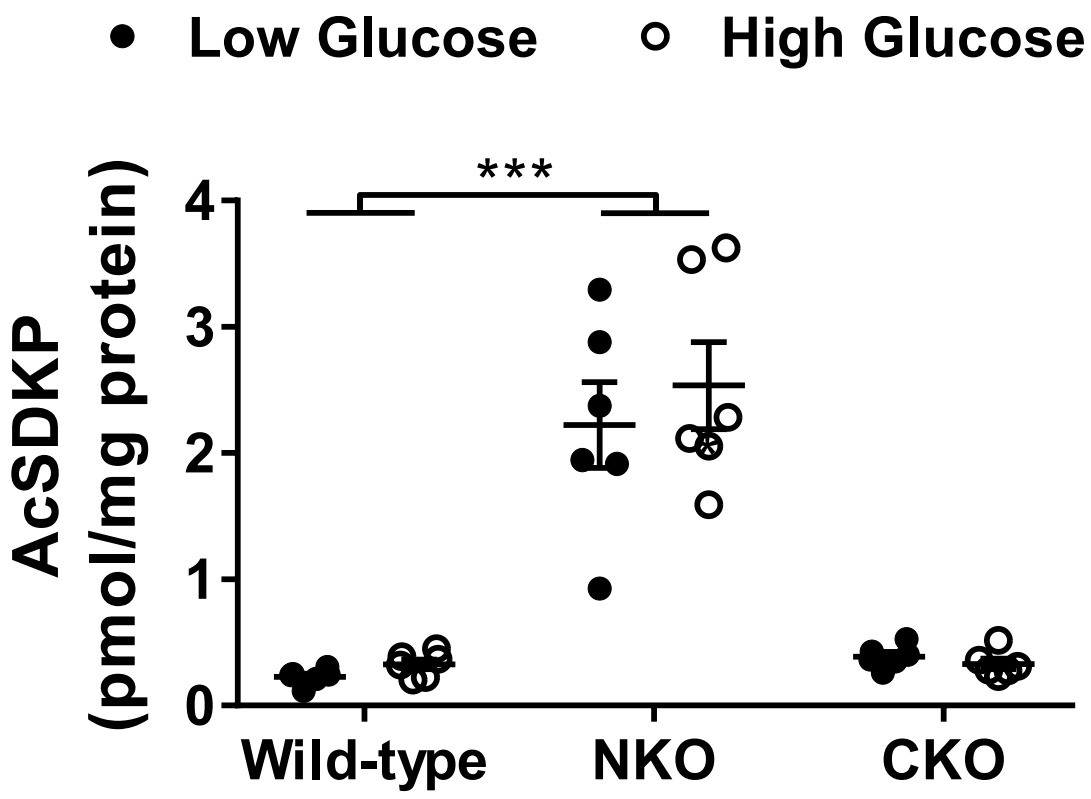

**Supplemental Figure 7** – (A) Urinary prostasin from Figure 8E and (B) urinary furin from Figure 8F were plotted against urinary AcSDKP from Figure 7A combining wild-type and NKO diabetic mice receiving either vehicle or S17092. Plots indicate a negative correlation between serine proteases and AcSDKP in the urine.




**Supplemental Figure 8** – Flow cytometry analysis revealed 75% of megalin-positive cells (proximal tubule marker) in a primary culture of wild-type renal tubular cells. Cells were detached with Accutase (Innovative Cell Technologies, San Diego, CA) and stained with an antibody against megalin.




**Supplemental Figure 9** – Different inflammatory markers were measured in the media of a primary culture of renal tubular epithelial cells from wild-type and NKO mice using a commercial cytokine array. After 7 days of culture, the glucose concentration of the culture media was increased from 5 (Low) to 30 mM (High) for 24 hours. Bars represent the abundance ratio (high glucose/low glucose) for each marker. Bars located to the right indicate an increase after high glucose. Bars located to the left indicate a decrease after high glucose. Bars located within the dotted lines (-1 to 1) indicate essentially no change after high glucose.




**Supplemental Figure 10** – Intracellular levels of AcSDKP were evaluated in tubular cells derived from WT, NKO and CKO mice using a commercial ELISA kit. After 7 days of culture, the glucose concentration of the culture media was increased from 5 (Low glucose) to 30 mM (High glucose) for 24 hours. $^{***}P<0.001$. Each dot represents an individual experiment. Horizontal bars represent the mean ± SEM.

**Supplemental Table 1** - Immunoblot protocol and antibody details.

| Antibody Target | ~kDa | µg protein /lane | Primary Ab supplier | Ab host | Dilution | Incubation time for Primary Ab | Secondary Ab supplier | Host and target | Dilution | Incubation time for Secondary Ab |
|---|---|---|---|---|---|---|---|---|---|---|
| ACE | 195 | 40 | Santa Cruz | Go | 1:1,000 | O/N | Li-Cor | DAG | 1:10,000 | 1 hr |
| β-actin | 42 | 40 | Sigma | Mo | 1:5,000 | 1 hr | Li-Cor | GAM | 1:10,000 | 1 hr |
| ENaC-α | 90<br>30 | 40 | Loffing (Zurich) | Rb | 1:5,000 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| ENaC-β | 90 | 40 | Loffing (Zurich) | Rb | 1:1,500 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| ENaC-γ | 75 | 50 | Loffing (Zurich) | Rb | 1:1,000 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| Furin | 60 | 10 (creatinine) | Abcam | Rb | 1:1,000 | O/N | Li-Cor | GAR | 1:5,000 | 1 hr |
| NCC | 140 | 40 | McDonough (USC) | Rb | 1:5,000 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| NCC pS71 | 140 | 40 | Loffing (Zurich) | Rb | 1:5,000 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| NHE3 | 100 | 40 | McDonough (USC) | Rb | 1:2,000 | O/N | Li-Cor | GAR | 1:5,000 | 1 hr |
| NKCC2 | 150 | 20 | C. Lytle (UCR) | Mo | 1:6,000 | O/N | Li-Cor | GAM | 1:10,000 | 1 hr |
| NKCC2 pT96T101 | 150 | 20 | Forbush (Yale) | Rb | 1:2,000 | O/N | Li-Cor | GAR | 1:10,000 | 1 hr |
| Prostasin | 40 | 10 (creatinine) | R&D | Mo | 1:1,000 | O/N | Li-Cor | GAM | 1:5,000 | 1 hr |
| Renin | 38 | 40 | Santa Cruz | Mo | 1:1,000 | O/N | Li-Cor | GAM | 1:10,000 | 1 hr |

Ab = antibody, Mo = mouse, Go = goat, Rb = rabbit, O/N = overnight, GAR = goat anti-rabbit, GAM = goat anti-mouse, DAG = donkey anti-goat.

**Supplemental Table 2** – Immunohistochemistry protocol and antibody details.

| Antibody Target | Primary Ab supplier | Ab host | Dilution | Incubation time for Primary Ab |
|---|---|---|---|---|
| CD31 | Abcam | Rat | 1:1000 | O/N |
| F4/80 | Bio-Rad | Ra | 1:50 | O/N |
| IL-1β | Abcam | Rb | 1:1,000 | O/N |
| IL-6 | Santa Cruz | Go | 1:1000 | O/N |
| TNFα | R&D Systems | Go | 1:5000 | O/N |
| TGFβ | Abcam | Rb | 1:1000 | O/N |

Ab = antibody, Go = goat, Ra = rat, Rb = rabbit, O/N = overnight.