Biochemical and Biophysical Research Communications 450 (2014) 312–317

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

High glucose modifies transient receptor potential canonical type 6 channels via increased oxidative stress and syndecan-4 in human podocytes

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ARTICLE INFO

Article history:
Received 20 May 2014
Available online 2 June 2014

Keywords:
Diabetic nephropathy
Podocytes
Transient receptor potential channels
Syndecan-4
High glucose
Oxidative stress

ABSTRACT

Transient receptor potential canonical (TRPC) channels type 6 play an important role in the function of human podocytes. Diabetic nephropathy is characterized by altered TRPC6 expression and functions of podocytes. Thus, we hypothesized that high glucose modifies TRPC6 channels via increased oxidative stress and syndecan-4 (SDC-4) in human podocytes.

Human podocytes were exposed to control conditions (5.6 mmol/L D-glucose), high glucose (30 mmol/L D-glucose or L-glucose), 100 μmol/L peroxynitrite, or high glucose and the superoxide dismutase mimetic tempol (100 μmol/L). TRPC6 and SDC-4 transcripts and protein expression were measured using RT-PCR and in-cell Western assay. Intracellular reactive oxygen species (ROS) and cytosolic calcium were measured using fluorescent dye techniques.

High D-glucose increased TRPC6 transcripts to 8.66 ± 4.08 ($p < 0.05$) and TRPC6 protein expression to 1.44 ± 0.07 ($p < 0.05$) without altering SDC-4 transcripts or protein expression. The D-glucose induced increase of TRPC6 expression was blocked by tempol. Increased oxidative stress using peroxynitrite significantly increased TRPC6 transcripts to 4.29 ± 1.26 ($p < 0.05$) and TRPC6 protein expression to 1.28 ± 0.05 ($p < 0.05$) without altering SDC-4 transcripts or protein expression. In human podocytes transfected with scrambled siRNA, high D-glucose increased ROS after 90 min to 3.55 ± 0.08 arbitrary units while 5.6 mmol/L D-glucose increased ROS to 2.49 ± 0.09 ($p < 0.001$) only. The increase in ROS was inhibited by tempol and by SDC-4 knockdown.

High glucose modifies TRPC6 channels and ROS production via SDC-4 in human podocytes.

1. Introduction

Increased transient receptor potential canonical (TRPC) channel type 6 and syndecan-4 (SDC-4) have been associated with altered podocyte function in diabetic nephropathy. Podocytes are specialized cells in kidney glomeruli that cover the urinary surface of the filtering capillaries, normally preventing protein leakage into the urinary space [1].

Transient receptor potential canonical 6 (TRPC6) channels in podocytes have been recognized to regulate the glomerular filtration barrier, thus serving as an important determinant of glomerular permeability [2–4]. Patients and mice with proteinuric kidney disease show an increased expression of native TRPC6 in podocytes [4,5]. We previously showed that TRPC6 expression is increased by high D-glucose-induced oxidative stress leading to increased calcium influx in human monocytes [6]. In a previous study our group also showed that patients with diabetic nephropathy have increased TRPC6 expression in renal cortex [7]. Other groups confirmed that high glucose induces apoptosis in podocytes by stimulating TRPC6 [8]. Finally it has been shown that high glucose-induced apoptosis in cultured podocytes involves TRPC6-dependent calcium entry via the RhoA/ROCK pathway [9].

Syndecan-4 (SDC-4), a member of the type I transmembrane heparan sulfate proteoglycan superfamily, is a major modulator of signal transduction and regulates localization and activity of proteins and channels [10–12]. Recent reports implicate changes in syndecan-4 with kidney diseases [13–15].

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http://dx.doi.org/10.1016/j.bbrc.2014.05.116
0006-291X/

It has been shown that syndecan-4 regulates TRPC6 channels, supporting the role of syndecan-4 for the regulation of functions in podocytes [16].

Since diabetic nephropathy is characterized by altered TRPC6 expression and functions of podocytes, in the present study we investigated the hypothesis, that high glucose modifies TRPC6 channels via increased oxidative stress and syndecan-4 in human podocytes.

2. Materials and methods

2.1. Preparation of cells

Conditionally immortalized human podocytes (podocyte cell line AB 8/13) cloned from the outgrowth of human glomeruli were a gift from Dr. Saleem (Bristol, UK) [17]. 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). Cells were cultivated at 33 °C (permissive condition) for propagation and at 37 °C (non-permissive condition resulting in the inactivation of the SV40 large T-antigen) for differentiation. Podocytes were seeded on plates and were subjected to the experimental treatment at subconfluence.

To evaluate the effects of high glucose and oxidative stress on TRPC6 and syndecan-4 expression podocytes were exposed to 5.6 mmol/L D-glucose (Control), 30 mmol/L D-glucose, or 100 μmol/L peroxynitrite for 4 h. Additional experiments were performed using 30 mmol/L L-glucose or 30 mmol/L D-glucose in the presence of the superoxiddismutase (SOD) mimetic tempol (100 μmol/L) [18].

siRNA knockdown of TRPC6 and syndecan-4. Podocytes were transfected with siRNA specific for TRPC6 or syndecan-4 using the silencer siRNA transfection kit (Ambion, Cambridgeshire, UK). The target sequence for TRPC6 in human podocytes was 5′-GGACUAUCUGCUCAUGGACtt-3′ (sense) and 5′-GUCCAUGAGC AGAUAGUCCtg-3′ (antisense). The target sequence for syndecan-4 in human podocytes was 5′-CUACUGCUCAUGUACCGUAtt-3′ (sense) and 5′-UACGGUACAUGAGCAGUAGga-3′ (antisense). Scrambled siRNA (Ambion) had no significant homology to any known human gene sequence.

RNA isolation and reverse transcription. 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.

2.2. 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), syndecan-4, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and TATA box binding protein (TBP) were performed using a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). The primers were as follows: TRPC6 (Reference Sequence (RefSeq) database accession number: NM_004621), forward, 5′GCCAATGAGCATCTGGAAAT3′; reverse, 5′TGGAGTCACATCA TGGGAGA3′; syndecan-4 (NM_002999.3) forward 5′TCTGTTCGCGC TGCTGCTGT3′; reverse 5′TTGGCTCCCAGACCCTGCCC3′; GAPDH (NM _002046), forward, 5′AACTGCTTAGCACCCCTGGC3′; reverse, 5′ATGA CCTTGCCCACAGCCTT3′; TBP (NM_003194.3) forward, 5′GAATATAAT CCCAAGCGGTTTG3′; reverse, 5′ACTTCACATCACAGCTCCCC3′.

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 μL. 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 69 °C (syndecan-4), at 57 °C (TRPC6) or at 58 °C (GAPDH, TBP), 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 the target genes (TRPC6, syndecan-4) relative to the housekeeping gene (GAPDH or TBP) was calculated. Control PCR was performed from samples containing RNA instead of cDNA.

For validation of the use of different housekeeping genes we plotted normalized ratios of GAPDH and TBP according to Bland and Altman [19]. We reasoned that plots depicting percentual differences between measurements of these two genes plotted against the mean of two measurements show a linear relationship in case the genes are directly related to each other, i.e. both genes represent housekeeping genes. There was a linear relationship when comparing GAPDH and TBP, indicating that GAPDH and TBP are both suitable housekeeping genes. In contrast plotting of GAPDH and TRPC6 according to Bland Altman showed a significantly skewed relationship, indicating that TRPC6 represents a true target gene in our experiments, not a housekeeping gene.

2.3. Immunofluorescence assay of TRPC6 channels and syndecan-4 protein

For the identification of TRPC6 channels and syndecan-4 proteins, quantitative in-cell Western assays of human podocytes were performed using the Odyssey infrared imaging system (Licor Biosciences, Bad Homburg, Germany). Human podocyte cell suspension was delivered to 96-well plates and incubated for 24 h, followed by fixation with 4% formaldehyde solution, permeabilization with Triton X-100 and blocking with Odyssey Blocking Buffer (Licor Biosciences, Bad Homburg, Germany). Afterwards, cells were incubated with rabbit anti-human syndecan-4 (1:200, Santa Cruz Biotechnology, USA), rabbit anti-human TRPC6 (1:200, alomone labs, Jerusalem, Israel) or goat anti-human GAPDH-primary antibodies (1:200) for 2 h, washed, incubated with IRDye 800 CW-or IRDye 680 RD-infrared fluorescent dye-conjugated secondary donkey anti-rabbit or anti-goat antibodies (1:200 for IRDye 680 RD or 1:800 for IRDye 800 CW; Licor Biosciences, Bad Homburg, Germany) for 1 h at Room temperature. Imaging was performed at 810 nm emission with an excitation wavelength of 780 nm. Control experiments were performed with omission of primary antibodies.

2.4. Measurements of reactive oxygen species using fluorescent dye technique

Podocytes were incubated with the dye 2′,7′-dichlorofluorescin diacetate (DCF-DA, 50 μmol/L) for 60 min and then washed and resuspended in HBSS. DCF-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of reactive oxygen species, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein which was monitored spectrophotometrically in a temperature-controlled 96-well-fluorescent plate reader at 37 °C (Varioskan Flash, Thermo Fisher Scientific, MA, USA) at 518 nm or 530 nm emission with an excitation wavelength of 485 nm.

2.5. Measurements of cytosolic calcium using fluorescent dye technique

For ratiometric imaging experiments podocytes were loaded with 10 μmol/L of the calcium-sensitive, cell-permeable, intracellular

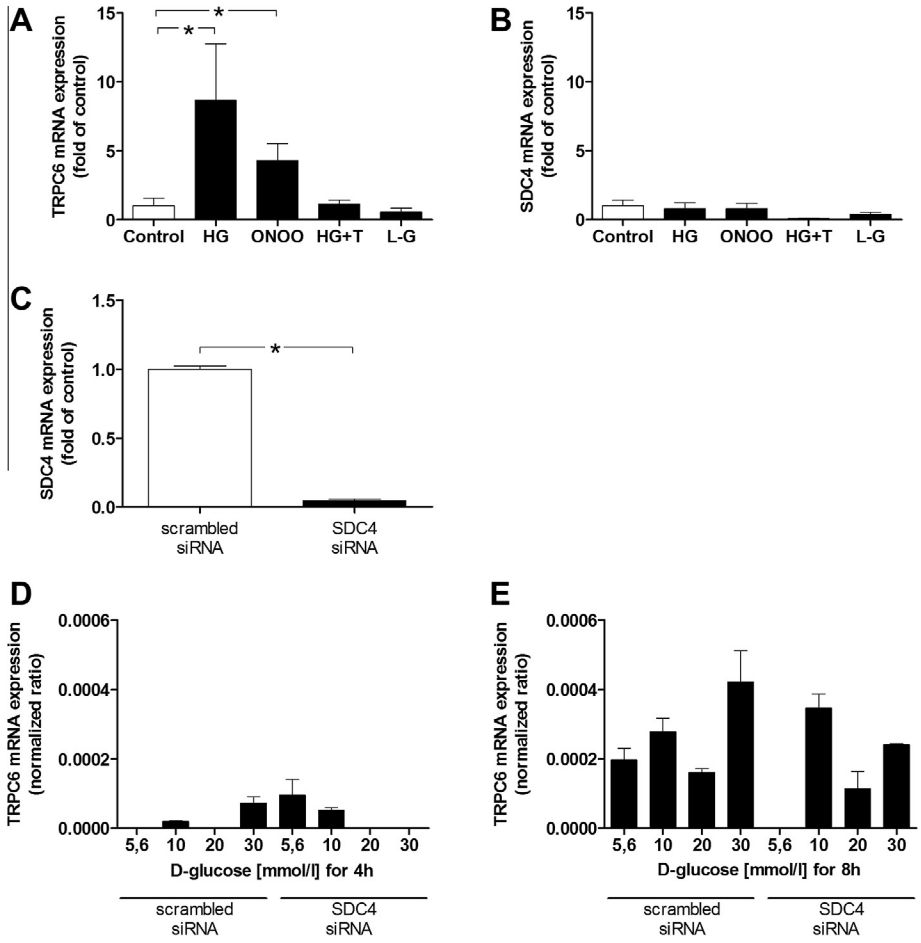

Fig. 1. (A) High D-glucose and oxidative stress increase TRPC6-mRNA in podocytes. Exposure of human podocytes to high glucose (HG; 30 mmol/L D-glucose) or peroxynitrite (100 μmol/L) for 4 h significantly increased TRPC6 mRNA expression. The effect of high glucose on TRPC6 expression is attenuated by the superoxide dismutase mimetic tempol (T, 100 μmol/L). Administration of L-glucose (L-G, 30 mmol/L, 4 h) showed no significant effect. Control (open bar) indicates control glucose (5.6 mmol/L D-glucose). Data are mean ± SEM from at least four independent experiments. (B) High D-glucose and oxidative stress do not alter syndecan-4-mRNA in podocytes. Exposure of human podocytes to high glucose (HG; 30 mmol/L D-glucose) or peroxynitrite (100 μmol/L) for 4 h did not change syndecan-4 mRNA expression. Specifically, compared to control conditions, 100 μmol/L peroxynitrite did not significantly change syndecan-4 transcripts. Data are mean ± SEM from at least four independent experiments. (C) Knockdown of syndecan-4 mRNA by Syndecan-4 siRNA. After transfection of human podocytes with syndecan-4 siRNA, expression of syndecan-4 mRNA was significantly lower compared to transfection with scrambled siRNA. Data are mean ± SEM from at least four independent experiments. (D) Expression of TRPC6 mRNA after 4 h of different D-glucose concentrations in podocytes transfected with syndecan-4 siRNA versus scrambled siRNA. (E) Expression of TRPC6 mRNA after 8 h of different D-glucose concentrations in podocytes transfected with syndecan-4 siRNA versus scrambled siRNA. Data are mean ± SEM from at least four independent experiments.

fluorescence dye fluo-4 AM (Merck Biosciences, Darmstadt, Germany) at room temperature for 45 min 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 measurements were performed in a temperature-controlled 96-well-fluorescent plate reader at 37 °C (Victor3, Helsinki, Finland) at 535 nm emission with excitation wavelengths of 485 nm. Baseline fluorescence was measured and stable fluorescence readings were obtained throughout. Calcium influx was induced by the known TRPC6 agonist flufenamic acid (FFA) [20,21]. The amplitudes were expressed as increase of the fluorescence ratio (F/F0).

All substances were obtained from Sigma–Aldrich (Deisenhofen, Germany) or Merck Millipore (Billerica, Massachusetts, USA) if not indicated otherwise.

2.6. Statistical analysis

All values are reported as mean ± SEM of at least 4 independent experiments. Comparisons between groups were analyzed using non-parametric Mann–Whitney test. Data from multiple groups were analyzed using the non-parametric Kruskal–Wallis test and Dunn's multiple comparison post hoc test. Each sample was tested in duplicate. The software GraphPad Prism 5.0 (LaJolla CA) was used. Two-sided p values below 0.05 were considered to indicate statistical significance. Where error bars do not appear on the figure, error was within the symbol size.

3. Results

3.1. High D-glucose and oxidative stress increased TRPC6- but not syndecan-4-mRNA and protein in podocytes

As shown in Fig. 1A, compared to control conditions, 30 mmol/L D-glucose significantly increased TRPC6-transcripts to 8.66 ± 4.08-fold of control ($n = 4$; $p < 0.05$). The administration of 30 mmol/L L-glucose did not affect TRPC6-transcripts. Concurrent administration of the superoxiddismutase mimetic tempol (final concentration, 100 μmol/L) blocked the effects of high D-glucose on TRPC6-transcripts to 1.14 ± 0.29-fold of control ($n = 4$; p = n.s.). Furthermore, compared to control conditions, 100 μmol/L peroxynitrite significantly increased TRPC6-transcripts to 4.29 ± 1.26 ($n = 4$; $p < 0.05$).

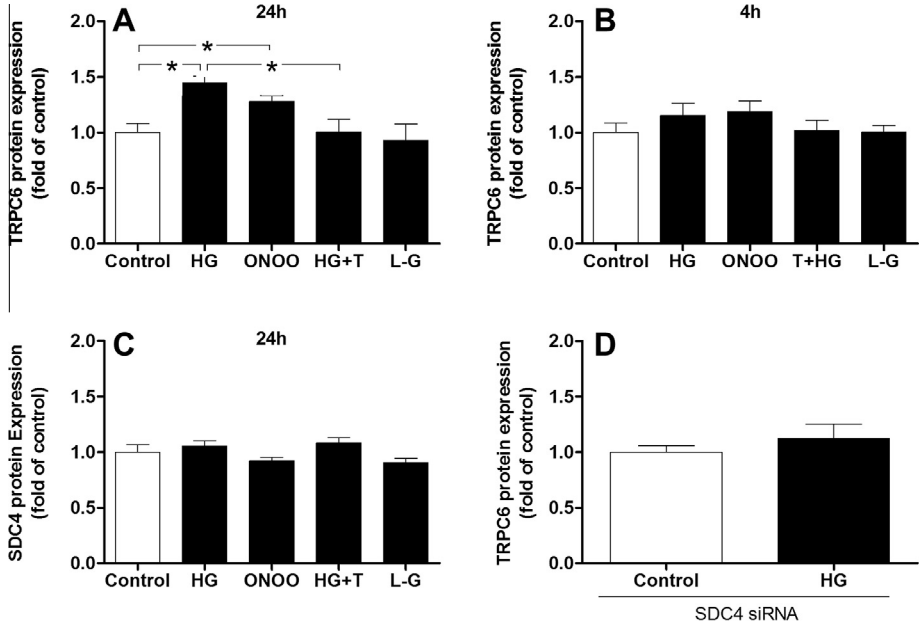

Fig. 2. (A) Exposure of human podocytes to high glucose (HG; 30 mmol/L D-glucose) or peroxynitrite (100 μmol/L) for 24 h significantly increased TRPC6 protein expression. The effect of high glucose on TRPC6 expression was attenuated by the superoxide dismutase mimetic tempol (T, 100 μmol/L). Administration of L-glucose (L–G, 30 mmol/L) showed no significant effect. Control (open bar) indicates control glucose (5.6 mmol/L D-glucose). Summary data are shown. Data are mean ± SEM from at least four independent experiments. (B) High D-glucose and peroxynitrite increased TRPC6 protein in podocytes only non-significantly after 4 h. Each $n = 4$. (C) High D-glucose or peroxynitrite do not alter expression of syndecan-4 protein. Even after 24 h there was no effect of high D-glucose or peroxynirite on expression of syndecan-4 protein. Summary data are shown. Data are mean ± SEM from at least four independent experiments. (D) After knockdown of syndecan-4 by syndecan-4 siRNA, TRPC6 protein expression failed to increase after 24 h of exposure to 30 mmol/L D-glucose. Data are mean ± SEM from at least four independent experiments.

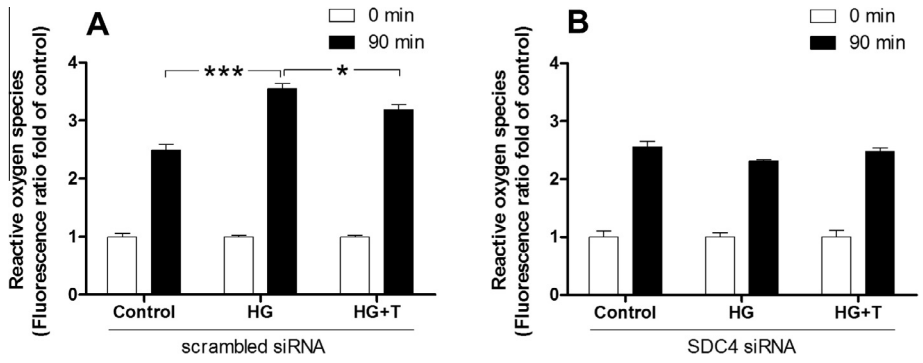

Fig. 3. High D-glucose increases reactive oxygen species in human podocytes via syndecan-4. (A) Administration of 30 mmol/L D-glucose for 90 min increased reactive oxygen species in human podocytes significantly compared to administration of 5.6 mmol/L D-glucose. The effect is inhibited by concurrent administration of tempol. (B) After knockdown of syndecan-4, 30 mmol/L D-glucose failed to increase reactive oxygen species.

Under the same conditions, syndecan-4-transcripts were not altered (Fig. 1B). Specifically, compared to control conditions, 100 μmol/L peroxynitrite did not significantly change syndecan-4 transcripts.

To evaluate whether the increase of TRPC6 expression occurred in a syndecan-4-dependent manner, we set out to knock down syndecan-4. As seen in Fig. 1C, expression of syndecan-4 was significantly downregulated in human podocytes after transfection of syndecan-4 siRNA compared to scrambled siRNA. As shown in Fig. 1D and E, knockdown of syndecan-4 by syndecan-4 siRNA changed the expression of TRPC6 mRNA in response to high glucose.

As shown in Fig. 2A, administration of 30 mmol/L D-glucose increased TRPC6 protein expression to 1.44 ± 0.07-fold of control at 24 h of expression ($n = 4$; $p < 0.05$). The administration of 30 mmol/L L-glucose did not affect TRPC6 protein. Concurrent administration of 100 μmol/L tempol blocked the effects of high D-glucose on TRPC6 protein expression. Compared to control conditions oxidative stress induced by 100 μmol/L peroxynitrite increased TRPC6 protein expression to 1.28 ± 0.05-fold of control at 24 h of expression ($n = 4$; $p < 0.05$). At 4 h of expression, high D-glucose and peroxynitrite increased TRPC6 protein in podocytes only non-significantly (Fig. 2B). Syndecan-4 protein expression was not significantly altered when analyzed after 24 h under the same conditions. Specifically, compared to control conditions, 100 μmol/L peroxynitrite did not significantly change syndecan-4 protein expression (Fig. 2C).

After knockdown of syndecan-4 by syndecan-4 siRNA, TRPC6 protein expression failed to increase after 24 h of exposure to 30 mmol/L D-glucose (1.00 ± 0.04 versus 1.21 ± 0.16, control versus high glucose; $n = 8$; p = n.s.) (Fig. 2D).

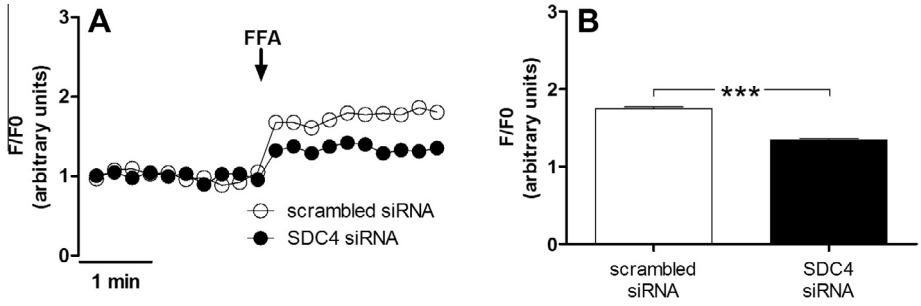

Fig. 4. FFA (100 μM) induced TRPC6-mediated calcium influx in fura2-loaded human podocytes under control conditions (scrambled siRNA) and after transfection with 30nM siRNA against syndecan-4. Original fluorescence tracings (A) and summary data (B) of peak calcium influx are shown. $n = 8$; $^{***}p < 0.001$.

3.2. High D-glucose increased reactive oxygen species in human podocytes via syndecan-4

To evaluate whether knockdown of syndecan-4 would alter the increase of reactive oxygen species after high glucose in podocytes we performed a knockdown of syndecan-4 (Fig. 3).

Administration of 30 mmol/L D-glucose for 90 min increased reactive oxygen species to 3.55 ± 0.08 arbitrary units in podocytes transfected with scrambled siRNA (Fig. 3A), while administration of 5.6 mmol/L D-glucose for 90 min increased reactive oxygen species to 2.49 ± 0.09 ($n = 8$; $p < 0.001$ 30 mmol D-glucose versus 5.6 mmol/L D-glucose after 90 min) only. The effect of high D-glucose was blocked by the concurrent administration of the superoxiddismutase mimetic tempol (100 μmol/L) to 3.19 ± 0.09 arbitrary units ($n = 8$; $p < 0.05$ 30 mmol D-glucose versus 30 mmol/L D-glucose and tempol after 90 min).

In podocytes transfected with siRNA directed against syndecan-4 (Fig. 3B), the administration of 30 mmol/L D-glucose for 90 min showed reactive oxygen species of 2.31 ± 0.03 arbitrary units which were not different compared to control conditions using 5.6 mmol/L D-glucose ($n = 8$; p = n.s.). Concurrent administration of the superoxiddismutase mimetic tempol (100 μmol/L) increased reactive oxygen species to 2.47 ± 0.06 arbitrary units ($n = 8$; p = n.s. 30 mmol D-glucose versus 30 mmol/L D-glucose and tempol after 90 min).

3.3. TRPC-mediated calcium influx in human podocytes occurs in a syndecan-4 dependent manner

Next, we evaluated whether knockdown of syndecan-4 by siRNA might lead to functional changes in podocytes, i.e. transplasmamembrane calcium influx. FFA-induced, TRPC6-mediated calcium influx was significantly decreased by 23% after transfection of podocytes with syndecan-4 siRNA compared to podocytes transfected with scrambled siRNA (scrambled siRNA, 1.75 ± 0.02; SDC4 siRNA, 1.35 ± 0.02; each $n = 8$; $p < 0.001$) (Fig. 4A and B).

4. Discussion

The experimental results show that both high D-glucose and oxidative stress increase TRPC6-mRNA and protein expression in podocytes. These findings are in accordance with recent data demonstrating that high glucose may regulate TRPC6 mRNA and protein expression in cultured podocytes [8,9].

High D-glucose and oxidative stress did not alter syndecan-4-mRNA or protein in podocytes. Syndecans belong to the type I transmembrane heparan sulfate proteoglycan superfamily, and are characterized as major modulators of signal transduction and regulators of the localization and activity of proteins and channels. Recent data from our group confirmed that syndecan-4 regulates TRPC6 channel expression [16]. This study adds that syndecan-4 itself is not regulated by high D-glucose. To the best of our knowledge there are no data analyzing a direct interaction of glucose with syndecan-4.

The experimental data indicate that expression of TRPC6 mRNA and protein in podocytes occurs in a syndecan-4 dependent manner. Changing TRPC6 expression by increased D-glucose concentration depends on the expression of syndecan-4. High glucose did not increase TRPC6 expression after syndecan-4 knock down.

This study demonstrated that high D-glucose increases reactive oxygen species in human podocytes via syndecan-4. Increased generation of reactive oxygen species in podocytes and altered function of podocytes have been described in patients with diabetes mellitus [22–26]. It has been shown before that reactive oxygen species (ROS), i.e. oxidative stress, is increased by high glucose and that the inhibition of ROS formation blocks the effect of high glucose on TRPC6 expression [8,9]. More specifically, it has been suggested that high glucose induces apoptosis in podocytes by stimulating TRPC6 via elevation of reactive oxygen species [8] and by involvement of TRPC6-dependent calcium entry via the RhoA/ROCK pathway [9]. Now we confirm that high D-glucose increases reactive oxygen species in podocytes in a syndecan-4 dependent manner. Furthermore, our data demonstrate that TRPC-mediated calcium influx in human podocytes occurs in a syndecan-4 dependent manner.

In conclusion, the present study indicates that high glucose modifies TRPC6 channels and ROS production via syndecan-4 in human podocytes. The increase in TRPC6 expression may be responsible for the deleterious effects of high levels of D-glucose on kidney function in diabetes mellitus. The data presented here point to a novel pathway for modulating the function of podocytes in patients with diabetes mellitus.

Conflict of interest

None.

Acknowledgments

The study was supported by the Deutsche Forschungsgemeinschaft and NovoNordisk-Fonden.

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