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

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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.

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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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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

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