Supplemental Data

Knockout of TRPC6 promotes insulin resistance and exacerbates glomerular injury in Akita mice

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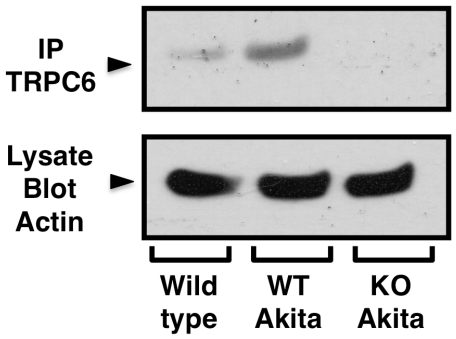
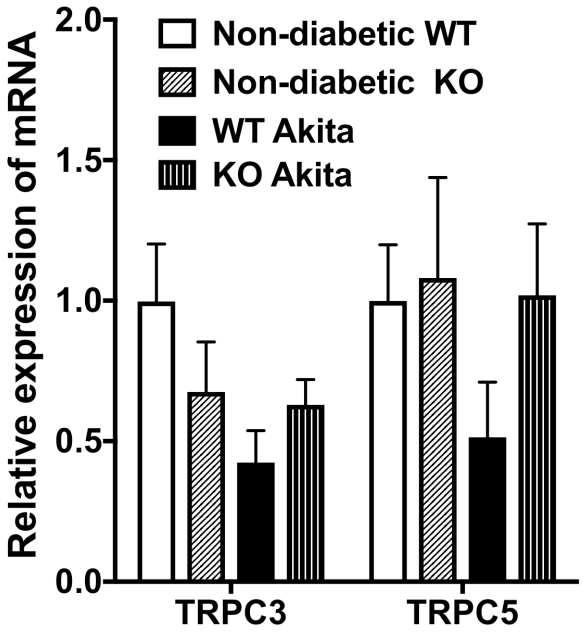
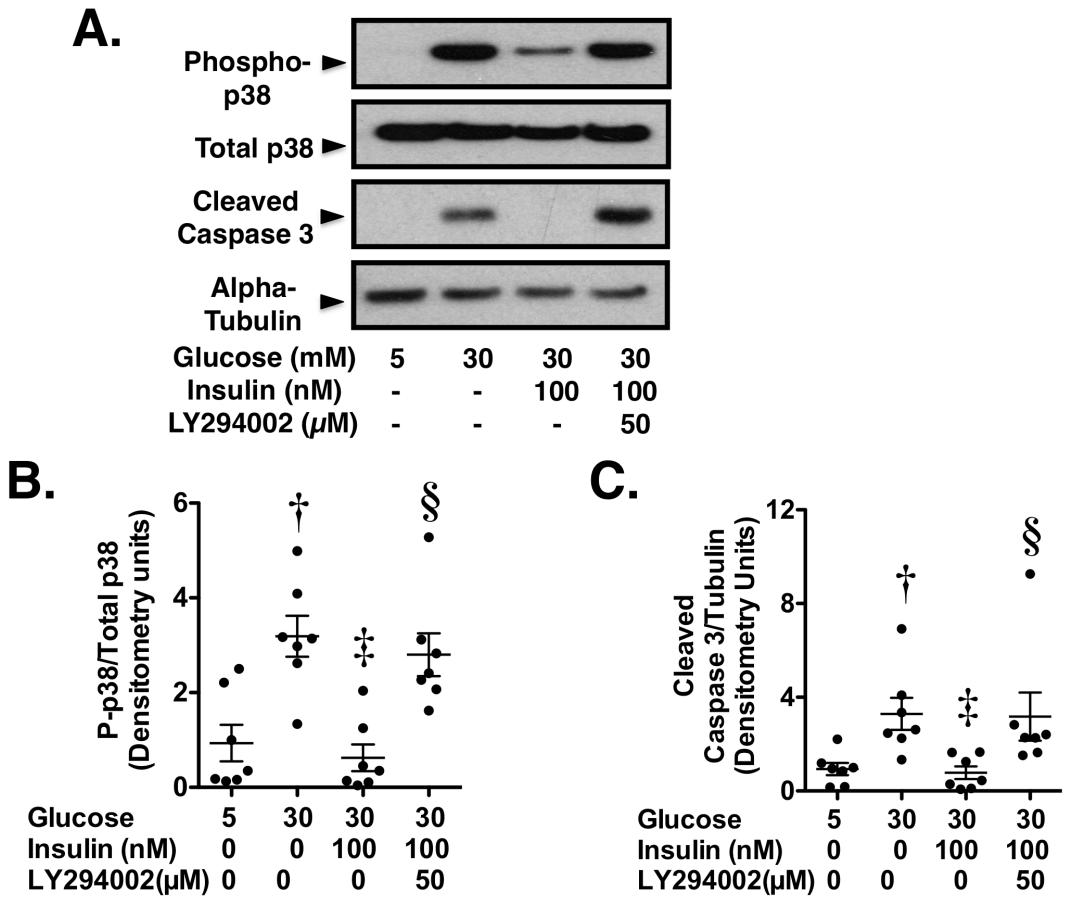


Supplementary Figure 1. Trpc6 was difficult to detect in kidney. We, therefore, immunoblotted for TRPC6 using lysates of brain as described in the Methods Section. TRPC6 protein levels tended to increase in WT Akita mice compared to non-diabetic WT controls. TRPC6 protein was not detected in the TRPC6 knockout (KO) Akita mice. Immunoblotting of the cell lysates for β-actin is shown in the lower panel. Similar results were obtained in 2 experiments.



Supplementary Figure 2. Expression of either TRPC3 or TRPC5 in glomerular preparations was not significantly altered in non-diabetic KO mice or in KO Akita mice. 5-6 mice were studied per group.



Supplementary Figure 3. (A & C) Hyperglycemia induced ASK1-p38 activation (enhanced levels of phosph-p38 or P-p38) and enhanced expression of the apoptosis marker cleaved caspase 3 in cultured podocytes. Both p38 activation and podocyte apoptosis were inhibited by insulin. In contrast, the PI3K-Akt inhibitor LY294002 blocked the anti-apoptotic effects of insulin treatment. †P<0.01 vs mM glucose, ‡P<0.01 vs 30 mM glucose, §P<0.05 vs 30 mM glucose and insulin

Materials and Methods

Experimental protocol: All experiments were performed using mouse strains on the FVB/NJ background. In previous studies (S1), we found that FVB/NJ Akita mice develop albuminuria and early histopathologic features of diabetic nephropathy (DN) in humans. To create Akita mice lacking TRPC6, we obtained TRPC6 KO mice through a collaboration with Dr. Michelle Winn at our institution, which were originally a gift of Dr. Lutz Birnbaumer (S2). For the experiments, we used male offspring because female Akita mice develop only mild hyperglycemia as well as modest functional and histologic features of diabetic kidney disease (S3).

For the experiments, the following groups were studied: diabetic mice expressing TRPC6 (WT Akita mice), diabetic mice lacking TRPC6 (KO Akita mice), non-diabetic mice expressing TRPC6 (WT controls), and non-diabetic mice lacking TRPC6 (KO controls). Fasting blood glucose measurements were made at 8, 12, and 16 and 20 weeks of age using the AlphaTRAK 2 testing system (Abbott Laboratories, Chicago, IL) calibrated for glucose measurements in mice according to directions of the manufacturer. Twenty-four hour urine collections were obtained at 12, 16 and 20 weeks of age using metabolic cages specifically designed for collection of mouse urine (Hatteras Instruments, Cary, NC). Systolic blood pressure (SBP) was measured at 12 and 20 weeks of age as described below. After the last urine collection, blood was obtained and then mice were sacrificed and kidneys removed. Kidneys were weighed and kidney tissue was saved in formalin, glutaraldehyde and OCT (Optimal Cutting Temperature compound) for light microscopy, transmission electron microscopy and immunofluorescence studies, respectively. The experiments conformed to the Guide for the Care and Use of Laboratory Animals (S4) and were approved by both the Duke and Durham Veterans Administration Medical Centers' Institutional Animal Care and Use Committees.

BP measurements: Systolic BP was measured using a computerized tail-cuff system (Hatteras Instruments, Cary, NC, USA) in conscious mice as previously described (S5). This technique has previously been shown to correlate closely with intra-arterial measurements (S6).

Histopathology: Light microscopic sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The slides were then evaluated by a pathologist (A.F.B.) blinded to genotype. Twenty-five glomeruli were evaluated in each tissue section to assess the average severity of mesangial expansion. Tubules and tubulointerstitial (TI) areas were examined for tubule injury, dilation and casts, and TI inflammation and fibrosis. No significant TI fibrosis was present in diabetic mice; so, this score was based on the severity of inflammation as described below. Abnormalities were graded using a semi-quantitative scale of 0-3 (0-normal, 1-mild, 2-moderate, 3-severe) as previously described (S5) based on the following criteria:

Mesangial expansion

1. Normal (baseline): Mesangial matrix occupies <10% of glomerular tuft area
2. Mild: 10-25% of the glomerular tuft area
3. Moderate: >25-50% of the glomerular tuft area
4. Severe: >50% of the glomerular tuft area

Tubule injury

1. Normal (baseline): None to minimal tubular dilation and casts without tubular degeneration or regeneration
2. Mild: Tubular degeneration and regeneration with/without tubular dilation and casts, involving <10% of cortex
3. Moderate: Tubular degeneration and regeneration with/without tubular dilation and casts, involving 10-25% of cortex
4. Severe: Tubular degeneration and regeneration with/without tubular dilation and casts, involving >25% of cortex

TI inflammation

1. Normal (baseline): One focus of inflammation (up to 15 mononuclear cells)
2. Mild: Two foci of 15+ mononuclear cells or 1 focus of 30+ mononuclear cells involving up to 5% of area of cortical parenchyma
3. Moderate: Inflammation involving >5-25% of the cortical parenchyma
4. Severe: Inflammation involving >25% of the cortical parenchyma

Kidney collagen content: Quantitation of kidney collagen content was performed using the Sirius Red/Fast Green Collagen Staining Kit from Chondrex (Redmond, WA). Briefly, deparaffinized tissue sections are stained with Sirius Red/Fast Green and the dye extracted from the tissue sections according to the manufacturer's directions. Optical density values were obtained at 540nm and 605nm and used to calculate the total collagenous and non-collagenous protein content, respectively.

Albuminuria: Urinary albumin concentrations were measured using a kit from AssayPro (St. Charles, MO) and urine creatinine levels were measured using a kit from Exocell (Philadelphia, PA). Urinary albumin excretion was expressed as the albumin/creatinine ratio.

Transmission electron microscopy (TEM) and GBM thickness: Electron microscopy was performed as previously described (S5). Analysis at the ultrastructural level was performed in a qualitative fashion and areas of interest selected in semithin sections for preparation of ultrathin sections for ultrastructural examination by a pathologist (A.F.B.) blinded to genotype. GBM width was measured using the orthogonal intercept method (S7) as previously described (S5).

Quantitation of glomerular volume, podocyte number and podocyte density: The mean glomerular volume (VGlom) was obtained from the arithmetic mean of the glomerular surface area using the method of Weibel and the equation described by Gundersen and coworkers (S8) as previously described (S5). To quantitate the number of podocytes per glomerular profile, podocyte nuclei in frozen tissue sections were stained with a rabbit polyclonal WT1 (Wilms tumor 1) antibody (rhodamine) (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-192), podocytes in the glomerular tuft were stained with a mouse monoclonal antibody to the podocyte marker synaptopodin (fluorescein) (Progen Biotechnik, catalog number 65194), and nuclei were countered stained with DAPI (4',6-diamidino-2-phenylindole) as previously

described (S5). Podocyte nuclei were then quantitated by counting nuclei that both co-localized with DAPI and were associated with synaptopodin staining. Using this technique, nuclear WT1 staining was consistently associated with synaptopodin stained cells. Podocyte number per glomerulus was quantitating using the methodology described by Wiggins and coworkers (S9) by converting the number of podocytes per glomerular profile into a podocyte density [Nv(P/Glom)] using an Excel spreadsheet supplied as supplementary data to the manuscript by Wiggins and colleagues (S9). An estimate of the total number of podocytes per glomerulus was then calculated by multiplying podocyte density by glomerular volume. For both the studies, all available glomeruli were evaluated in each frozen tissue sections (average number of glomeruli evaluated 23 ± 0.8 glomeruli with a range of 12-28).

Quantitative RT-PCR (Q-RTR-PCR): To evaluate expression of CN target genes, Q-RT-PCR was performed using the following primers: TRPC6 forward, 5'- TGC TTG ACT TTG GAA TGC TG -3'; reverse, 5'- GTT GTC CCC CAG TGT GAC TT -3'; COX2 forward, 5'- TGC AGA ATT GAA AGC CCT CT -3'; reverse, 5'- CCC CAA AGA TAG CAT CTG GA -3'; RCAN1 forward, 5'- CTC CTC CCG TTG GCT GGA AA -3'; reverse, 5'- CTG GGA GTG GTG TCT GTC GC; insulin receptor substrate 1 (IRS1) forward, 5' – GCCAGTCTTCATCCAGTTGC - 3'; reverse 5'- GGTTTCCCACCCACCATACT - 3'; IRS2 forward, 5'- CCC CAG TGT CCC CAT CCT - 3'; reverse, 5'- TTT CCT GAG TGA GAC ATT TTC CA - 3'; Cyclophilin, 5' – GGC CGA TGA CGA GCC C – 3'; reverse, 5' – TGT CTT TGG AAC TTT GTC TGC AA – 3'.

Immunofluorescence studies: Expression of COX2 in kidney sections was detected by indirect immunofluorescence using a rabbit monoclonal antibody to COX2 from Cell Signaling Technology (catalog number 12282) and a mouse monoclonal antibody to the podocyte marker synaptopodin (Progen Biotechnik), respectively. For the studies, frozen tissue sections were fixed in 2% paraformaldehyde for 5 minutes, air-dried, treated with 0.1% Triton-X in Dulbecco's

phosphate buffered saline (D-PBS) for 5 minutes and then blocked for 1 hour in D-PBS with 5% bovine serum albumin (BSA). The COX2 and synaptopodin primary antibodies were then added at a 1:200 dilution and 1:50, respectively in D-PBS with 5% BSA. After an overnight incubation, slides were washed 3 times in D-PBS and then incubated for 1 hour with an Alexa Fluor 488 labeled donkey anti-rabbit antibody (Life Technologies, A21206) and a rhodamine labeled goat anti-mouse antibody (Life Technologies, catalog number A6393) both at a dilution of 1:1000 in D-PBS with 5% BSA. Sides were then washed 3 times in D-PBS and then examined by confocal microscopy using a Zeiss 510 inverted confocal fluorescent microscope.

Culture of mouse glomerular podocytes: The immortalized mouse podocyte cell line was maintained in culture as previously described (S10).

Measurements of podocyte apoptosis: For the apoptosis studies, podocytes were plated in 6 well tissue culture clusters (Corning-Costar, Corning, NY) and then differentiated for 5-7 days (S10). After differentiation, podocytes were made quiescent by incubation in serum free medium overnight. Cells were then changed to either 5 mM glucose or 30 mM glucose in the presence or absence of 25 mM mannitol or the indicated concentrations of the mitogen-activated kinase kinase (MEK) inhibitor PD98059 (S11), the apoptosis signal regulated kinase 1(ASK1) inhibitor NQD1 (S12) or the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (S13) all from Tocris BioScience (Bristol, United Kingdom). After the overnight incubation, cells were harvested and apoptotic cells were identified by annexin V staining using a kits from BD Pharmingen (San Diego, CA) according to the directions of the manufacturer. Quantitation of the apoptotic cells was performed by flow cytometric analysis at the Duke Comprehensive Cancer facility. For the annexin V studies, apoptotic podocytes were differentiated from necrotic cells by counting cells that stained with both the annexin V antibody and 7-amino-actinomycin

D. In parallel studies, podocytes were harvested in lysis buffer for immunoblotting as described below.

Immunoblotting studies: Immunoblotting was performed as previously described (S5) using the following antibodies: 1. Rabbit polyclonal antibodies to total p38, phospho-p38, IRS2 and cleaved caspase 2. Rabbit monoclonal antibodies to IRS1, ERK (extracellular regulated kinase), phospho-ERK, Akt, Phospho-Akt (T308), phospho-Akt (S473) and COX2 (Cell Signaling Technology), and 3. A rabbit polyclonal antibody from Alomone Labs (Jerusalem Israel), 4. A mouse monoclonal antibody to actin (Chemicon International, Temecula, CA), and 5. A mouse monoclonal antibody to α-tubulin (Santa Cruz Biotechnology, Dallas, TX). Isolation of enriched glomerular pellets and preparation of cellular lysates were performed using previously described techniques (S5, S10). For the IRS protein studies using cultured podocytes, cells were made quiescent by incubation overnight in serum free Dulbecco's Modified Eagle Medium (DMEM), and then incubated in serum free DMEM with either 5 mM or 30 mM glucose and a 10 μM concentration of the proteasome inhibitor MG132 (S14) for 6 hours at 37°C in a humidified tissue culture incubator. (Note: Longer periods of incubation resulted in podocyte apoptosis as described in the apoptosis experiments above). After the incubation period, cells were solubilized in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 2mM ethylenediaminetetraacetic acid (EDTA), 1% IGEPAL CA-630 [NP-40]) with protease inhibitors (Sigma-Aldrich) as described below. For the IRS studies *ex vivo*, ~2 mm^3 of renal cortex was isolated sterilely and then incubated in serum free DMEM with either 5 mM or 30 mM glucose and 10μM of the proteasome inhibitor MG132 (S14). After incubation at 37°C for 6 hours in a humidified tissue culture incubator, cortices were homogenized in NP-40 lysis buffer using a Pro Scientific homogenizer (Oxford, CT) and then processed as described below.

For the immunoblotting studies, glomerular pellets, mouse brain or cultured podocytes were solubilized in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM sodium chloride, 2mM ethylenediaminetetraacetic acid (EDTA), 1% IGEPAL CA-630 [NP-40]) with protease inhibitors (Sigma-Aldrich) by sonication and frozen at -70°C until the time of study. For immunoblotting, proteins were separated using the XCell SureLock Bis-Tris Mini-Cell Electrophoresis System (Thermo Fisher Scientific, Waltham, MA) and transferred to PVDF (polyvinylidene fluoride) membranes according to the directions of the manufacturer. Immunoblots were then blocked in 20 mM Tris-HCl, 137 mM NaCl, pH 7.6 (TBS) with 0.2% Tween 20 (T-TBS) and 2% bovine serum albumin. The primary antibody was added at a concentration of 0.1 µg/ml in blocking buffer and incubated overnight. After washing, the HRP (horseradish peroxidase) linked secondary antibody to goat (Santa Cruz Biotechnology) was added a 1:2000 in blocking solution and incubated for 1 hour prior to washing. Protein detection was performed using enhanced chemiluminescence (ECL) (Thermo Scientific, Waltham, MA) according to the directions of the manufacturer. To assess protein-loading immunoblots were stripped according to the directions of the manufacturer and immunoblotting was performed using mouse monoclonal antibodies to either β-actin (0.5µg/ml) or α-tubulin (1:500) in blocking solution and an HRP-linked anti-mouse secondary antibody (1:2000) from Santa Cruz Biotechnology. For densitometry, the immunoblots were converted into a digital format using an Epson Perfection scanner 1670 (Seiko Epsom Corporation) and then analyzed using ScanAnalysis 2.5 software (Biosoft). For the analyses, the densitometric data for the renin protein signal were divided by the matched signal for β-actin. To compare separate immunoblots, densitometric data were normalized to non-diabetic mice.

Development of TRPC6 Knockout (KO) podocytes and insulin signaling studies: KO of TRPC6 was generated using CRISPR [(Clustered regularly interspaced short palindromic

repeats)/CAS9 (CRISPR associated protein 9)] methodologies (S15), and constructs from Origene Technologies (Rockville, MD). Transfection of the constructs was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Podocytes were then selected using puromycin (1μg/ml) added to complete medium. Control cells were transfected with a scrambled construct and selected in a similar fashion. After selection, 2 clones of control podocytes (TRPC6+/+) and 2 KO clones (TRPC-/-) were selected for study. For the insulin signaling studies, podocytes were plated in 6-well tissue culture clusters (Corning-Costar, Corning, NY) and then differentiated as previously described (S10). Cells were treated with the indicated doses of insulin, harvested in NP40 lysis buffer as described above with the modification that calyculin (Cell Signaling Technology) was added to the lysis buffer (50 nM concentration). Immunoblotting was then performed as described above.

Insulin tolerance test (ITT): The test was performed on random-fed mice as described by Bruning et al (S16). For the studies, 8-week old mice were injected with regular insulin (0.75 U/kg, IP) in ~0.1 ml 0.9% sterile saline. The insulin syringe was precoated with an insulin solution prior to the first injection to prevent absorption of insulin to the plastic of the syringe. A drop of blood is taken from the tail vein before the injection of insulin and after 15, 30, 45, 60, 90 and 120 min for the determination of blood glucose levels using a glucometer as described above.

Glucose tolerance test (GTT): The test was performed after fasting mice for 6 hours at the end of their dark (feeding) cycle. During the fasting period, mice are permitted free access to water. For the study, 8-week old mice are weighed and a 6-hour fasting blood glucose level is obtained from the tail vein using a glucometer (see above). After measuring fasting blood glucose levels, mice are injected with glucose (1 mg/, IP) in 0.9% sterile saline. Blood glucose values are then obtained at the 15 30, 60, and 120 minute time points. At the end of the study, a second blood

sample is obtained and the serum saved at -80°C to measure serum insulin levels using an ELISA kit for mice (ThermoFisher Scientific, Waltham, MA).

Statistical analysis: Data are presented as the mean ± standard error of the mean (SEM) and statistical analyses were performed using the Prism computer program (GraphPad Software, Inc.). For comparison of continuous variables, the following statistical methods were used: 1. A t-test for comparisons between two groups, or 2. A one-way Analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons post-test for comparisons between more than two groups. For non-continuous variables, data was analyzed using a Fishers Exact test. When more than 2 categories were present (such as diabetes and genotype), data was analyzed using a 2-way ANOVA followed a Bonferroni multiple comparisons post-test.

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