Title page

Supplementary information

Sildenafil prevents podocyte injury via PPAR-γ-mediated TRPC6 inhibition

Ramon Sonneveld[1], Joost G. Hoenderop[2], Andrea M. Isidori[3], Carole Henique[4,5], Henry B. Dijkman[6], Jo H. Berden[1], Pierre-Louis Tharaux[4,5,7], Johan van der Vlag[1], Tom Nijenhuis[1]

[1]Department of Nephrology, Radboud university medical center, PO Box 9101 6500 HB, Nijmegen, the Netherlands

[2]Department of Physiology, Radboud university medical center, PO Box 9101 6500 HB, Nijmegen, the Netherlands

[3]Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy

[4]Paris Cardiovascular Centre (PARCC), Institut National de la Santé et de la Recherche Médicale (INSERM)

[5]Université Paris Descartes; Sorbonne Paris Cité, Paris, France

[6]Department of Pathology, Radboud university medical center, PO Box 9101 6500 HB, Nijmegen, the Netherlands

[7]Hôpital Européen Georges Pompidou, Service de Néphrologie, Paris, France

Running title: Sildenafil prevents podocyte injury

Correspondence to: Tom Nijenhuis, M.D., Ph.D.
Radboud university medical center
Department of Nephrology (464)
PO Box 9101
6500 HB, Nijmegen, the Netherlands
T: +31 24 3614761
F: +31 24 3540022
E: Tom.Nijenhuis@Radboudumc.nl

Complete methods

Animal studies

Mice, 12-14 weeks of age, with a podocyte-specific deletion of PPARy were created using the NPHS2-Cre recombinase (podocin-Cre) mouse [16]. Podocyte-specific disruption of PPARy alleles was achieved by crossing podocin-Cre positive mice with the B6.129S6-Ppargtm1.1Mgn/Mmmh strain obtained from the Mutant Mouse Regional Resource Center at the University of Missouri (stock number 012035-MU) on a C57BL6/J background. For the induction of adriamycin nephropathy, 9-11-week-old mice were treated with a single intravenous injection of adriamycin (15 mg/kg body wt diluted in 0.9% saline). Control mice received saline solution alone. Subsequently, mice were treated for 3 weeks with vehicle or sildenafil (1.6mg/kg bodyweight). Wild-type littermates were used as age-matched controls. Hereafter, urine and kidneys were collected.

Hyperglycemia was induced in 12 week old CD1 mice by a single injection of STZ (150mg/kg bodyweight; Sigma-Aldrich, St. Louis, Missouri, USA) as described before [35]. After the development of hyperglycemia, mice were treated 3 weeks with vehicle or sildenafil (1.6 mg/kg bodyweight). A drop of tail blood was used to monitor glucose concentrations by MediSense Precision Plus kit (Abbott Diagnostics, Melbourne, Victoria, Australia).

Wistar rats (Charles River Laboratories, Wilmington, USA), 8 – 10 weeks of age were intravenously injected with vehicle or 5mg/kg bodyweight adriamycin to induce adriamycin nephropathy. Treatment consisted of daily oral supplementation of 5mg/kg bodyweight sildenafil or 12mg/kg bodyweight pioglitazone for 6 weeks, or every other day intra-peritoneal injections with 2.5mg/kg bodyweight GW9662 for 3 weeks. After treatment rats were housed in metabolic cages for 24 hours, hereafter urine and kidneys were collected.

All animal procedures were approved by the Animal Ethics Committee of the Radboud University, the INSERM local Ethic Review Board at Paris Descartes University and by the Sapienza University's Animal Research Ethics Committee (165/2016-PR), in accordance with the guidelines of the Dutch, French and Italian Council for Animal Care and the European Communities Council Directive (86/609/EEC).

Urinary parameters

Urinary IgG (Antibodies-online, Aachen, Germany) levels were measured using ELISA according to the manufactures protocol. Creatinine concentrations were determined by the

Architect C16000 (Abbott Diagnostics, Chicago, USA). Urinary albumin was measured by radial immunodiffusion.

Immunohistochemistry

Frozen kidney cortex was cut into 2 micrometer sections and stained for TRPC6 (Rat: Abcam, Cambridge, UK, Mice: Alomone, Jerusalem, Israel), nephrin (R&D, Minneapolis, USA), synaptopodin (Progen Biotechnik GmbH, Heidelberg, Germany), desmin and PDE5A (Santa Cruz, Santa Cruz, USA). Subsequently the mean fluorescent intensity in 30-40 glomeruli per animal was determined, as described previously [10, 11] .

Cell culture studies

Conditionally immortalized mouse podocytes (MPC-5) cells were cultured as described previously [36]. Differentiated cells were injured using adriamycin (0.25µg/mL) or PAN (100µg/mL), in combination with transfection with scrambled, PPAR-γ or PKG-1 siRNA using Dharmafect (GE lifesciences, Marlborough, USA) and treated with vehicle or various concentrations of sildenafil, pioglitazone, rosiglitazone, 8-Br-cGMP, KT5823 or GW9662 (Sigma-Aldrich, St. Louis, Missouri, USA). Part of the experiments were performed in TRPC6 KD cells as described previously [11].

Intracellular Ca^{2+} was visualized using fura-2 fluorescent Ca^{2+} as previously described [11]. MPC-5 cells were fixed and stained for TRPC6. Then, TRPC6 expression and cellular localization were studied.

TRPC6 promoter activity luciferase reporter assay

The 5′-promoter region of the mouse TRPC6 gene (−1500/+32; +1 designates the transcription start site, NM_013838.2) was obtained by amplification of genomic DNA using primers 5′-GACGCTCGAGTGTTGCTTCTGCAGCCCGAGTG-3′ and 5′-GATCAAGCTTAGCCGCGAAAGGAACCTTGACC-3′. The PCR product was cloned into the pGL3-basic luciferase reporter vector, and the cloned promoter sequence was verified by sequence analysis. The pRL-CMV vector encoding Renilla luciferase under the control of a CMV promoter was used as control for transfection efficiency (Promega Corp., Fitchburg, USA).

The TRPC6 promoter activity assay was performed as described previously [10]. Briefly, Opossum Kidney (OK) cells were transfected with the TRPC6 promoter construct, PKG-1,

PPAR-γ or empty vector, after which cells were treated with vehicle, sildenafil, pioglitazone, rosiglitazone, 8-Br-cGMP, KT5823 or GW9662. After 24 hours, cells were harvested and luciferase activity was measured according to the manufacturers protocol (Promega Corp., Fitchburg, USA).

Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} was visualized using fura-2 fluorescent Ca^{2+} as previously described [11]. Briefly, mouse podocytes with a tetracycline-inducible stably transfected TRPC6 siRNA or scrambled construct were differentiated in 96-well plates for 2 weeks. We induced silencing of TRPC6 4 days prior to the experiments using tetracycline (Sigma-Aldrich). Subsequently cells were pretreated with vehicle, sildenafil, pioglitazone, 8-Br-cGMP, KT5823 and/or GW9662 for 24 hours. Thereafter, cells were loaded with fura-2 and put in a Ca^{2+}-containing buffer that did not contain the aforementioned pretreatment compounds. Subsequently, 100 µmol/L OAG (Sigma-Aldrich, St. Louis, MO) was added to activateTRPC6. Fluorescent intensity was measured using the Pathway 855 microscope (BD Biosciences, San Jose, CA).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the manufacturers protocol. Briefly, OK cells were transfected using lipofectamin with TRPC6 promoter construct and untransfected podocytes were treated with 10µM pioglitazone for 4h, after which ChIP was performed (Magna ChIP A Chromatin Immunoprecipitation kit, Upstate Millipore) using an antibody directed against PPAR-γ (Santa Cruz, Santa Cruz, USA), positive control (H3) and the appropriate isotype control (IgG). The enrichment of the TRPC6 promoter compared to the isotype control was evaluated using real-time PCR with specific primers corresponding to the TRPC6 promoter region (5'-CTCAACGCATGTCCCCATAC-3' and 5'-GTAACACCAAGGGAGGGC-3').

Real-time quantitative PCR analysis

RNA was isolated from kidney cortex or cultured MPC-5 cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) and was processed for cDNA synthesis by reverse transcription (Transcriptor Kit; Roche Diagnostics, Mannheim, Germany). Real-time PCR was performed using SYBR Green SuperMix (Roche Diagnostics) on a CFX96 C1000 Touch real-time PCR detection system (Bio-Rad Laboratories, Hercules, USA). Glyceraldehyde-3-phosphate dehydrogenase was used as housekeeping gene.

Statistical analyses

All results are depicted as mean ± SEM. Student t-test or ANOVA was used to test for significance using SPSS software (IBM, New York, USA) followed by a Bonferroni *post-hoc* test. P values less than 0.05 were considered significant.

Supplementary Figure legends

Supplementary Figure 1: Representative images of TRPC6 expression in cultured podocytes

Representative immunofluorescent images of TRPC6 expression in adriamycin-injured or uninjured cultured podocytes treated with vehicle, sildenafil or pioglitazone, or inhibition of PKG-1 or PPAR-γ in uninjured podocytes. The arrows indicate enhanced TRPC6 membrane localization.

Supplementary Figure 2: TRPC channel mRNA expression by cultured podocytes treated with sildenafil or pioglitazone

Cultured podocytes were treated with vehicle, sildenafil, pioglitazone or rosiglitazone, mRNA was isolated and expressional changes were determined. We detected expression of TRPC1 (A), TRPC2 (B), TRPC3 (C), TRPC5 (D) and TRPC7 (E) by cultured podocytes. Statistical significance was determined using ANOVA followed by Bonferroni *post hoc* test. * $p < 0.05$ vs vehicle-treated cells.

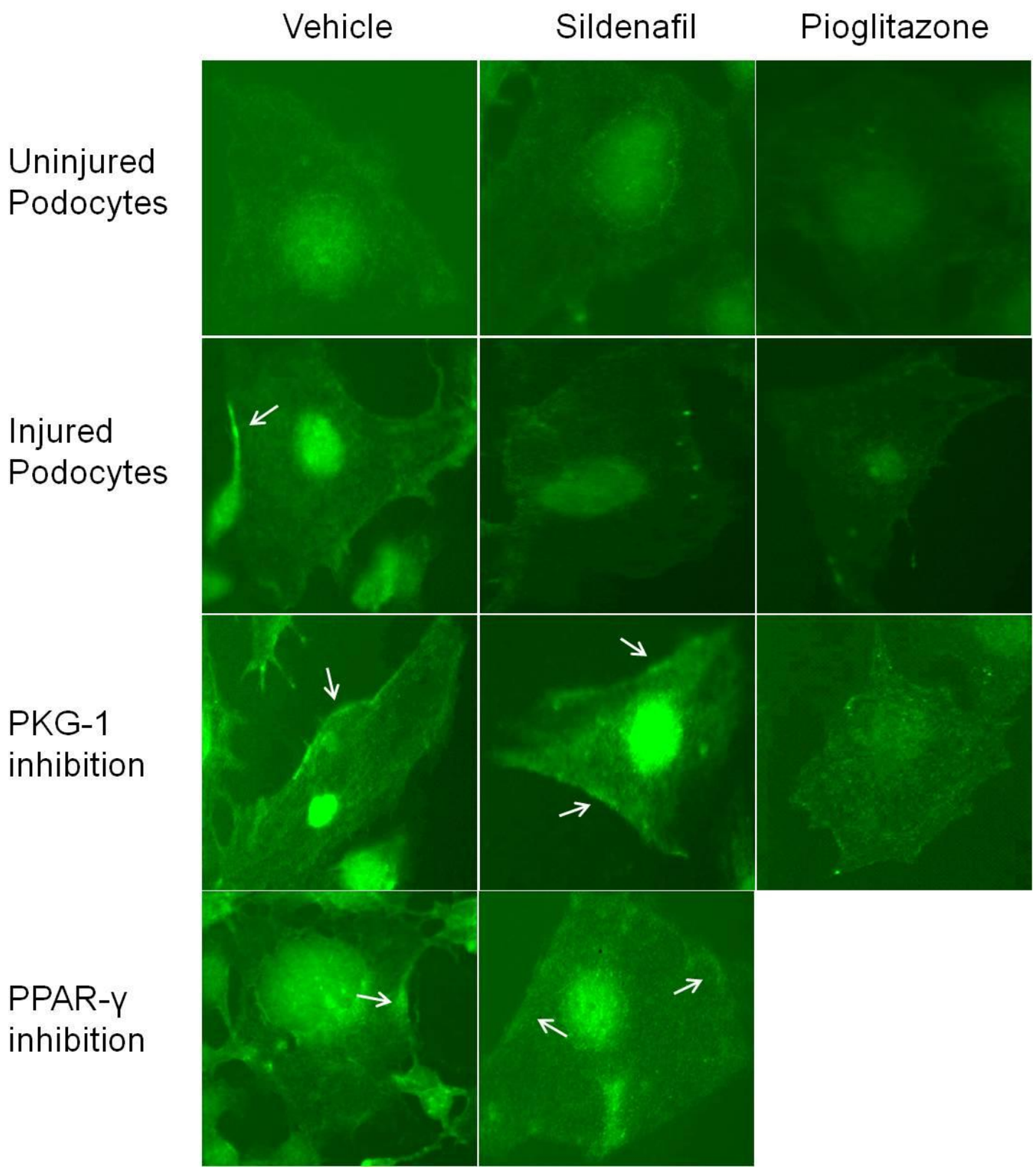
Supplementary Figure 3: Pilot study on the effect of different doses of GW9662 on glomerular TRPC6 expression and proteinuria

To determine the concentration GW9662 to induce TRPC6 transcriptional changes and proteinuria, Wistar rats were treated with 0, 0.1, 1.0 or 2.5mg/kg bodyweight GW9662. After the animals were sacrificed, TRPC6 protein expression (A) and proteinuria (B) was determined. Statistical significance was determined using ANOVA followed by Bonferroni *post hoc* test. * $p < 0.05$ vs vehicle-treated rats.

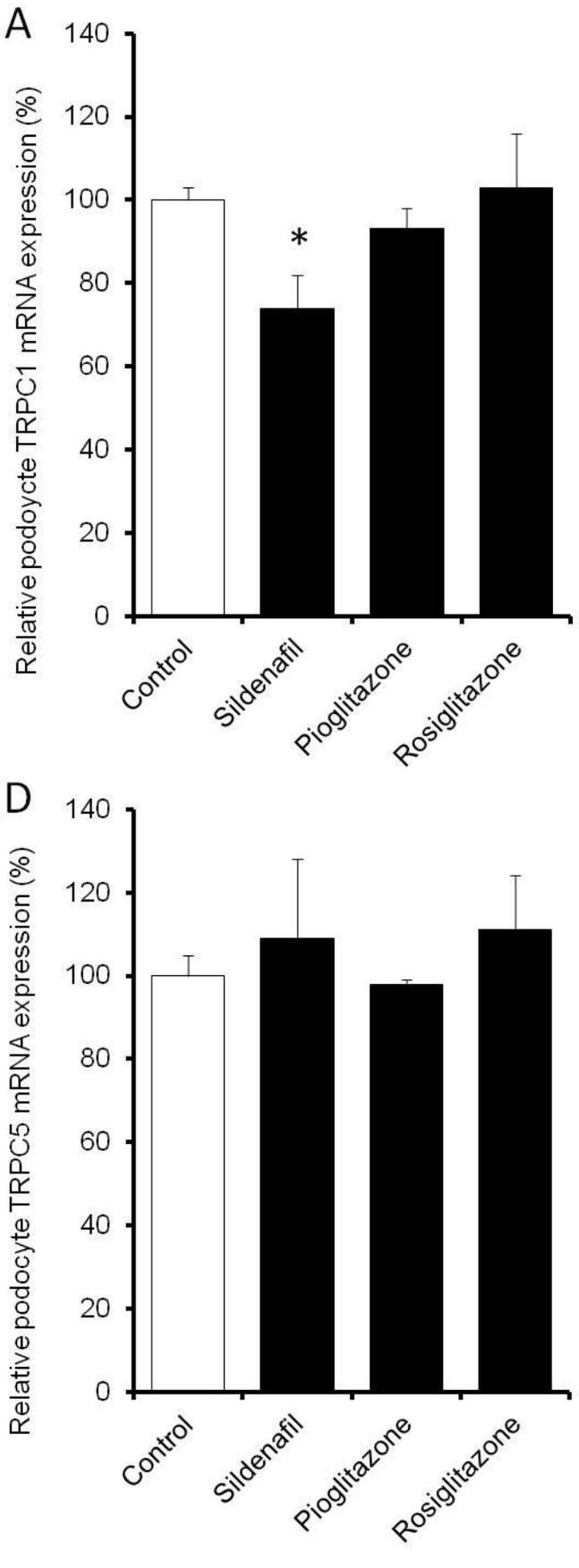
Supplementary Figure 4: Sildenafil does not cause glomerular injury

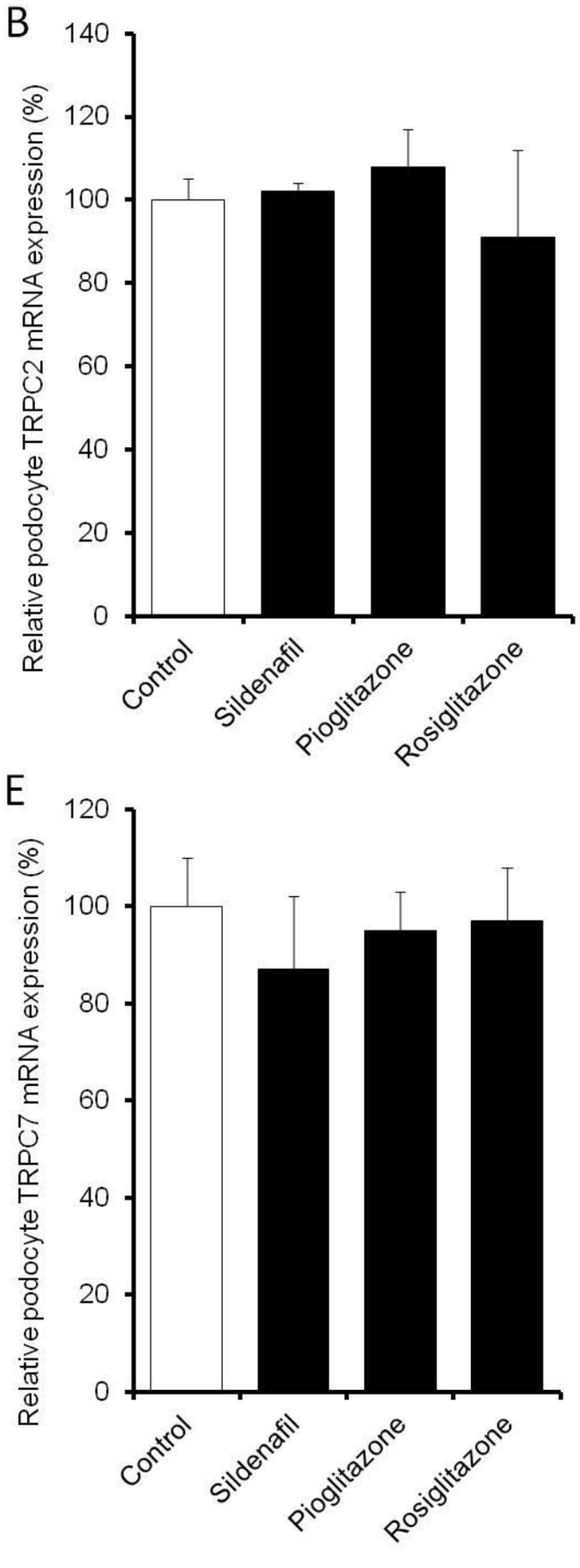
Healthy mice were treated with vehicle or sildenafil. After mice were sacrificed TRPC6 protein (A), mRNA (B), desmin protein (C) and nephrin protein expression (D) was determined. Statistical significance was tested using the student-T-test.

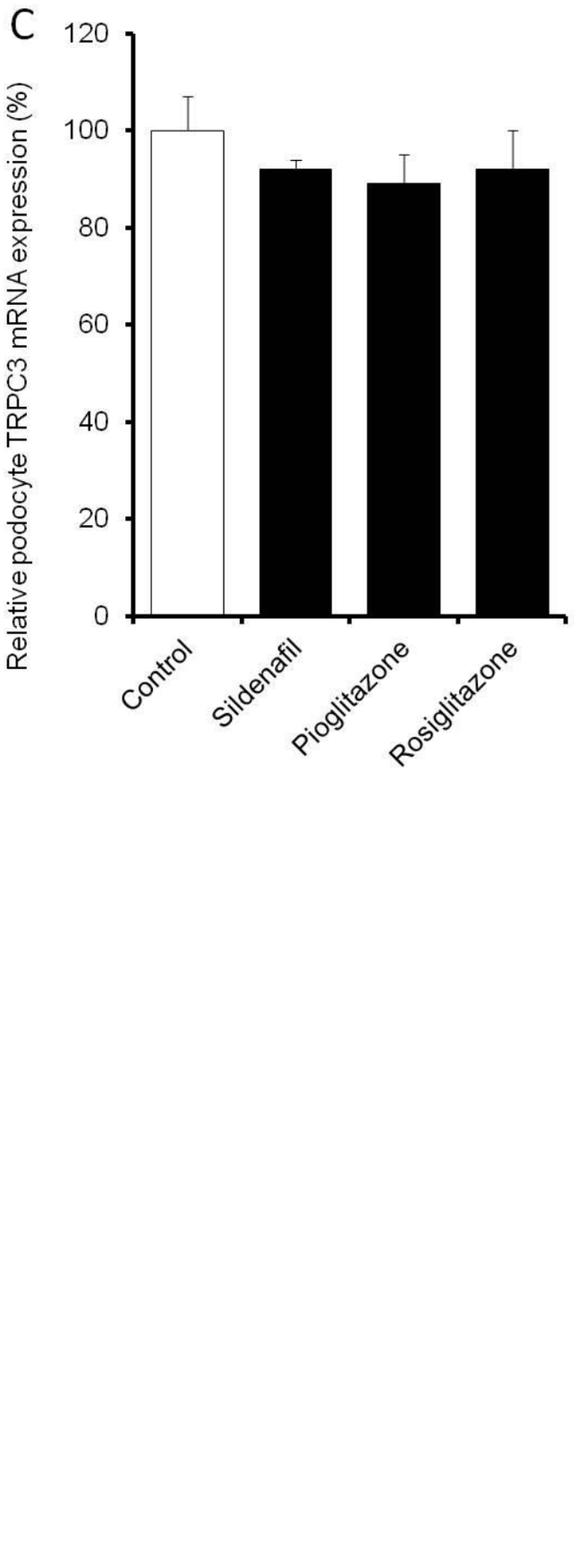
Supplementary Figure 1



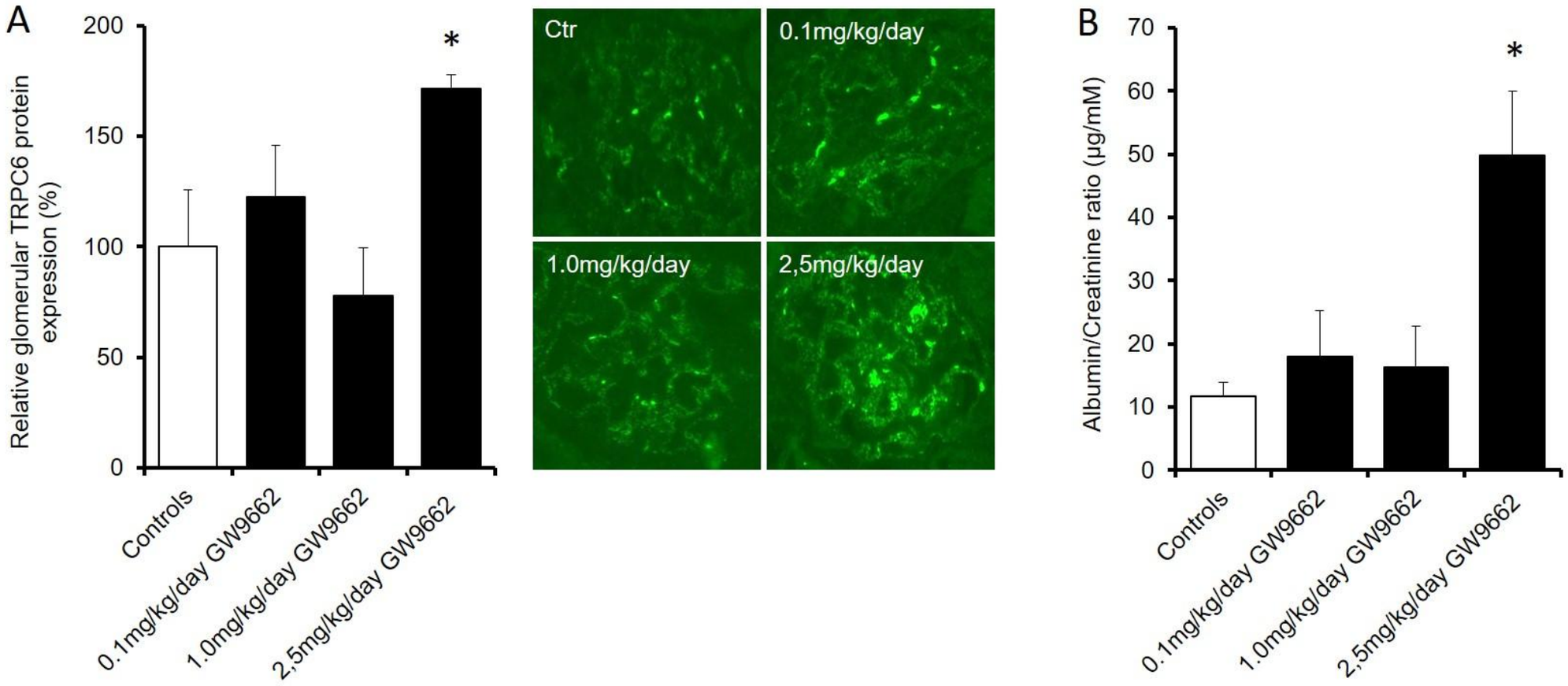
Supplementary Figure 2







Supplementary Figure 3



Supplementary Figure 4