

A NOX4/TRPC6 Pathway in Podocyte Calcium Regulation and Renal Damage in Diabetic Kidney Disease

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Abstract

Background Loss of glomerular podocytes is an indicator of diabetic kidney disease (DKD). The damage to these cells has been attributed in part to elevated intrarenal oxidative stress. The primary source of the renal reactive oxygen species, particularly H₂O₂, is NADPH oxidase 4 (NOX4). We hypothesized that NOX4-derived H₂O₂ contributes to podocyte damage in DKD *via* elevation of podocyte calcium.

Methods We used Dahl salt-sensitive (SS) rats with a null mutation for the *Nox4* gene (SS^{Nox4^{-/-}}) and mice with knockout of the nonselective calcium channel TRPC6 or double knockout of TRPC5 and TRPC6. We performed whole animal studies and used biosensor measurements, electron microscopy, electrophysiology, and live calcium imaging experiments to evaluate the contribution of this pathway to the physiology of the podocytes in freshly isolated glomeruli.

Results Upon induction of type 1 diabetes with streptozotocin, SS^{Nox4^{-/-}} rats exhibited significantly lower basal intracellular Ca²⁺ levels in podocytes and less DKD-associated damage than SS rats did. Furthermore, the angiotensin II–elicited calcium flux was blunted in glomeruli isolated from diabetic SS^{Nox4^{-/-}} rats compared with that in glomeruli from diabetic SS rats. H₂O₂ stimulated TRPC-dependent calcium influx in podocytes from wild-type mice, but this influx was blunted in podocytes from *Trpc6*-knockout mice and, in a similar manner, in podocytes from *Trpc5/6* double-knockout mice. Finally, electron microscopy revealed that podocytes of glomeruli isolated from *Trpc6*-knockout or *Trpc5/6* double-knockout mice were protected from damage induced by H₂O₂ to the same extent.

Conclusions These data reveal a novel signaling mechanism involving NOX4 and TRPC6 in podocytes that could be pharmacologically targeted to abate the development of DKD.

Keywords: calcium, diabetic nephropathy, podocyte, reactive oxygen species, NADPH oxidase, ion channel

Abstract

Malfunction of podocytes (glomerular visceral epithelial cells of the kidney) is a major contributor to the pathogenesis of diabetic kidney disease (DKD) because it leads to albuminuria, an early defining feature of this condition. Current DKD therapies target the renin-angiotensin system (RAS) to reduce hypertension, glomerular permeability to urinary albumin, and renal interstitial fibrosis. Angiotensin II (AngII) is one of the bioactive members of the RAS shown to have various physiologic effects in podocytes, including podocyte calcium signaling^{1,3} and production of reactive oxygen species (ROS).⁴ Increased levels of AngII have been found in the diabetic kidney.^{5,6}

Dysfunction of calcium signaling regulation in podocytes leads to podocyte cytoskeletal disorganization, foot process (FP) effacement, disruption of the slit diaphragm, and proteinuria.^{7,8} Importance of podocytic calcium handling was highlighted in subjects with the gain-of-function mutation in the nonselective calcium channel TRPC (transient receptor potential canonical) that caused FSGS.^{9,10} We and others found that AngII-evoked calcium increases are primarily mediated *via* TRPC6 channels and proposed that this pathway might be critical in the development of DKD.^{2,3} Furthermore, it was reported that this pathway required the generation of ROS¹¹ which are produced concurrently with AngII signaling.

Renal ROS production appears to be pivotal in the pathogenesis of DKD.¹² A recent study by Jha *et al.*¹³ performed on human samples and transgenic mice reported that ROS could promote renal injury in DKD. The NADPH oxidase NOX4 is one of the dominant renal sources of ROS in rodents and humans, and is highly expressed in the kidneys of diabetic models.^{14,15} The pharmacologic inhibition and both the global and podocyte-specific genetic deletions of Nox4 all attenuate albuminuria and podocyte damage in mouse models of DKD.^{16,17} Hydrogen peroxide (H₂O₂), rather than superoxide (O₂⁻), is commonly used to assess NOX4 activity.¹⁸ It is recognized that exogenous H₂O₂ can increase the plasma membrane abundance and activity of *Trpc6* channels, whereas the knockdown of *Trpc6* attenuates H₂O₂-induced apoptosis.^{19,20} On the basis of these observations we hypothesized that H₂O₂ produced from NOX4 may play an important role in AngII-induced glomerular H₂O₂ production, alteration of TRPC channels, and podocyte calcium dysfunction in the development of DKD.

Methods

Experimental Protocol and Animals

The animal use and welfare adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals following protocols reviewed and approved by the Medical College of Wisconsin IACUC (AUA1061 and 1641). Seven-week-old male Dahl salt-sensitive rats (SS; SS/JrHsdMewi) and SS^{Nox4^{-/-}} rats were provided food (0.4% NaCl AIC-76 purified rodent chow; Dyets, Bethlehem, PA) and water *ad libitum*. SS^{Nox4^{-/-}} rats were previously described.²¹ Rats were housed individually in cages with bedding except during 24-hour urine collections when they were housed in metabolic cages. SS and SS^{Nox4^{-/-}} rats were injected intraperitoneally (i.p.) with streptozotocin (STZ, 75 mg/kg; Sigma-Aldrich, St Louis, MO) to induce type 1 diabetes or vehicle (50 mM sodium citrate, pH 4.5) as a control. At 7 days post-i.p. injection, a slow-release insulin (LinShin, Canada) or blank pellet was implanted s.c. into diabetic and control rats, respectively, to maintain moderate hyperglycemia in STZ-treated rats (approximately 300 mg/dl). Wild-type (129S/c57 strain), *Trpc6^{-/-}*, and *Trpc5/6^{-/-}* mice were provided by L.B. (Neurobiology Laboratory, National Institute of Environmental Health Sciences) and housed at the Medical College of Wisconsin. *Trpc6^{-/-}* and *Trpc5^{-/-}* mice were previously described^{22,23} and *Trpc5/6^{-/-}* mice were generated at the Comparative Medicine Branch of the National Institute of Environmental Health Sciences by backcrossing of *Trpc5^{-/-}* and *Trpc6^{-/-}* mice to obtain the desired genotypes (wild-type *Trpc5/6^{+/+}* and double KO *Trpc5/6^{-/-}*).

Kidney Isolation

Rats were anesthetized and their kidneys were flushed with PBS *via* aortic catheterization (3 ml/min per kidney until blanched). For each rat, one kidney was used for glomeruli isolation, and the other kidney was coronally halved for Western blot and immunohistochemistry analyses.

Electrolyte Measurements

Whole blood and urine electrolytes and creatinine were measured with a blood gas and electrolyte analyzer (ABL system 800 Flex; Radiometer, Copenhagen, Denmark). Kidney function was determined by albuminuria (measured using a fluorescent assay [Albumin Blue 580 dye; Molecular Probes, Eugene, OR] and read by a fluorescent plate reader [FL600; Bio-Tek, Winooski, VT]) and BUN levels (Alfa Wassermann, West Caldwell, NJ).

Histologic Staining and Analysis

Rat kidneys were cleared from blood, formalin fixed, paraffin embedded, sectioned, and mounted on slides, as previously described.²⁴ Slides were stained with Masson's trichrome stain. A double-blind glomerular injury score was assessed using a 0–4 scale as previously used. Protein cast analysis was performed using a color thresholding method using Metamorph (Molecular Devices, Sunnyvale, CA) software.

Isolation of Glomeruli and Calcium Measurements

Isolation and basal podocyte intracellular calcium ($[Ca^{2+}]_i$) measurements in rats and mice were done as described previously.^{25, 27} Isolated decapsulated glomeruli were incubated with Fura-2TH, AM and Fluo-4, AM (5 μ M; Thermo Fisher Scientific) for 40 minutes at room temperature. Calcium imaging was performed as previously described.^{3, 25, 27} A laser scanning confocal microscope system (Nikon A1-R, NIS Elements; Nikon Instruments Inc., Tokyo, Japan) was used to collect images in time series (xyt, 4 seconds per frame). Basal $[Ca^{2+}]_i$ concentrations of podocytes were measured after stable fluorescence intensities were established using a common protocol with ionomycin and $MnCl_2$.

Patch-Clamp Electrophysiology and Data Analysis

The cover glass with attached glomeruli was placed into a perfusion chamber and mounted on an inverted Nikon Ti-S microscope. After a high-resistance seal was obtained, the cell-attached recording was performed immediately and analyzed as previously described.^{27, 28}

H₂O₂ Level Measurements with Enzymatic Biosensors

Biosensors for H₂O₂ analysis were obtained from Sarissa Biomedical Inc. and used previously to assess extracellular H₂O₂ concentrations *ex vivo* and *in vivo*.²⁹

Western Blotting

Western blot analysis of the blood-free kidney cortex was performed as reported previously^{24, 30} using an antibody against the intracellular epitope of the Trpc6 channel (ACC-017; Alomone Labs).

Electron Microscopy

For the EM experiments glomeruli were fixed in 2% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.4) and processed as described previously.³¹ Sections were viewed in a Hitachi HS600 transmission electron microscope and images recorded *via* an AMT 1K digital imaging camera.

Statistical Analyses

Data are presented as box plots, where all data points are shown; the box denotes SEM, error bars are St. Dev., and horizontal line is the mean value. Data using the four groups of rats (SS, Nox4^{-/-}, STZ-SS, STZ-Nox4^{-/-}) were compared using the two-factor ANOVA for repeated measures followed by the Holm–

Sidak *post hoc* test. Podocyte calcium imaging analysis used the values of $[Ca^{2+}]_i$ at every moment of time for individual cells and averaged the number of regions registered in each experiment. An independent-samples *t* test was used to compare H_2O_2 production by isolated glomeruli.

Results

SS^{Nox4^{-/-}} Rats Are Protected from the Development of Microalbuminuria and Kidney Injury in Type 1 Diabetes

We tested here the contribution of NOX4 in the development of DKD, where type 1 diabetes was induced by a low dose of STZ in Dahl SS hypertensive rats with a null mutation for the *Nox4* gene (SS^{Nox4^{-/-}}).²¹ After a single i.p. injection of STZ or vehicle the development of type 1 diabetes was monitored in Dahl SS and SS^{Nox4^{-/-}} rats over 11 weeks (Figure 1A). Attenuation of body growth and strong hypertrophy of the kidneys was observed in both SS and SS^{Nox4^{-/-}} diabetic groups compared with control animals (Figure 1B), along with the development of hyperglycemia in rats injected with STZ as well as a significant increase in BUN value in the diabetic rats (Figure 1C). Electrolyte levels were assessed in the terminally collected blood samples; data are shown in Supplemental Figure 1. Consistent with our previous work²⁶, STZ-injected rats developed polyuria (Figure 1D). Urinary electrolyte/creatinine analysis (see Supplemental Figure 2) did not reveal any difference between SS and SS^{Nox4^{-/-}} groups in control or under diabetic conditions. Microalbumin levels in the urine confirmed kidney disease development in the SS-STZ group compared with control SS and SS^{Nox4^{-/-}} rats and demonstrated a progressive increase in 24-hour microalbumin excretion in the diabetic SS rats; microalbuminuria was attenuated in the STZ-treated SS^{Nox4^{-/-}} group (Figure 1E).

We further assessed kidney damage using Masson's trichrome staining; as seen in Figure 2A, control SS^{Nox4^{-/-}} rats showed dramatically less protein cast formation than wild-type SS rats in both control and STZ-treated groups. Scoring of glomerular damage (Figure 2B) revealed aggravated glomerular injury in STZ-SS rats. Protein cast analysis and glomerular injury were ameliorated in both diabetic and nondiabetic SS^{Nox4^{-/-}} rats.

Diabetes-Induced Calcium Mishandling Is Attenuated in SS^{Nox4^{-/-}} Rat Podocytes

In addition to confirming the development of type 1 diabetes, the above-described data revealed reduced kidney damage in the STZ-treated SS^{Nox4^{-/-}} group. We further isolated glomeruli from the kidney cortex of each studied group and performed confocal imaging to assess the basal level of calcium and calcium influx in response to AngII. A representative glomerulus loaded with fluorescent calcium dyes Fluo4 and Fura-2TH is shown in Figure 3A. We observed that in the STZ-SS animals the response to AngII was significantly higher than in the control preparation; however, in line with our data pertaining to renal tissue damage, the response was blunted in the SS^{Nox4^{-/-}} rats (see Figure 3, B and C). Additionally, the increase in basal calcium previously observed by us in the diabetic SS rat podocytes was absent in the STZ-treated SS^{Nox4^{-/-}} group (Figure 3D). This would suggest a protective effect of lower levels of H_2O_2 associated with the *Nox4^{-/-}* phenotype, counteracting the pathologic increase in $[Ca^{2+}]_i$.

According to our previous findings,²⁵ podocytic TRPC6 is the channel that mainly contributes to changes in $[Ca^{2+}]_i$ in normal and pathologic conditions. Here, we tested the expression level of TRPC6 in the kidney cortex in normal and diabetic conditions; as summarized in Figure 3E, DKD development resulted in a significant increase in TRPC6 expression in both STZ-treated wild-type SS and SS^{Nox4^{-/-}} strains. Therefore, the diminished response to AngII and suppressed increase in basal calcium we report for the STZ-treated SS^{Nox4^{-/-}} animals could be due to changes in the channel open probability (P_o); however, we do not exclude the effects on TRPC6 channel expression, because in cell-attached mode we are not disrupting fast increase in surface expression of the channel. Next, we looked at the podocyte ultrastructure using electron microscopy (Figure 3F); these studies revealed diminished FP damage in the STZ-SS^{Nox4^{-/-}} group, as opposed to the effacement and deterioration observed in the STZ-treated wild-type group.

NOX4 Is the Major Source for H₂O₂ Production in the SS Rat Glomeruli in Response to AngII

NOX4 is reported to be one of the main sources of H₂O₂ production in the kidney¹⁵; however, H₂O₂ release in glomeruli has not been shown. To test the contribution of NOX4 to H₂O₂ release in glomeruli, we employed here a novel approach featuring enzymatic biosensors capable of detecting specific substances and dynamic changes in their concentrations in fresh tissues.^{32,33} In order to test H₂O₂ release, we isolated glomeruli from the wild-type SS rats and age-matched SS^{Nox4^{-/-}} animals and washed them by spinning; next, glomeruli were placed into a 100- μ l tube. A biosensor was then placed into the solution (see [Figure 4A](#)) and, after allowing time for equilibration, stable basal levels of H₂O₂ were recorded for several minutes. AngII (1 μ M) was then added to the tube while recording the subsequent changes in H₂O₂ production. To ensure specificity of the signal, a catalase block was used at the end of each experiment. [Figure 4, B and C](#) feature the representative traces and summary of H₂O₂ release from wild-type SS and SS^{Nox4^{-/-}} rat glomeruli. As seen from the data, H₂O₂ production from rat glomeruli in response to AngII was significantly blunted in the SS^{Nox4^{-/-}} strain. These findings allow us to conclude that Nox4 is the predominant source of H₂O₂ production in glomeruli.

H₂O₂ Activates Calcium Influx via TRPC6 Channels in the Podocytes

Having identified that Nox4 knockout potentially plays a protective role during type 1 diabetes, we decided to investigate the involvement of TRPC channels, and specifically TRPC6, in this mechanism. First, using patch-clamp analysis on freshly isolated mouse glomeruli, we found that H₂O₂ acutely activates TRPC channels in the podocytes ([Figure 5A](#)). Next, we tested the effect of H₂O₂ on total calcium influx in mouse podocytes using confocal microscopy; as summarized in [Figure 5B](#), H₂O₂ evokes a dose-dependent increase in calcium level within the podocytes, with an EC₅₀ of 56.4 \pm 6.2 μ M. Interestingly, application of lower (<50 μ M) concentrations of H₂O₂ results in a short calcium transient, whereas H₂O₂>50 μ M produces a transient followed by a long-term plateau-like increase in calcium, that should likely be attributed to apoptotic rather than signaling events (see [Figure 5C](#)). Next, we asked whether the observed calcium transient results from the influx of calcium from the extracellular space, release from intracellular stores, or both; to test this, we removed Ca²⁺ from the bath solution, and performed calcium imaging. As shown in [Figure 5D](#), in the absence of Ca²⁺ the transient is abolished, which allows us to conclude that H₂O₂ causes mainly Ca²⁺ influx from the extracellular space—presumably, *via* TRPC channels.

Lack of TRPC6 Channel Protects Podocytes from H₂O₂-Induced Damage

We next investigated the hypothesis that high H₂O₂ levels can lead to podocyte damage through the activation of calcium influx *via* TRPC channels. First, we used *Trpc6*^{-/-} mice because it was reported that mutations in *Trpc6* cause development of FSGS and podocyte damage^{9,10} and also that the TRPC6 channel can be activated by H₂O₂.³⁴ We also generated a double knockout for both *Trpc5* and *Trpc6* to test the contribution of TRPC5 because previous studies proposed that this channel also promotes podocyte injury.^{35,36} [Supplemental Figures 3–5](#) provide some basic phenotyping of renal function of the *Trpc5/6*^{-/-} mice. We did not observe significant changes in the renal function except for decreased creatinine level ([Supplemental Figure 3](#)), which is consistent with the analysis of *Trpc6*^{-/-} mice.²² Data shown in [Figure 6A](#) revealed a moderate increase in basal calcium level in the podocytes in both knockout strains, which was surprising but could be attributed to some compensatory regulation. We next tested calcium influx in the podocytes of these knockout strains in response to H₂O₂ (50 μ M). As shown in [Figure 6B](#), H₂O₂ produced a robust increase in calcium level in wild-type mouse podocytes, as was observed earlier ([Figure 5C](#)). This response was attenuated in a similar manner in both *Trpc6*^{-/-} and *Trpc5/6*^{-/-} mice. These results suggest that it is TRPC6 rather than the TRPC5 channel that reduces the robust increase in calcium influx triggered by a high concentration of H₂O₂. These observations led us to a hypothesis that absence of TRPC6 could play a protective role in the H₂O₂-induced podocyte damage. To test this idea, glomeruli were isolated from wild-type, TRPC6^{-/-}, and TRPC5/6^{-/-} mice subjected to acute treatment with 100 μ M

H₂O₂ or vehicle (for 30 minutes). As shown in [Figure 6C](#), no differences in FP structure were observed in control conditions (vehicle treatment) among the tested strains. Upon H₂O₂ application, however, wild-type podocytes showed dramatic effacement and apparent damage, whereas neither *Trpc6*^{-/-} nor *Trpc5/6*^{-/-} podocytes revealed such changes. Taken together, these data indicate that lack of *Trpc6* plays a protective role in the H₂O₂-triggered podocyte damage.

Discussion

This study provides evidence that *Nox*-derived ROS production promotes podocyte injury by enhancing calcium influx *via* TRPC6 channels. We utilized a null mutation of *Nox4* in a model of type 1 diabetes in the Dahl SS rat background to test whether H₂O₂ contributes to the mechanism of the calcium handling dysfunction as we previously reported in the diabetic SS rats.²⁶ We show, for the first time, that the lack of *Nox4* attenuates the diabetes-induced potentiation of calcium handling in podocytes, and reveal the mechanism that may be targeted to prevent the development of DKD. This attenuation was a consequence of reduced H₂O₂ production in SS^{*Nox4*^{-/-}} rats because we showed that AngII-mediated H₂O₂ production was greatly reduced in *Nox4*^{-/-} glomeruli.

The study by Liu *et al.*¹⁹ in podocyte cell culture showed that H₂O₂ stimulates podocytic TRPC6 channels. This was validated by our observations in *ex vivo* isolated glomeruli in which dose-dependent changes of H₂O₂-induced calcium flux were examined. Whereas a low dose was sufficient to activate TRPC channels, a high dose induced a sustained calcium flux in podocytes that was abolished in a manner similar to the glomerular podocytes obtained from *Trpc6* and *Trpc5/6* knockout mice. Because only minor differences were observed between the *Trpc6* and *Trpc5/6* mice, these data indicate that TRPC6 is the primary calcium channel stimulated by H₂O₂. Supporting this conclusion were the observations that after acute H₂O₂ treatment glomeruli from *Trpc6* knockout mice did not exhibit the podocyte damage observed in the wild-type animals and the observation that knockout of both *Trpc5* and *Trpc6* together did not provide additional protection from the damaging effects of ROS.

The role of TRPC5 in podocyte injury is currently debated. A study by Zhou *et al.*³⁷ reported that TRPC5 activity drives FSGS and that chronic TRPC5 inhibition suppressed severe proteinuria and prevented podocyte loss in a transgenic rat model of FSGS. However, studies by Reiser and colleagues found in *Trpc5* transgenic mice that neither overexpression nor activation of the TRPC5 ion channel caused kidney barrier injury or aggravated such injury under pathologic conditions.³⁸ As with many contradictory studies, it is not possible to explain such differences because they could result from methodologic differences. This study clearly supports the view that the TRPC5 channel does not have an additive effect to TRPC6 and contributes little to podocyte FP effacement, disruption of the glomerular filtration barrier, and conduction of calcium signal in response to ROS.

Trpc6 null mice were shown by others to be protected from proteinuric effects of AngII.³⁹ AngII was found to cause podocyte injury, induce H₂O₂ production, and activate podocyte TRPC channels.^{1,26} This study found that AngII induces H₂O₂ production derived from *Nox4* that in turn activates calcium influx *via* TRPC6 channels, which triggers downstream apoptotic cascades (see [Figure 7](#)). The significant, but not complete, reduction in AngII-elicited glomerular H₂O₂ production in the *Nox4*^{-/-} rat glomeruli suggests the presence of other sources of H₂O₂. However, the absence of *Nox4* mostly prevented albuminuria in diabetic rats, which is consistent with results of targeting of *Nox4* in other models of DKD that reduced but did not prevent albuminuria.

Knockdown of *Nox4* in an STZ-treated Sprague Dawley rat was previously shown to reduce kidney hypertrophy 2 weeks post-STZ injection.⁴⁰ However, in our STZ-SS model, the absence of *Nox4* did not attenuate renal hypertrophy. This fact could be due to the greater basal renal ROS produced in SS rats versus Sprague Dawley rats, and the difference in the acute versus long-term effects (2 versus 11 weeks post-STZ treatment). Cowley *et al.*⁴¹ observed that kidneys of SS rats exhibit greater levels of oxidative stress compared with SS^{*Nox4*^{-/-}} rats even when fed a low-salt diet. Previous results have shown that ROS, as well as AngII,^{41,42} affect glomerular TRPC6 expression.⁴³ In our hands, the absence of *Nox4* did not attenuate the increased cortical TRPC6 expression, which may indicate that additional factors are controlling this mechanism. However, the data from this study indicate that the podocyte calcium

dysfunction in diabetic SS rats resulted from reduced ROS stimulation of TRPC6 rather than an increased TRPC6 channel abundance. A recent manuscript by Cowley *et al.*²¹ demonstrated the importance of NOX4 in the development of hypertension in Dahl SS rats, and reported a significant reduction in salt-induced BP, albuminuria, tubular casts, and glomerular injury in the SS^{Nox4^{-/-}} rats in comparison to wild-type controls. Taken together, this suggests that ROS generated by Nox4 may be a part of a universal mechanism triggering kidney damage in different pathologic conditions characterized by albuminuria and glomerular injury.

AngII and ACE show increased activity and circulating levels during DKD.^{44, 46} On the basis of this study, we hypothesize that AngII stimulates Nox4 to produce excess H₂O₂, which in turn activates TRPC6 channels. However, in the diabetic model, other mechanisms could also contribute to the nephrotic changes, such as glucose, which could increase ROS and thereby increase protein expression of TRPC6 channels in podocytes as reported by others.^{2, 19, 47} There is also an emerging concept that TRPC6 channels in podocytes are activated by inflammatory mediators (in sera from patients with nephrotic syndromes)⁴⁸; this interesting pathway requires further investigation in DKD in association with AngII signaling.

In conclusion, using unique *in vivo* models and *ex vivo* techniques we provide evidence that Nox4-derived ROS contribute to podocyte injury *via*, at least in part, podocyte TRPC6 channels. Furthermore, Nox4 produces the majority of AngII-elicited H₂O₂ in the glomerulus and its absence attenuates the podocytic calcium signaling dysfunction that occurs in diabetes.

Disclosures

None.

Supplementary Material

Supplemental Data:

Acknowledgments

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Footnotes

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Figures and Tables

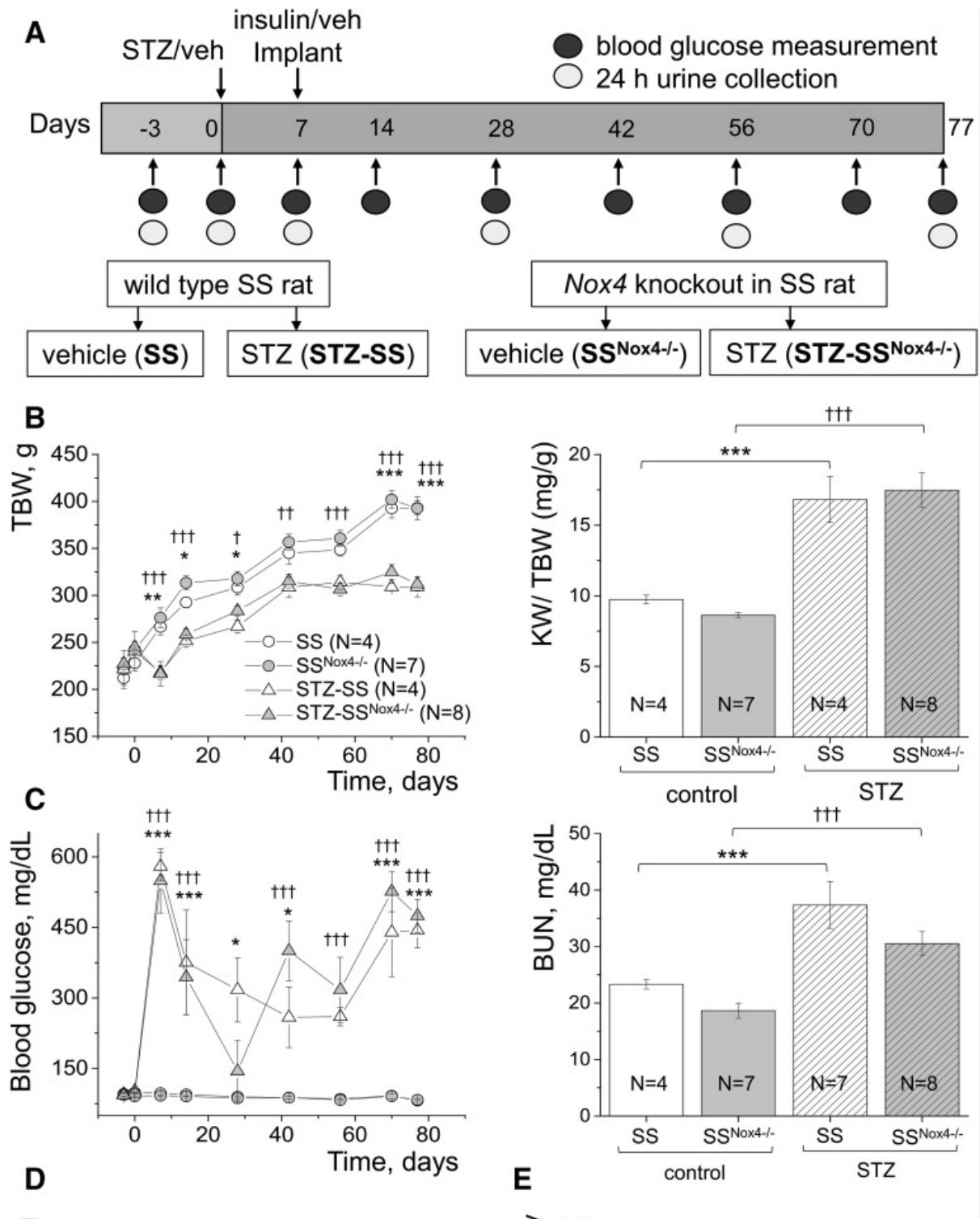


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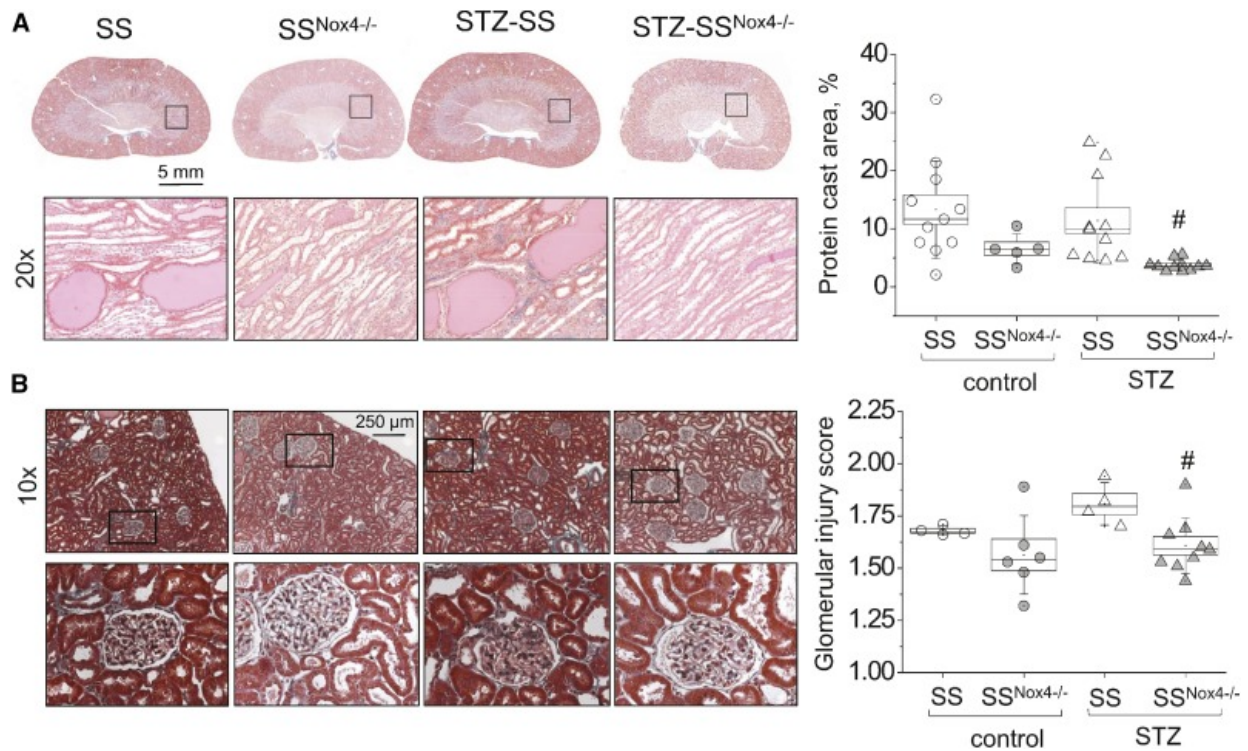
Figure 1.



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Nox4 knockout protects Dahl SS rats from the injury associated with the development of type 1 diabetes. (A) Schematic representation of the experimental protocol is shown: type 1 diabetes was induced in Dahl SS and $SS^{Nox4-/-}$ rats with a single i.p. injection of STZ after insulin implant at day 7, and key characteristics of the disease were monitored during 11 weeks post-diabetes induction. Studied experimental groups: control SS and $SS^{Nox4-/-}$ animals treated with vehicle, as well as STZ-treated SS and $SS^{Nox4-/-}$ rats. (B) Total body weight (TBW, left panel) and kidney to body weight ratio (KW/TBW, right panel) in control and diabetic rats throughout the experimental protocol. (C) Blood glucose level (left panel) as measured in nonfasting conditions over the 11 weeks of diabetes development. Right panel in (C) shows BUN levels tested in the terminal plasma samples collected from all four groups of animals. (D and E) The 24-hour urinary volume (normalized to TBW) and daily microalbumin excretion, respectively. #Denotes statistically significant difference between STZ-SS and STZ- $SS^{Nox4-/-}$ groups, †denotes $SS^{Nox4-/-}$ versus STZ- $SS^{Nox4-/-}$, and *denotes SS versus STZ-SS animals. Veh, vehicle.

Figure 2.



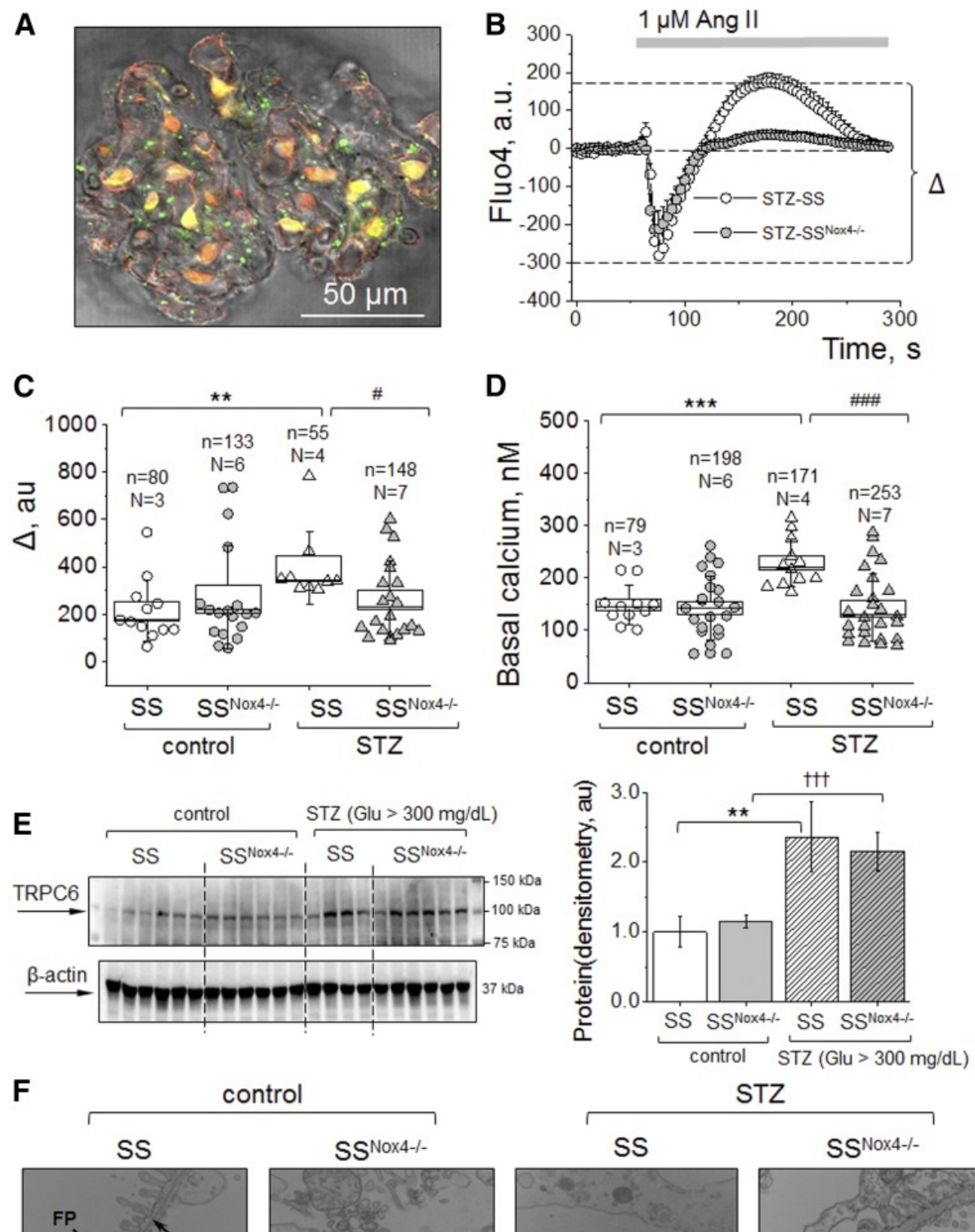
Kidney damage after type 1 diabetes induction with streptozotocin is attenuated in Dahl SS rats lacking NOX4. (A) Representative images of the kidney tissues stained with Masson's trichrome (left panel, whole kidney images and expanded areas [20× magnification]) and then analyzed for protein cast formation. Right panel in (A) shows a summary graph of protein cast area (percent of total kidney area) for each studied group. (B) Images of the representative cortical renal tissues containing glomeruli (upper row, 10×) and magnified examples of single glomeruli from each group.

Glomerular injury score is summarized in the right panel in (B); 100 random glomeruli were scored for each kidney.

#Denotes statistically significant difference between STZ-SS and STZ-SS^{Nox4^{-/-}} groups; scale bar shown is common for all of the images in the same row; each point on the graph illustrates an average value from 100 glomeruli randomly scored per kidney.



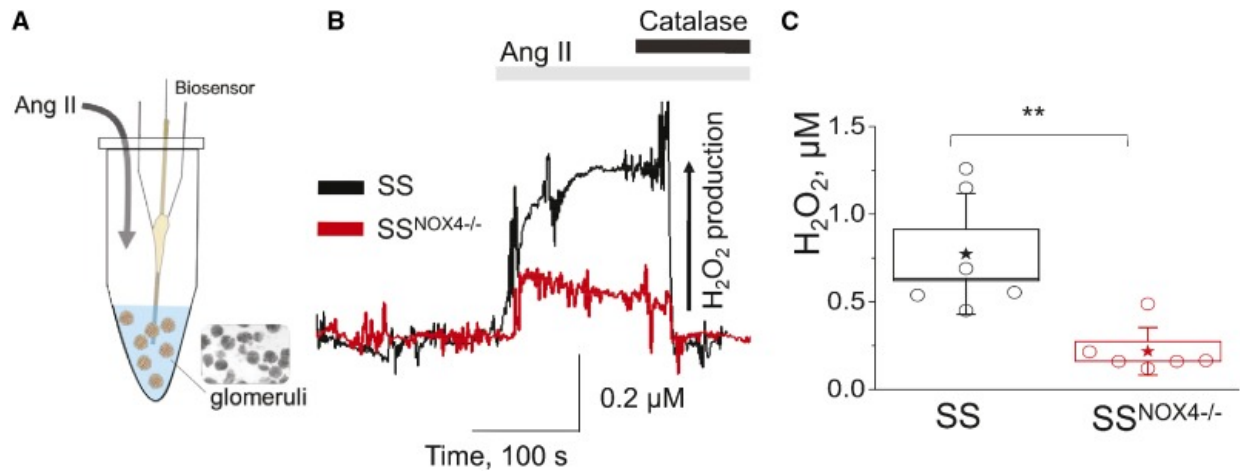
Figure 3.



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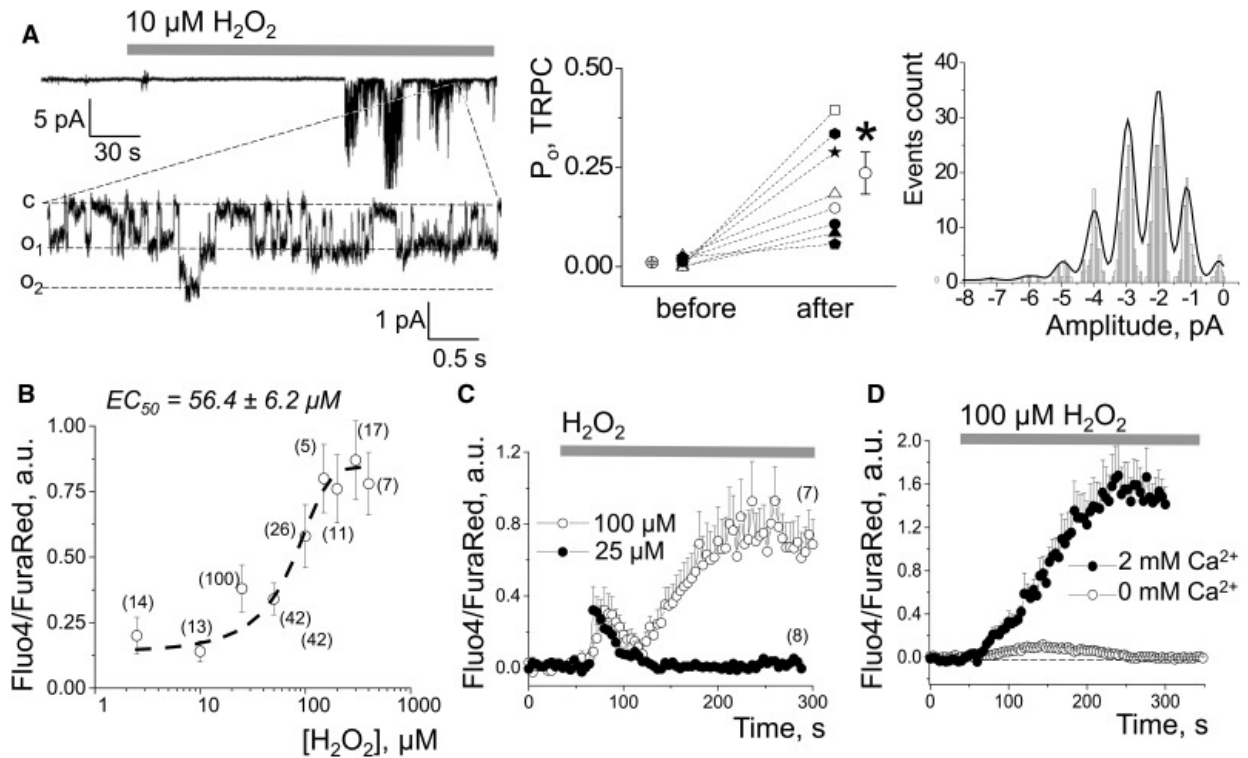
Type 1 diabetes-associated podocyte damage and intracellular calcium increase are blunted in Dahl SS rats lacking *Nox4*. (A) A representative image of a glomerulus loaded with Fluo4 (green pseudocolor) and Fura-2TH (red pseudocolor) for confocal imaging of $[Ca^{2+}]_i$ handling; scale bar is shown. (B) Typical fluorescence transients demonstrating calcium influx in response to 1 μ M of AngII obtained from STZ-treated SS and SS^{Nox4-/-} rats. (C) Graph summarizing the peak amplitude of the response (Δ , arbitrary units of Fluo4 intensity). (D) Basal calcium levels in the studied samples (before AngII application) are summarized. Number of animals (N) and number of podocytes (n) tested for each group are shown on the graphs; each point on the graph represents an averaged peak amplitude or calcium level from one glomerulus. (E) Representative western blotting image and summary densitometry graph illustrating the changes in *Trpc6* channel expression in the kidney cortex of diabetic (STZ-injected) and control (vehicle-injected) SS and SS^{Nox4-/-} rats. (F) Representative electron microscopy images demonstrating podocyte FPs in control and diabetic SS and SS^{Nox4-/-} rat strains. Scale bar is shown; $n=3$ rats per group. a.u., arbitrary units; FP, podocyte foot processes; GBM, glomerular basement membrane; Glu, blood glucose.

Figure 4.



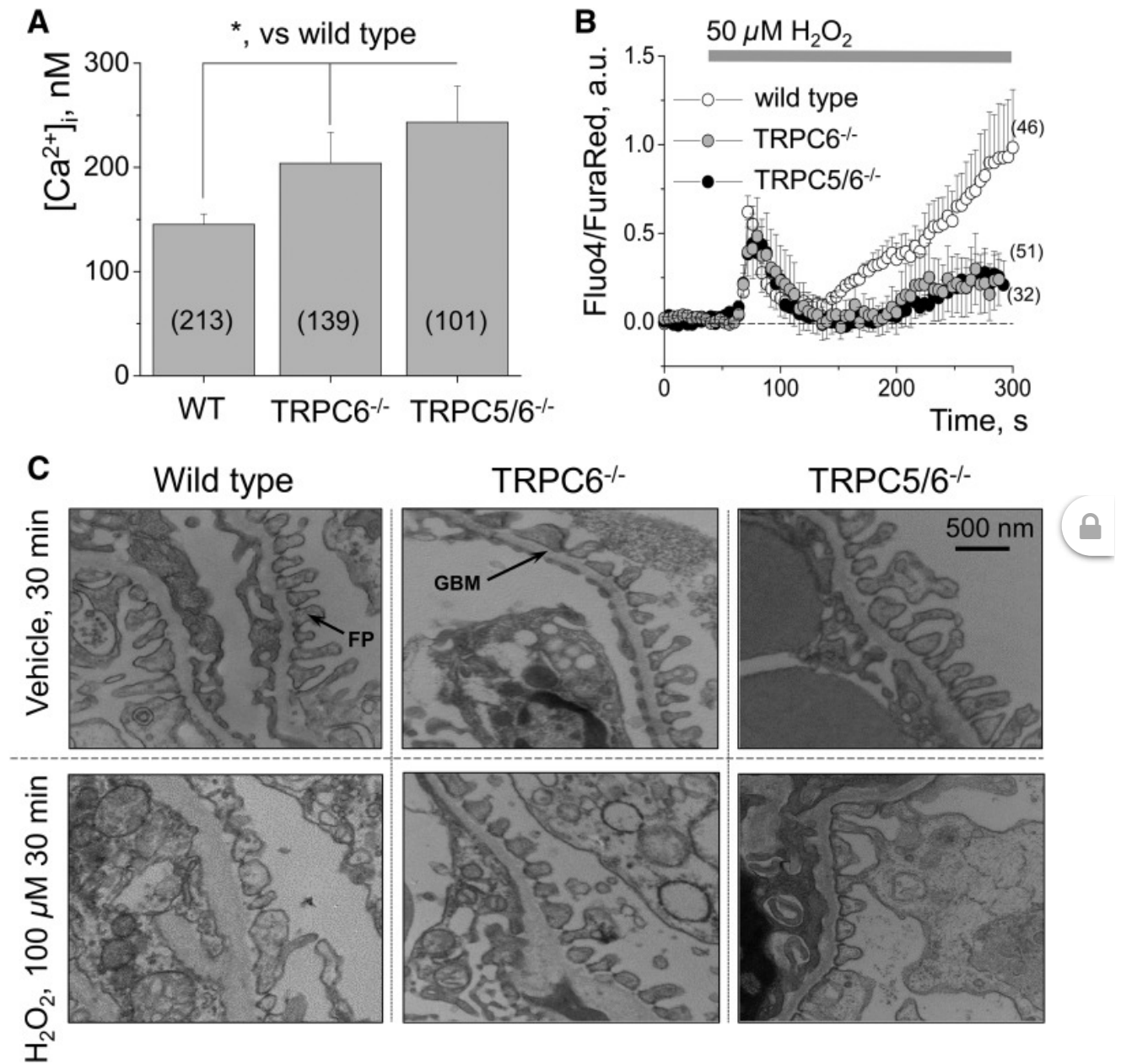
H_2O_2 production in response to Ang II is decreased in the freshly isolated glomeruli from *Nox4* knockout rats compared to wild type animals. (A) Schematic of the biosensors setup for the measurement of H_2O_2 production. (B) Representative transients of H_2O_2 release from the glomeruli isolated from the control (nondiabetic) SS and $SS^{Nox4-/-}$ rat strains; the release was measured in response to an acute addition of 1 μM AngII. Note application of catalase (2 mg/ml; $\geq 10,000$ U/mg protein) at the end of the experiment. (C) Summary graph showing the cumulative release of H_2O_2 in SS and $SS^{Nox4-/-}$ groups as measured by curve integration.

Figure 5.



TRPC6 channels are the main drivers of podocyte calcium influx in response to H_2O_2 . (A) Representative trace (left panel) and summary of the single TRPC channels' open probability (P_o) recorded in the podocytes of the freshly isolated mouse glomeruli in response to an acute application of H_2O_2 . Recording was obtained at a holding potential of -60 mV; shown is a full trace and an expanded fragment, "c" denotes closed state of the channel, and o_i is an open state (inward openings are downward). Right panel shows a typical amplitude histogram used for the single event detection and analysis of the recordings. (B) Dose-response curve illustrating the effect of different concentrations of H_2O_2 on calcium influx in freshly isolated mouse glomeruli. EC_{50} and number of podocytes analyzed per point on the graph are shown (minimum three animals were used to test each H_2O_2 concentration). (C) Representative calcium transients in response to low (25 μM) and high (100 μM) concentrations of H_2O_2 recorded from the podocytes of the freshly isolated mouse glomeruli. (D) Representative calcium transients obtained from the podocytes in the absence (0 mM) and presence (2 mM) of Ca^{2+} in the extracellular solution in response to H_2O_2 . a.u., arbitrary units; pA, picoAmperes.

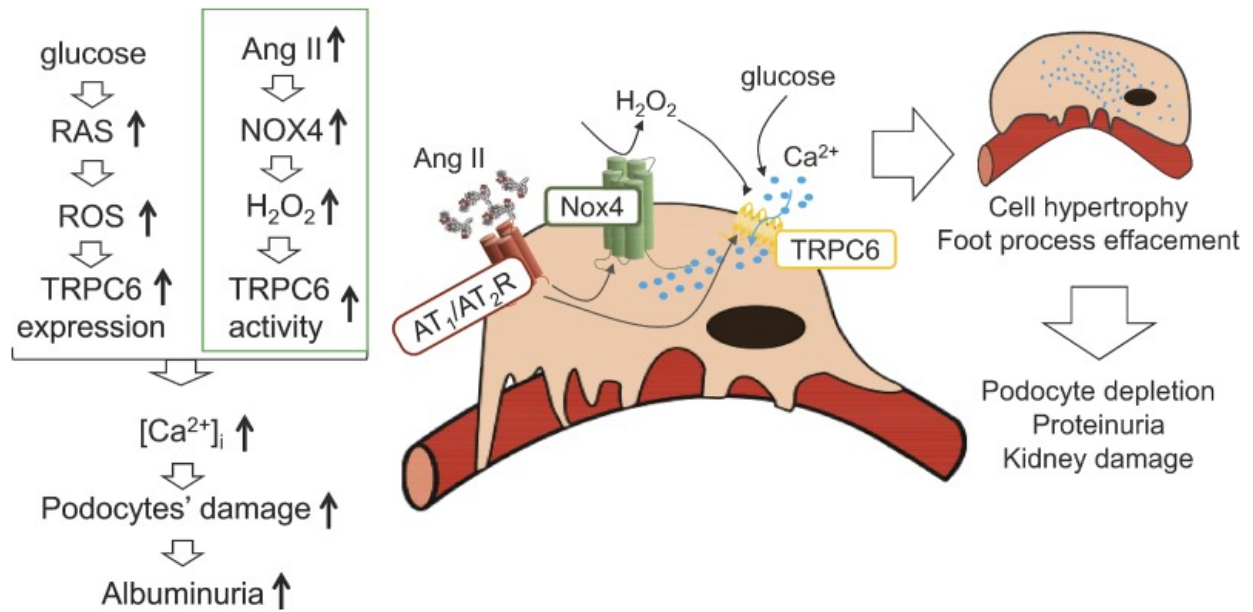
Figure 6.



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Trpc6^{-/-} and *Trpc5/6*^{-/-} podocytes exhibit protection from H_2O_2 -induced damage. (A) Basal calcium concentration measured in wild-type (129S/c57 strain), *Trpc6*^{-/-}, and *Trpc5/6*^{-/-} podocytes of the freshly isolated mouse glomeruli. Each point on the graph represents an averaged basal calcium level from one glomerulus; number of individual podocytes analyzed is shown in brackets. (B) Summarized calcium influx in response to a high concentration of H_2O_2 observed in wild-type, *Trpc6*^{-/-}, and *Trpc5/6*^{-/-} podocytes of the freshly isolated mouse glomeruli. Number of podocytes analyzed per group is shown on the graph; a minimum of three different mice were tested in each set. (C) Representative electron microscopy images featuring the podocyte FP and glomerular basement membrane (GBM) of the wild-type, *Trpc6*^{-/-}, and *Trpc5/6*^{-/-} glomeruli treated with vehicle or H_2O_2 . a.u., arbitrary units Vs, versus.

Figure 7.



NOX4/TRPC6 pathway is important in podocyte calcium regulation and renal damage progression in diabetic kidney disease. The hypothesis featured in this manuscript offers elevated AngII levels as one of the potential stimuli that could lead to activation of NOX4 in diabetic conditions. This would lead to an augmented production of H₂O₂, which in turn would activate TRPC6 channels and stimulate calcium influx into the cells, eventually leading to podocyte hypertrophy and apoptosis. An alternative hypothesis suggests that high glucose in DKD can by itself activate RAS and result in ROS production and oxidative stress, which results in increased TRPC6 expression. Both pathways would lead to increased calcium influx in the podocytes.