

Original Paper

Insulin Increases Expression of TRPC6 Channels in Podocytes by a Calcineurin-Dependent Pathway

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Key Words

Podocytes • Insulin • Transient receptor potential channel • Calcineurin-pathway

Abstract

Background/Aims: Insulin signaling to podocytes is relevant for the function of the glomerulus. Now, we tested the hypothesis that insulin increases the surface expression of canonical transient receptor potential canonical type 6 (TRPC6) channels in podocytes by a calcineurin-dependent pathway. **Methods:** We used quantitative RT-PCR, immunoblotting, immunofluorescence and fluorescence spectrophotometry in cultured podocytes. Activation of Nuclear Factor of Activated T-cells (NFATc1) was measured using a specific calorimetric assay. **Results:** Insulin increased the expression of TRPC6 transcripts and protein in podocytes. Insulin increased TRPC6 transcripts in a time and dose-dependent manner. The insulin-induced elevation of TRPC6 transcripts was blocked in the presence of tacrolimus, cyclosporine A, and NFAT-inhibitor (each $p < 0.01$ by ANOVA and Bonferroni's multiple comparison test). Transcripts of NOX4, another target gene of the calcineurin-NFAT pathway, were affected in a similar way. Immunoblotting showed that the administration of 100 nmol/L insulin increased TRPC6-proteins 2-fold within 48 hours. Insulin increased the activity of NFATc1 in nuclear extracts ($p < 0.001$) whereas tacrolimus, cyclosporine A, and NFAT-inhibitor blocked that insulin effect ($p < 0.001$; two way ANOVA). Immunofluorescence showed that insulin increased TRPC6-expression on the cell surface. Fluorescence-spectrophotometry and manganese quench experiments indicated that the increased TRPC6-expression after insulin administration was accompanied by an elevated transplasmamembrane cation influx. Insulin-stimulated surface expression of TRPC6 as well as transplasmamembrane cation influx could be reduced by pretreatment with tacrolimus. **Conclusion:** Insulin increases the expression of TRPC6 channels in podocytes by activation of the calcineurin-dependent pathway.

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Introduction

Diabetic nephropathy is a major cause of chronic kidney disease. Functional podocytes are important for the integrity of the glomerular filtration barrier to prevent albuminuria which is an early symptom of diabetic nephropathy [1]. As reviewed by Welsh & Coward, the two fundamental cellular changes that occur in diabetes mellitus are a failure of insulin to signal to cells and an environment of hyperglycemia [2]. Several recent studies investigated the effects of glucose on podocytes [3-7]. A recent study indicated that mice with specific deletion of the insulin receptor from their podocytes develop albuminuria together with histological features that resemble diabetic nephropathy, but in a normoglycemic environment. These studies pointed to some involved insulin signaling pathways in podocytes, e.g. the activation of the mitogen-activated protein kinases and phosphatidylinositol-4, 5-bisphosphate 3-kinase pathways [8]. Other groups showed that insulin increases the expression and mobilization of large-conductance Ca(2+)-activated K(+) channels as well as transient receptor potential canonical (TRPC6) channels in podocytes [9, 10]. Using biotinylation assays, Kim et al. showed that insulin increased surface expression of TRPC6 [10]. Kim et al. showed that insulin may increase the surface expression of TRPC6 channels in podocytes due to changes of reactive oxygen species [10]. Changes of TRPC6 channels have been associated with proteinuric diseases [11]. Recently, we showed an increased TRPC6 channel protein expression in podocytes from patients with diabetic nephropathy compared to control subjects [12]. We also reported that the expression of TRPC6 channels in podocytes can be modified by growth factors [12, 13]. These findings supported the view that insulin-dependent changes of TRPC6 channels may be involved in the pathogenesis of diabetic nephropathy. However, the underlying pathways are only partly known. Since the calcium-calmodulin-nuclear factor of activated T cell (NFAT)-pathway has been associated with diabetic nephropathy [14, 15], in the present study we investigated the objective that insulin-induced increase of TRPC6 in podocytes may be blocked by calcineurin inhibition. We present evidence that insulin modulates TRPC6 channels in cultured podocytes. Treatment with insulin increased TRPC6 transcripts, increased TRPC6 protein expression and transplasmamembrane cation influx into podocytes. Insulin-stimulated surface expression of TRPC6 could be reduced by pretreatment with the calcineurin inhibitors, tacrolimus and cyclosporine A as well as by NFAT-inhibitor.

Material and Methods

Preparation of cells

Conditionally immortalized human podocytes (podocyte cell line AB 8/13) were used as described [12, 16]. The cell preparation procedures had also been described by our group earlier [12, 13]. The podocytes were maintained in RPMI 1640 medium without glucose (Gibco, Life Technologies, CA, USA) supplemented with 5.6 mmol/L D-glucose, 10 % fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (Biochrom AG, Berlin, Germany). Podocytes were seeded on plates and were subjected to the experimental treatment at subconfluence. Podocytes were exposed to insulin (final concentration, 100 nmol/L) [10]. Tacrolimus, cyclosporine and NFAT-inhibitor were used at final concentrations of 15 ng/mL, 500 nmol/L and 1 µmol/L, respectively [17, 18]. TRPC6 knockdown using siRNA was performed as reported previously by our group [13].

RNA isolation and reverse transcription

The RNA isolation and reverse transcription procedures had been described by our group earlier [12, 13]. Minor modifications were due to different genes, primers or suppliers of reagents or equipment. Total RNA was isolated from podocytes using the RNeasy mini kit including RNase-free DNase set (Qiagen, Hilden, Germany). Using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany), cDNA was synthesized from 2 µg of total RNA using oligo dT (12-18) and 5 U AMV reverse transcriptase at 50°C for 60 min, followed by heating to 85°C for 5 min.

Quantitative real-time reverse transcriptase polymerase chain reactions

Quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCR) for transient receptor potential canonical type 6 (TRPC6), NOX4, and beta-actin were performed using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) and the following primers:

TRPC6 (Reference Sequence (RefSeq) database accession number: NM_004621); forward, 5'GCC AAT GAG CAT CTG GAAAT3'; reverse, 5'TGG AGT CAC ATC ATG GGAGA3';

Beta-actin (NM_001101.3); forward GGA CTT CGA GCA AGA GATGG; reverse AGC ACT GTG TTG GCG TACAG.

NADPH oxidase 4 (NM_001143836); forward AAT TTA GAT ACC CAC CCTCC, reverse TCT GTG GAA AAT TAG CTTGG.

LightCycler-Fast Start DNA SYBR Green I mix (Roche Diagnostics) and 500 nmol/L of each primer were used in a final volume of 20 μ L. The reaction was initiated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 10 s at 57°C, and extension at 72°C for 15 s. Melting curve analysis was performed from 65°C to 95°C with a heating rate of 0.1°C/s. Data were recorded on a LightCycler 2.0 Instrument using LightCycler Software Version 4.0 (Roche Diagnostics). The relative quantification method was used whereby the change in expression of target relative to the housekeeping gene was calculated. Control PCR was performed from samples containing RNA instead of cDNA.

PCR products were size fractionated on 1% agarose gels, and were visualized by ethidium bromide staining using an imaging analyzer (GelDoc 2000; BioRad Laboratories, Munich, Germany). The expected product size was 109 bp for TRPC6.

Immunoblotting of proteins

The immunoblottings had been described by our group earlier [12, 13, 19, 20]. Minor modifications were due to different proteins, suppliers of reagents or equipment. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond-ECL nitrocellulose membranes (NEN Life Science, Boston, MA, USA). Membranes were incubated with primary antibodies and were subsequently incubated with secondary IRDye800CW-infrared fluorescent dye-labeled sheep anti-rabbit antibodies (1:1000, Biomol, Hamburg, Germany) and Alexa Fluor 680-allophycocyanin-fluorescence-labeled donkey anti-goat antibodies (1:1000; Invitrogen). Imaging was performed using the Odyssey infrared imaging system (Licor Biosciences, Bad Homburg, Germany). Podocytes' plasma membrane proteins were biotinylated and isolated using a commercially available cell surface protein isolation kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's recommendations. The isolated proteins were identified by immunoblotting.

Immunofluorescence

Immunofluorescence had been described by our group earlier [13]. Minor modifications were due to different proteins, suppliers of reagents or equipment. Cells were fixed in 3.7% formaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 20 min. Samples were then blocked and incubated with primary TRPC6 antibodies from rabbit (1:100; Alomone Labs, Jerusalem, Israel) or GAPDH antibodies from goat (1:100; Santa Cruz Biotechnology, Inc., USA) overnight at 4°C. Samples were subsequently incubated with secondary Alexa Fluor 488-conjugated anti-rabbit antibodies (1:100; Invitrogen, Carlsbad, CA, USA), or secondary Alexa Fluor 594-conjugated anti-goat antibodies (Invitrogen). Nuclei were counterstained with 4', 6-diamidino -2-phenylindole (DAPI; Roche, Mannheim, Germany, 1:1500). For staining of actomyosin bundles (stress fibers), cells were fixed in 3.7% formaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, washed, and incubated in blocking buffer for 30 minutes. Samples were incubated with Alexa Fluor 488-conjugated phalloidin antibody (5 μ g/mL; Invitrogen) for 30 min, washed and nuclei were counterstained with DAPI. Cell images were taken using Fluid Cell Imaging Station (Life Technologies, Grand Island, NY, USA). Mean values of actomyosin bundles (stress fibers) from 20 areas of interest were determined.

Activation of Nuclear Factor of Activated T-cells (NFAT)

To evaluate the activation of NFAT in podocytes we used Nuclear Extract kit and TransAM NFATC1 transcription factor assay kit according to the manufacturer's instructions (Active Motif, Carlsbad, California, USA). Briefly, cultured podocytes were harvested, washed, lysed in hypotonic buffer and centrifuged for

30 seconds at 14,000 x g in a microcentrifuge. The nuclear fraction was resuspended in complete lysis buffer with dithiothreitol, lysis buffer AMI, and protease inhibitor cocktail and centrifuged at 14,000 x g in a microcentrifuge. The nuclear extract in complete lysis buffer was added to a microwell plate coated with the oligonucleotide 5'-AGGAAA-3', containing a NFAT consensus binding site, incubated for 1 hour, washed, primary NFATc1 antibody was added for 1 hour, washed, and secondary antimouse horseradish peroxidase conjugated antibody was added for 1 hour. The absorbance of the calorimetric reaction was measured after addition of a development solution and a stopping solution at 450 nm with a reference wavelength of 655 nm.

Measurements of cytosolic calcium using fluorescent dye technique

Measurements of cytosolic calcium using fluorescent dye technique had been described by our group earlier [12, 13, 19-21]. Minor modifications were due to different suppliers of reagents or equipment. For ratiometric imaging experiments podocytes were loaded with 10 $\mu\text{mol/L}$ of the calcium-sensitive, cell-permeable, intracellular fluorescence dye fluo-4 acetoxymethylester (Merck Biosciences, Darmstadt, Germany) at room temperature for 1h and then washed with physiological saline solution containing 134 mmol/L NaCl, 6 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L glucose, and 10 mmol/L HEPES (pH 7.4 with NaOH) to remove extraneous dye. Fluorescence was measured using a plate reader (Perkin Elmer EnSpire Multimode Plate Reader; Denmark) at 515nm emission with 488nm excitation. Baseline fluorescence was measured and stable fluorescence readings were obtained throughout. Calcium influx was induced by the known TRPC6 agonist oleoyl-2-acetyl-*sng*lycerol (OAG). The amplitudes were expressed as increase of the fluorescence ratio (F/F₀). To report unidirectional cation fluxes manganese quenching experiments were performed as described by our group [21].

Substances

Tacrolimus (molecular weight, 822 Da), cyclosporine A (molecular weight, 1202 Da), and NFAT-inhibitor were purchased from Sigma-Aldrich, St. Louis, MO, USA. The NFAT-inhibitor is a high-affinity calcineurin-binding peptide that inhibits Nuclear Factor of Activated T cells (NFAT) activation, has the amino acid sequence, Met-Ala-Gly-Pro-His-Pro-Val-Ile-Val-Ile-Thr-Gly-Pro-His-Glu-Glu, and has a molecular weight of 1683 Da. All substances were obtained from Sigma-Aldrich (St. Louis, MO, USA) if not indicated otherwise.

Statistical Analysis

All data were expressed as mean \pm SD. Comparisons between groups were analyzed using the t-test or ANOVA and Bonferroni's multiple comparison test as appropriate. The variation of NFATc1 activity in nuclear extracts caused by insulin and calcineurin inhibitors and NFAT-inhibitor was determined using two-way ANOVA (GraphPad Prism 5.0, La Jolla, CA, USA). A two-tailed probability value less than 0.05 was considered statistically significant. Where error bars do not appear on the figure, error was within the symbol size.

Results

Figure 1 shows the effect of insulin on TRPC6 transcripts in podocytes. Using quantitative real time RT-PCR, we detected TRPC6 transcripts in podocytes. Compared to control conditions the administration of insulin for 15 minutes increased TRPC6 transcripts by 3.2 ± 0.3 fold ($p < 0.01$). Increased TRPC6 transcript levels could be observed for up to 48 hours (Fig. 1A). The dose-dependent effect of insulin on TRPC6 transcripts is shown in Fig. 1B. We investigated the effects of calcineurin inhibitors, tacrolimus and cyclosporine A, and the NFAT-inhibitor (Fig. 1C). In the presence of tacrolimus, cyclosporine A, and NFAT-inhibitor the insulin-induced elevation of TRPC6 transcripts was blocked to 1.1 ± 0.2 , 0.9 ± 0.2 , and 0.8 ± 0.2 , respectively (each $p < 0.01$ by ANOVA and Bonferroni's multiple comparison test). According to literature, NOX4 may be another target gene of the calcineurin-NFAT pathway [10]. Compared to control conditions the administration of insulin increased NOX4 transcripts by 1.7 ± 0.5 fold ($p < 0.05$), whereas addition of tacrolimus, cyclosporine A,

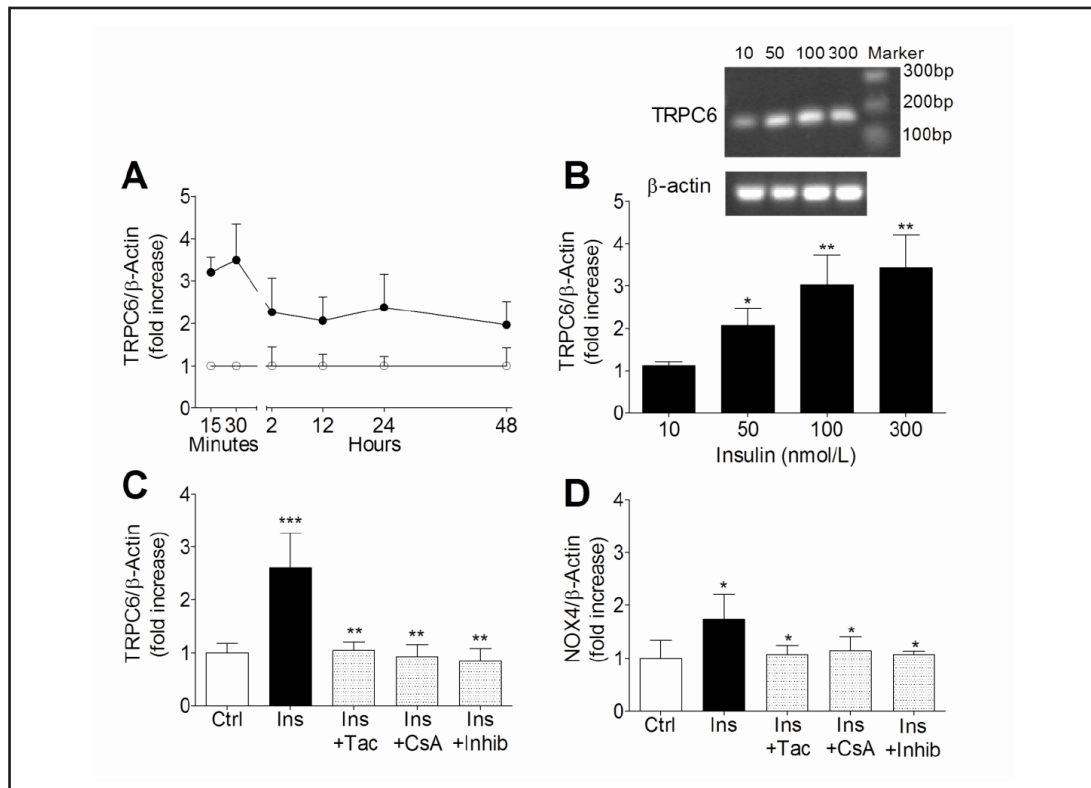


Fig. 1. Insulin increases transient receptor potential channels type 6 (TRPC6) transcripts in podocytes. (A) Quantitative real-time RT-PCR showing the time-dependent effect of insulin on TRPC6 transcripts. Mean \pm SD are given for the normalized ratio of TRPC6 expression compared to β -actin. (B) Dose-response curve of the effects of insulin on TRPC6 transcripts. Gel electrophoresis of PCR products from TRPC6 transcripts after administration of increasing doses of insulin is shown. β -Actin is shown for comparison. (C) Summary data of quantitative real-time RT-PCR for TRPC6 transcripts. *** $p < 0.001$ for the comparison insulin (Ins) and control (Ctrl). ** $p < 0.01$ for the comparison Ins vs insulin + tacrolimus (Ins + Tac), insulin + cyclosporine A (Ins + CsA), and insulin + NFAT-inhibitor (Ins + NFAT-Inhib), respectively (ANOVA and Bonferroni's multiple comparison test). (D) Summary data of quantitative real-time RT-PCR for NOX4 transcripts. * $p < 0.05$ for the comparison insulin (Ins) and control (Ctrl), Ins vs insulin + tacrolimus (Ins + Tac), insulin + cyclosporine A (Ins + CsA), and insulin + NFAT-inhibitor (Ins + NFAT-Inhib), respectively (ANOVA and Bonferroni's multiple comparison test).

and NFAT-inhibitor blocked the insulin-induced elevation of NOX4 transcripts to 1.1 ± 0.2 , 1.1 ± 0.3 , and 1.1 ± 0.1 , respectively (each $p < 0.05$ by ANOVA and Bonferroni's multiple comparison test; Fig. 1D).

Next, we established that the administration of 100 nmol/L insulin increased TRPC6-proteins in podocytes. Immunoblotting showed that insulin increased TRPC6 protein 2.2 ± 0.1 fold within 48 hours (Fig. 2A). In the presence of tacrolimus, the insulin-induced elevation of TRPC6 proteins was blocked to 1.3 ± 0.1 ($p < 0.01$ by ANOVA and Bonferroni's multiple comparison test; Fig. 2B).

After activation, calcineurin binds and dephosphorylates cytoplasmic NFAT, which then translocates to the nucleus of activated cells to induce expression of several genes. It is well known that tacrolimus, cyclosporine A, as well as selective inhibitors of NFAT-calcineurin-association, block downstream NFAT effectors [22]. Using a specific calorimetric assay we showed that insulin activated the activity of NFATc1 in nuclear extracts from podocytes (Fig. 3A). Two-way ANOVA showed that insulin accounted for 40% of the observed total variation activity of NFATc1 activity in nuclear extracts ($p < 0.001$). Furthermore, tacrolimus,

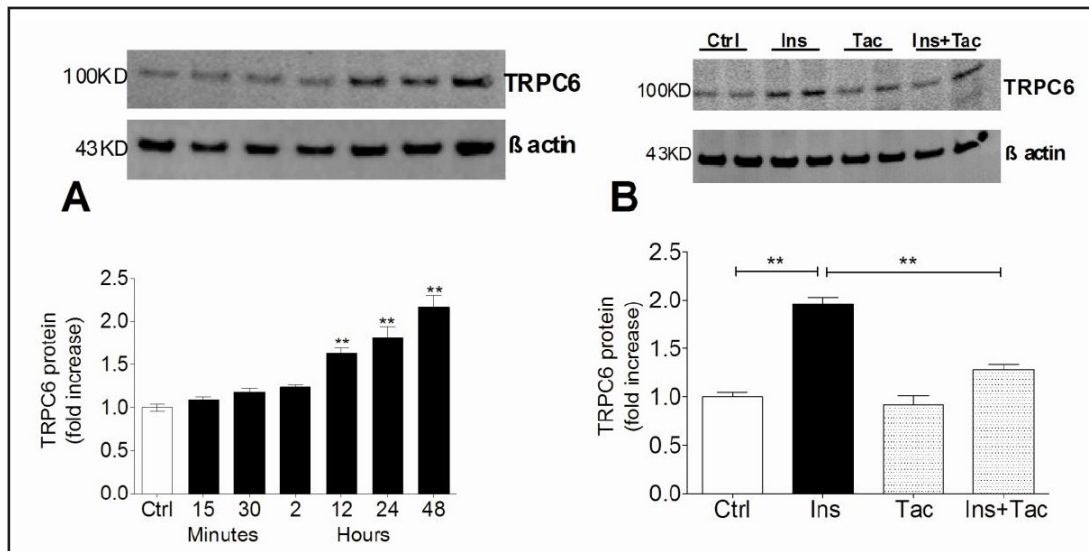


Fig. 2. Insulin increases transient receptor potential channels type 6 (TRPC6) protein in podocytes. (A) Representative immunoblottings (top panels) and summary data of TRPC6 protein in podocytes after administration of insulin. Data are mean \pm SD, ** $p < 0.01$ compared to control conditions by ANOVA and Bonferroni's multiple comparison test. (B) Representative immunoblottings and summary data (bar graphs) of TRPC6 protein under control conditions (Control) and in the presence of insulin (Ins), tacrolimus (Tac) or both, insulin plus tacrolimus (Ins + Tac). ** $p < 0.01$ compared to control conditions by ANOVA and Bonferroni's multiple comparison test.

cyclosporine A, and NFAT-inhibitor accounted for 42% of the observed total variation of the activity of NFATc1 in nuclear extracts ($p < 0.001$; Fig. 3A).

Immunofluorescence indicated that insulin mainly increased TRPC6-expression on the cell surface. This insulin-induced TRPC6 surface expression was blocked in the presence of tacrolimus, cyclosporine A, or NFAT-inhibitor (Fig. 3B). Morphology may be variable, however, no large morphological changes were observed.

Plasma membrane biotinylation assays showed that the administration of insulin increased the expression of TRPC6 in the plasma membrane (Fig. 3C and D).

Manganese quenching experiments indicated that an increased TRPC6-expression on the cell surface was accompanied by an increased transplasmamembrane cation influx into podocytes (Fig. 3E). Furthermore, the administration of insulin elevated the oleoyl-2-acetyl-snglycerol (OAG)-induced calcium increase by 22% (Fig. 3F and G). The elevated TRPC6-mediated cation influx was reduced after tacrolimus. It should be noted that OAG is not specific for activation of the TRPC6 isoform, therefore activation of other isoforms may contribute to the observed effects.

To clarify the effects of insulin on TRPC6 expression we used siRNA for TRPC6 knockdown in podocytes. Compared to control conditions with scramble RNA the administration of siRNA caused a reduction of TRPC6 transcripts to $32 \pm 5\%$ (Fig. 4A). Immunofluorescence showed that siRNA against TRPC6 reduced insulin-induced TRPC6-expression on the cell surface (Fig. 4B). We showed that insulin increased the activity of NFATc1 in nuclear extracts from podocytes from 0.25 ± 0.01 to 0.44 ± 0.03 arbitrary units ($p < 0.01$), whereas after administration of siRNA against TRPC6 the activity of NFATc1 was reduced to 0.31 ± 0.03 arbitrary units ($p < 0.01$ by ANOVA and Bonferroni's multiple comparison test; Fig. 4C). Furthermore, both tacrolimus as well as siRNA against TRPC6 attenuated the insulin-induced expression of stress-fibers in podocytes (Fig. 4D and E). These results underscore that the insulin-induced expression of TRPC6 channels in podocytes further enhances the calcium-calcineurin-NFAT pathway.

Fig. 3. Insulin activates NFAT in podocytes and increases TRPC6 protein localized in the plasma membrane. (A) Summary data of a specific calorimetric assay of NFATc1 in nuclear extracts from podocytes. Insulin accounted for 40% ($p < 0.001$), whereas tacrolimus, cyclosporine A, and NFAT-inhibitor accounted for 42% of the observed total variation of the activity of NFATc1 in nuclear extracts ($p < 0.001$). (B) Representative immunofluorescence of TRPC6 protein under control conditions (Ctrl) and in the presence of insulin (Ins), tacrolimus (Tac), insulin + tacrolimus (Ins + Tac), insulin + cyclosporine A (Ins + CsA), and insulin + NFAT-inhibitor (Ins + Inhib). Scale bar = 10 μm . Immunofluorescence data may only be interpreted semi-quantitatively. (C) Plasma membrane biotinylation assays of plasma membrane and intracellular TRPC6 expression. (D) The ratios of plasma membrane to intracellular TRPC6 were summarized ($n = 3$ in each group; $* p < 0.05$ between indicated groups by ANOVA with Bonferroni's multiple comparison test). (E) Representative manganese quenching experiments showing oleoyl-2-acetyl-snglycerol (OAG)-induced and TRPC6-mediated electrolyte influx into podocytes. Fluorescence values are shown after stimulation with OAG under control conditions (Control) and in the presence of insulin (Ins), tacrolimus (Tac) or both, insulin plus tacrolimus (Ins + Tac). Representative fluorescence measurements (F) and summary data (G) of oleoyl-2-acetyl-snglycerol (OAG)-induced and TRPC6-mediated calcium increase in podocytes. The F/F₀ ratio is given after stimulation with OAG under control conditions (Control) and in the presence of insulin (Ins), tacrolimus (Tac) or both, insulin plus tacrolimus (Ins + Tac). $* p < 0.05$.

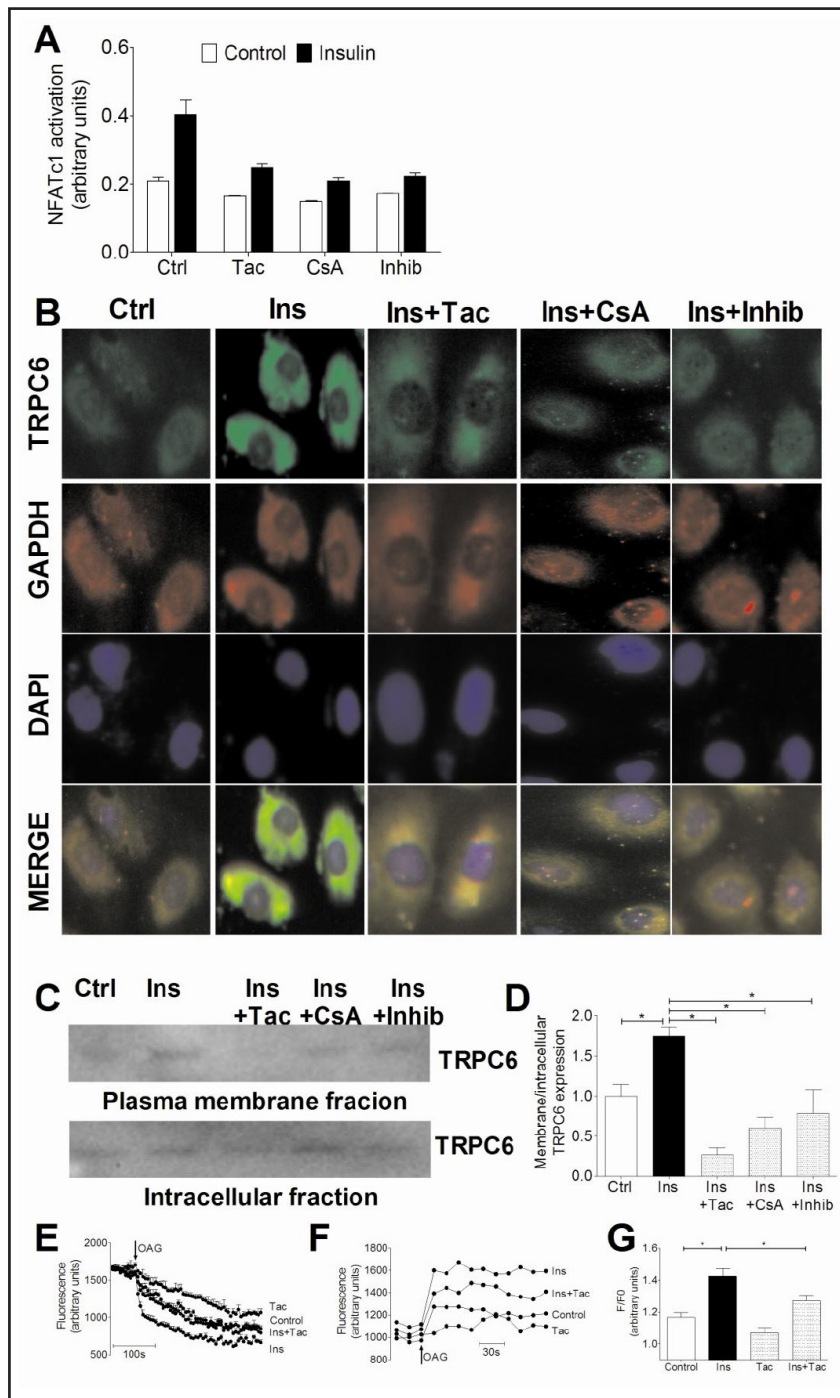
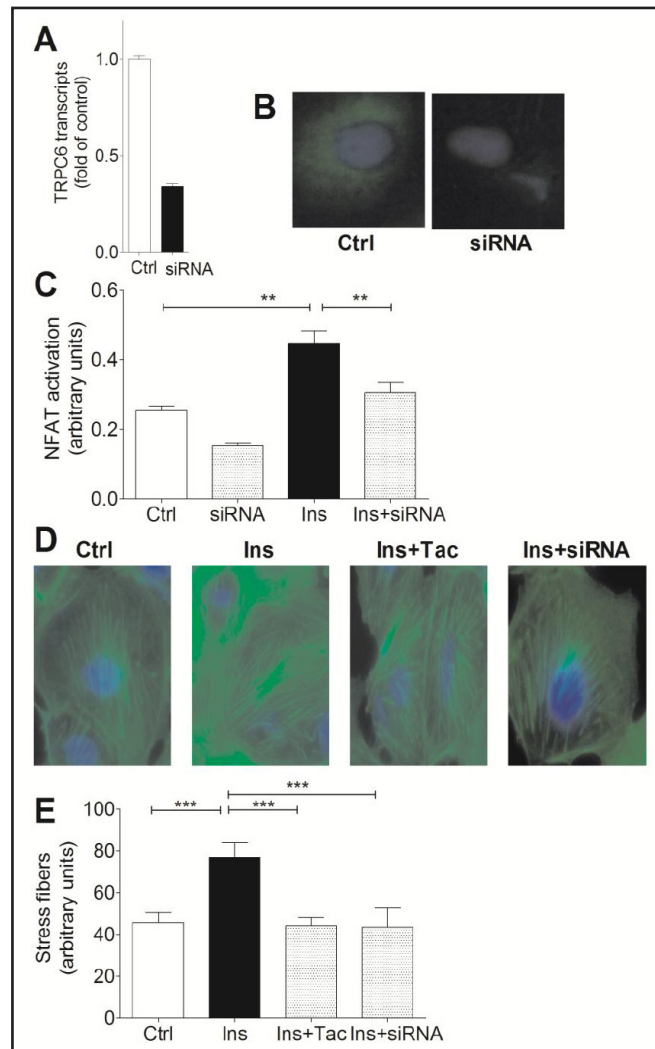


Fig. 4. siRNA against TRPC6 modulates insulin-dependent NFATc1 activity and podocyte activation. (A) Effect of siRNA on TRPC6 transcripts. Ctrl indicates scramble RNA. (B) Representative immunofluorescence of insulin-induced TRPC6 protein in podocytes under control conditions (Ctrl) and after administration of siRNA against TRPC6 (siRNA). (C) Bar graph of NFATc1 activity in nuclear extracts from podocytes under control conditions (Ctrl), in the presence of siRNA against TRPC6 (siRNA), after administration of insulin (Ins), and after administration of both siRNA against TRPC6 and insulin (Ins + siRNA). ** $p < 0.01$ between indicated groups (ANOVA and Bonferroni's multiple comparison test). (D) Expression of stress-fibers in podocytes after stimulation with insulin for 24 hours in the absence and presence of tacrolimus and siRNA against TRPC6. (E) Summary data of stress fiber analyses which were performed in 20 areas of interest for each condition. The coefficient of variation was 11%, 10%, 9%, and 21% for Ctrl, Ins, Ins + Tac, and Ins + siRNA, respectively. *** $p < 0.001$ for the comparison between the indicated groups by ANOVA and Bonferroni's multiple comparison test.



Discussion

The main finding of our present study is that insulin increases the expression of TRPC6 channels in podocytes by activation of the calcineurin-dependent pathway. We observed that insulin increased TRPC6 transcripts, TRPC6 proteins, TRPC6 localized in the plasma membrane of podocytes, as well as transplasmamembrane cation influx.

Several investigations showed that TRPC6 plays an important role in podocyte function. The upregulation of TRPC6 has been associated with acquired proteinuric kidney diseases [23], whereas TRPC6-deficient mice have been reported to be resistant to angiotensin II-induced proteinuria [24]. Furthermore our group reported increased TRPC6 channel protein in podocytes from patients with diabetic nephropathy compared to control subjects [12].

We showed increased TRPC6 transcripts for up to 48 hours after activation. Others also showed that the activation of podocytes increases TRPC6 transcripts within 12 to 24 hours [25]. An increased TRPC6 expression may activate the calcium-calmodulin-dependent serine/threonine protein phosphatase, calcineurin, which dephosphorylates nuclear factor of activated T cells which then subsequently translocates to the cell nucleus. Earlier studies indicated that activation of this calcium-calmodulin-dependent calcineurin-nuclear factor of activated T cells-pathway causes podocytes damage [26]. In cardiac cells TRPC6 has been described as a positive regulator of calcium-calmodulin-nuclear factor of activated T-cells-signaling during pathologic cardiac remodeling [27]. The present study in podocytes showed

that siRNA against TRPC6 reduced NFATc1 activity, indicating that the insulin-induced expression of TRPC6 channels in podocytes further enhances the calcium-calcineurin-NFAT pathway probably by a positive regulation loop. In podocytes, Nijenhuis et al. reported that the angiotensin-II-induced TRPC6 expression required the activation of the calcineurin as well as the nuclear factor of activated T cells [28]. It should be noted that other substances, i.e. angiotensin-II, which stimulate podocytes also activated the calcium-calcineurin-NFAT pathway [28]. They also showed that a podocyte-specific inducible expression of a constitutively active NFAT cells-mutant increased TRPC6 expression and induced severe proteinuria [28]. Schlöndorff et al. described that TRPC6 mutations which enhance channel activity caused an enhanced basal nuclear factor of activated T cells-mediated transcription in podocytes [29]. In support of these findings, Liu et al. showed that the administration of the calcineurin inhibitor, tacrolimus, reduced enhanced glomerular TRPC6 protein expressions in adriamycin-treated rats [30].

Our present study now indicated that inhibition of calcineurin blocks the insulin-induced expression of TRPC6 channels in podocytes. Our results are in line with previous studies, showing that the inhibition of calcineurin reduced renal hypertrophy and blocked glomerular hypertrophy in kidneys from diabetic rats [31]. In addition, whole kidney and glomerular hypertrophy were also reduced in diabetic calcineurin A- β knockout mice [32]. It may be concluded that the insulin-TRPC6-calcium/calmodulin-calcineurin-nuclear factor of activated T cells-pathway may be important for the pathogenesis of diabetic nephropathy. In summary, we show that insulin increased the expression of TRPC6 channels in podocytes by a calcineurin-dependent pathway.

Acknowledgements

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

References

- 1 Brinkkoetter PT, Ising C, Benzing T: The role of the podocyte in albumin filtration. *Nat Rev Nephrol* 2013;9:328-336.
- 2 Welsh GI, Coward RJ: Podocytes, glucose and insulin. *Curr Opin Nephrol Hypertens* 2010;19:379-384.
- 3 Chen T, Zheng LY, Xiao W, Gui D, Wang X, Wang N: Emodin ameliorates high glucose induced-podocyte epithelial-mesenchymal transition in-vitro and in-vivo. *Cell Physiol Biochem* 2015;35:1425-1436.
- 4 Gui D, Wei L, Jian G, Guo Y, Yang J, Wang N: Notoginsenoside R1 ameliorates podocyte adhesion under diabetic condition through $\alpha 3\beta 1$ integrin upregulation *in vitro* and *in vivo*. *Cell Physiol Biochem* 2014;34:1849-1862.
- 5 Wang C, Li C, Peng H, Ye Z, Zhang J, Liu X, Lou T: Activation of the Nrf2-ARE pathway attenuates hyperglycemia-mediated injuries in mouse podocytes. *Cell Physiol Biochem* 2014;34:891-902.
- 6 Guo J, Xia N, Yang L, Zhou S, Zhang Q, Qiao Y, Liu Z: GSK-3 β and vitamin D receptor are involved in β -catenin and snail signaling in high glucose-induced epithelial-mesenchymal transition of mouse podocytes. *Cell Physiol Biochem* 2014;33:1087-1096.
- 7 Kong F, Ma L, Zou L, Meng K, Ji T, Zhang L, Zhang R, Jiao J: Alpha1-Adrenergic receptor activation stimulates calcium entry and proliferation via TRPC6 channels in cultured human mesangial cells. *Cell Physiol Biochem* 2015;36:1928-1938.

- 8 Welsh GI, Hale LJ, Eremina V, Jeansson M, Maezawa Y, Lennon R, Pons DA, Owen RJ, Satchell SC, Miles MJ, Caunt CJ, McArdle CA, Pavenstädt H, Tavaré JM, Herzenberg AM, Kahn CR, Mathieson PW, Quaggin SE, Saleem MA, Coward RJ: Insulin signaling to the glomerular podocyte is critical for normal kidney function. *Cell Metab* 2010;12:329-340.
- 9 Kim EY, Dryer SE: Effects of insulin and high glucose on mobilization of slo1 BKCa channels in podocytes. *J Cell Physiol* 2011;226:2307-2315.
- 10 Kim EY, Anderson M, Dryer SE: Insulin increases surface expression of TRPC6 channels in podocytes: role of NADPH oxidases and reactive oxygen species. *Am J Physiol Renal Physiol* 2012;302:F298-F307.
- 11 Dryer SE, Reiser J: TRPC6 channels and their binding partners in podocytes: role in glomerular filtration and pathophysiology. *Am J Physiol Renal Physiol* 2010;299:F689-F701.
- 12 Thilo F, Liu Y, Loddenkemper C, Schuelein R, Schmidt A, Yan Z, Zhu Z, Zakrzewicz A, Gollasch M, Tepel M: VEGF regulates TRPC6 channels in podocytes. *Nephrol Dial Transplant* 2012;27:921-929.
- 13 Liu Y, Echtermeyer F, Thilo F, Theilmeyer G, Schmidt A, Schülein R, Jensen BL, Loddenkemper C, Jankowski V, Marcussen N, Gollasch M, Arendshorst WJ, Tepel M: The proteoglycan syndecan 4 regulates transient receptor potential canonical 6 channels via RhoA/Rho-associated protein kinase signaling. *Arterioscler Thromb Vasc Biol* 2012;32:378-385.
- 14 Gooch JL, Pèrgola PE, Guler RL, Abboud HE, Barnes JL: Differential expression of calcineurin A isoforms in the diabetic kidney. *J Am Soc Nephrol* 2004;15:1421-1429.
- 15 Zhang L, Li R, Shi W, Liang X, Liu S, Ye Z, Yu C, Chen Y, Zhang B, Wang W, Lai Y, Ma J, Li Z, Tan X: NFAT2 inhibitor ameliorates diabetic nephropathy and podocyte injury in db/db mice. *Br J Pharmacol* 2013;170:426-439.
- 16 Saleem MA, Zavadil J, Bailly M, McGee K, Witherden IR, Pavenstadt H, Hsu H, Sanday J, Satchell SC, Lennon R, Ni L, Bottinger EP, Mundel P, Mathieson PW: The molecular and functional phenotype of glomerular podocytes reveals key features of contractile smooth muscle cells. *Am J Physiol Renal Physiol* 2008;295:F959-F970.
- 17 Liao R, Liu Q, Zheng Z, Fan J, Peng W, Kong Q, He H, Yang S, Chen W, Tang X, Yu X: Tacrolimus protects podocytes from injury in lupus nephritis partly by stabilizing the cytoskeleton and inhibiting podocyte apoptosis. *PLoS One* 2015;10:e0132724.
- 18 Li X, Zhang X, Li X, Wang X, Wang S, Ding J: Cyclosporine A protects podocytes via stabilization of cofilin-1 expression in the unphosphorylated state. *Exp Biol Med (Maywood)* 2014;239:922-936.
- 19 Liu Y, Thilo F, Kreutz R, Schulz A, Wendt N, Loddenkemper C, Jankowski V, Tepel M: Tissue expression of TRPC3 and TRPC6 in hypertensive Munich Wistar Frömter rats showing proteinuria. *Am J Nephrol* 2010;31:36-44.
- 20 Thilo F, Baumunk D, Krause H, Schrader M, Miller K, Loddenkemper C, Zakrzewicz A, Krueger K, Zidek W, Tepel M: Transient receptor potential canonical type 3 channels and blood pressure in humans. *J Hypertens* 2009;27:1217-1223.
- 21 Tepel M, Bachmann J, Schluter H, Zidek W: Diadenosine polyphosphate-induced increase in cytosolic free calcium in vascular smooth muscle cells. *J Hypertens* 1995;13:1686-1688.
- 22 Yu H, Bot I, Sliedregt K, Xu X, Bot M, van Heiningen SH, van der Marel GA, Bennett MR, Overkleeft H, van Berkel TJ, Biessen EA: Selective modulation of nuclear factor of activated T-cell function in restenosis by a potent bipartite peptide inhibitor. *Circ Res* 2012;110:200-210.
- 23 Möller CC, Wei C, Altintas M M, Li J, Greka A, Ohse T, Pippin JW, Rastaldi MP, Wawersik S, Schiavi S, Henger A, Kretzler M, Shankland S J, Reiser J: Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. *J Am Soc Nephrol* 2007;18:29-36.
- 24 Eckel J, Lavin PJ, Finch EA, Mukerji N, Burch J, Gbadegesin R, Wu G, Bowling B, Byrd A, Hall G, Sparks M, Zhang Z S, Homstad A., Barisoni L, Birbaumer L, Rosenberg P, Winn MP: TRPC6 enhances angiotensin II-induced albuminuria. *J Am Soc Nephrol* 2011;22:526-535.
- 25 Yang H, Zhao B, Liao C, Zhang R, Meng K, Xu J, Jiao J: High glucose-induced apoptosis in cultured podocytes involves TRPC6-dependent calcium entry via the RhoA/ROCK pathway. *Biochem Biophys Res Commun* 2013;434:394-400.
- 26 Wang Y, Jarad G, Tripathi P, Pan M, Cunningham J, Martin DR, Liapis H, Miner JH, Chen F: Activation of NFAT signaling in podocytes causes glomerulosclerosis. *J Am Soc Nephrol* 2010;21:1657-1666.
- 27 Kuwahara K, Wang Y, McAnally J, Richardson J A, Bassel-Duby R, Hill JA, Olson EN: TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. *J Clin Invest* 2006;116:3114-3126.

- 28 Nijenhuis T, Sloan AJ, Hoenderop JG, Flesche J, van Goor H, Kistler AD, Bakker M, Bindels RJ, de Boer RA, Möller CC, Hamming I, Navis G, Wetzels JF, Berden JH, Reiser J, Faul C, van der Vlag J: Angiotensin II contributes to podocyte injury by increasing TRPC6 expression via an NFAT-mediated positive feedback signaling pathway. *Am J Pathol* 2011;179:1719-1732.
- 29 Schlöndorff J, Del Camino D, Carrasquillo R, Lacey V, Pollak MR: TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription. *Am J Physiol Cell Physiol* 2009;296:C558-C569.
- 30 Liu Y, Ji Z: FK506 alleviates proteinuria in rats with adriamycin-induced nephropathy by down-regulating TRPC6 and CaN expression. *J Nephrol* 2012;25: 918-925.
- 31 Gooch JL, Barnes JL, Garcia S, Abboud HE: Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation. *Am J Physiol Renal Physiol* 2003;284:F144-F154.
- 32 Reddy RN, Knotts TL, Roberts BR, Molkentin JD, Price SR, Gooch JL: Calcineurin A- β is required for hypertrophy but not matrix expansion in the diabetic kidney. *J Cell Mol Med* 2011;15:414-422.