Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca\(^{2+}\) signaling in glomerular mesangial cells in diabetes

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\(^1\)Department of Integrative Physiology, \(^2\)Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas; \(^3\)Department of Physiology, Anhui Medical University, Hefei, People’s Republic of China; and \(^4\)Department of Medicine, Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

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Graham S, Ding M, Sours-Brothers S, Yorio T, Ma J-X, Ma R. Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca\(^{2+}\) signaling in glomerular mesangial cells in diabetes. Am J Physiol Renal Physiol 293: F1381–F1390, 2007. First published August 15, 2007; doi:10.1152/ajprenal.00185.2007—The present study was performed to investigate whether transient receptor potential potential (TRPC6) participated in Ca\(^{2+}\) signaling of glomerular mesangial cells (MCs) and expression of this protein was altered in diabetes. Western blots and real-time PCR were used to evaluate the expression level of TRPC6 protein and mRNA, respectively. Cell-attached patch-clamp and fura-2 fluorescence measurements were utilized to assess angiotensin II (ANG II)–stimulated membrane currents and Ca\(^{2+}\) responses in MCs. In cultured human MCs, high glucose significantly reduced expression of TRPC6 protein, but there was no effect on either TRPC1 or TRPC3. The high glucose-induced effect on TRPC6 was time and dose dependent with the maximum effect observed on day 7 and at 30 mM glucose, respectively. In glomeruli isolated from streptozotocin-induced diabetic rats, TRPC6, but not TRPC1, was markedly reduced compared with the glomeruli of control rats. Furthermore, TRPC6 mRNA in MCs was also significantly decreased by high glucose as early as 1 day after treatment with maximal reduction on day 4. Patch-clamp experiments showed that ANG II–stimulated membrane currents in MCs were significantly attenuated or enhanced by knockdown or overexpression of TRPC6, respectively. Fura-2 fluorescence measurements revealed that the ANG II–induced Ca\(^{2+}\) influxes were markedly inhibited in MCs with TRPC6 knockdown, reminiscent of the impaired Ca\(^{2+}\) entry in response to ANG II in high glucose-treated MCs. These results suggest that the TRPC6 protein expression in MCs was downregulated by high glucose and the deficiency of TRPC6 protein might contribute to the impaired Ca\(^{2+}\) signaling of MCs seen in diabetes.

Ca\(^{2+}\) influxes; glomeruli; diabetic nephropathy; hyperfiltration

Diabetic nephropathy (DN) is a major and devastating complication of diabetes. At the very onset of diabetes, a dominant pathophysiological characteristic is the development of renal glomerular hyperfiltration (2, 39). This early hemodynamic phenotype provokes the subsequent demise of a diabetic kidney. Diabetic hyperfiltration is derived from a combination of decreased responsiveness of both the renal afferent arterioles and glomerular mesangial cells (MCs) to vasoconstrictors (2, 3, 20, 29, 42). MCs, located within glomerular capillary loops, physiologically regulate glomerular hemodynamics (26, 37). In diabetes, mesangial contractile function is impaired, and reduced Ca\(^{2+}\) influx is believed to be a major contributing factor to the hypocontractility (29, 42). However, the underlying molecular mechanism(s) responsible for the impaired Ca\(^{2+}\) signaling remains poorly understood.

Hyperglycemia is the main determinant of initiation and progression of diabetic microvascular complications including nephropathy (13, 38). In vitro and in vivo studies demonstrated that high glucose or hyperglycemia directly stimulates MCs, which subsequently results in mesangial dysfunction or mal-function (20, 23, 24, 29, 42). However, the mechanisms by which hyperglycemia causes nephropathy are still not well defined.

Recently, canonical transient receptor potential (TRPC) proteins have been proposed as Ca\(^{2+}\)-permeable cation channels that are activated in response to stimulation of G protein-coupled receptors (1, 16, 19, 30). The TRPC family includes seven related members, designated as TRPC1–7 (28). As a member of the TRPC family, TRPC6 has been reported in vascular smooth muscle cells and regulates myogenic tone or agonist-stimulated constriction of blood vessels (5, 7, 19, 31, 40, 41). TRPC6 is also present and functional in renal microcirculation. In freshly isolated rat renal preglomerular resistance vessels, TRPC6 mRNA and protein were detected at significantly greater levels than in conduit vessels (aorta) (9), implying that TRPC6 may have an important role in renal microcirculation. Recently, TRPC6 in glomerular podocytes has been reported to be tightly linked to hereditary familial focal segmental glomerulosclerosis and acquired forms of proteinuric kidney diseases (27, 43). In a recent study, we demonstrated that TRPC6 is also abundantly expressed in human and rat MCs (36), which possess similar contractile phenotype and biophysical properties to those in vascular smooth muscle cells. However, the physiological and pathophysiological relevance of TRPC6 in MCs is completely unknown.

In the present study, we tested the hypothesis that the TRPC6 channel protein mediates vasoconstrictor-induced Ca\(^{2+}\) responses in MCs and the expression of this protein is downregulated by high glucose or hyperglycemia in diabetes.

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MATERIALS AND METHODS

Generating diabetic rats. The study protocol was approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (~8 wk) were purchased from Harlan (Indianapolis, IN). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) at 65 mg/kg body wt in sodium citrate buffer (0.01 M, pH 4.5). An equivalent amount of sodium citrate buffer alone was used as a vehicle control. Blood glucose levels were monitored 24 h later and periodically thereafter (LifeScan One Touch glucometer, Johnson & Johnson, Milpitas, CA) by rat-tailed blood sampling. STZ-injected rats with sustained elevation of blood glucose above 300 mg/dl were designated as diabetic rats. All rats had unrestricted access to food and water and were maintained in accordance with Institutional Animal Care and Use Committee procedures of the University of North Texas Health Science Center.

Isolation of glomeruli. On day 14 after STZ or vehicle injection, all rats were euthanized, and both kidneys were removed. Glomeruli were isolated by differential sieving of minced renal cortex. Finely chopped kidney cortex in Hank’s balanced salt solution (pH 7.4) was pressed through sequentially smaller metal sieves and collected on a final sieve of 63-μm pore size (mini-sieve set, Scienceware, Pequannock, NJ). Following three alternate washes and centrifugations, the pellets of glomeruli were solubilized in a lysis buffer and the supernatants were collected for Western blot.

MC culture and transient transfection. Human MCs were purchased from Cambrex. MCs were subcultured to no more than 10 generations by standard methods (22) except the concentrations of d-glucose in the culture medium that were indicated in the text, figure legends, or in figures. Appropriate concentration of α-mannitol was supplemented in the culture medium as an osmotic control. All plasmids were transiently transfected into MCs using Lipofectamine and Plus reagent (Invitrogen-BRL, Carlsbad, CA) following the protocols provided by the manufacturer.

Patch-clamp procedure. The conventional cell-attached voltage clamp was employed as described in our previous study (22). Single-channel analysis was made with a Warner PC-505B amplifier (Warner Instrument, Hamden, CT) and pClamp 9.2 (Axon Instrument, Foster City, CA). The extracellular solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 2 MgCl2, 1 CaCl2, and 10 glucose. The pipette solution contained (in mM) 135 NaCl, 5 KCl, 0.1 CaCl2, 10 HEPES. At the time of the experiment, the pipette solution was supplemented with 100 μM niflumic acid, 10 mM TEA, and 100 nM tetrodotoxin to block Ca2+-activated Cl− channels and K+ channels, respectively. To exclude the influence of fluid flow on channel activity upon ANG II infusion, the bathing solution continuously flowed throughout the experiments. The flow rate was adjusted by gravity and controlled by an additional block Ca2+ -activated Cl− channel (Zeiss LSM410).

Fluorescence measurement of [Ca2+]i. Measurements of [Ca2+]i in MCs using fura-2 were performed using dual excitation wavelength fluorescence microscopy. MCs, grown on a coverslip (22 × 22 mm), were loaded with fura-2 by incubation for ~50 min at room temperature in the dark in physiological saline solution containing 2 μM acetoxymethyl ester of fura-2 (fura-2/AM), 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 (Molecular Probes, Eugene, OR) followed by washing three times with physiological saline solution. The cells were then incubated with fura-2 free physiological saline solution for an additional ~20 min. The coverslip was then placed in a perfusion chamber (Warner, model RC-2OH) mounted on the stage of a Nikon Diaphot inverted microscope. Fura-2 fluorescence was monitored by a ratio technique (excitation at 340 and 380 nm, emission at 510 nm) using Metaflour software (Universal Imaging, West Chester, PA).

Western blot. Western blot was performed as described in our previous publication (36). In brief, protein extracts of glomeruli or MC lysates were fractionated by 10% SDS-PAGE, transferred to PVDF membranes, and probed with primary TRPC antibodies. Bound antibodies were visualized with Super Signal West Femu Luminol/Enhancer Solution (Pierce Biotechnology, Rockford, IL).

Fluorescent immunohistochemistry. Fluorescent immunohistochemistry was performed as described in our previous publication (36). In brief, adult male Sprague-Dawley rats (weighing 200–250 g) were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). Kidneys were perfused with physiological saline solution via a catheter inserted into the abdominal aorta followed by 4% paraformaldehyde and then excised from the animals. The kidneys were fixed in 2% paraformaldehyde in K3PO4 free PBS over 2 h at 4°C, immersed in 30% sucrose overnight at 4°C, and cryosectioned at 6-μm thickness (Cryostat 2800 Frigocut-E, Leica Instruments). The sections were washed with K3PO4-free PBS and treated with blocking buffer containing 50 mM NH4Cl, 2% BSA, 0.05% saponin in K3PO4-free PBS for 20 min at room temperature for permeabilization. The sections were then incubated overnight at 4°C in blocking buffer containing rabbit polyclonal anti-TRPC6 antibody. To label glomerular MCs, we also incubated the sections with mouse monoclonal anti-desmin antibody overnight at 4°C. The concentrations for all primary antibodies were 2–5 μg/ml. The sections were rinsed and incubated for 30 min at room temperature with Alexa Fluor 488 goat anti-rabbit IgG or donkey anti-mouse IgG (Molecular Probes), depending on the primary antibodies. The concentrations of the secondary antibodies were 2 μg/ml. In control slides, the equal amounts of rabbit IgG or mouse IgG were used instead of the primary antibodies. All stainings were visualized using confocal laser-scanning microscopy (Zeiss LSM410).

Quantitative real-time RT-PCR. The total RNA was isolated from MCs or glomeruli using a Versagene RNA kit following the manufacturer’s protocol (Gentra System). Tagman primers and probes for trpc1, trpc4, trpc6, and β-actin were designed according to their respective human DNA sequences (Table 1) using Beacon designer software and

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer and Probe Sequence (5’-3’)</th>
<th>GeneBank Accession Number</th>
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<td>F TGGTTGCTTTGACAGATGTCG</td>
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<tr>
<td></td>
<td>R AAACGAGTGAGGACCTATCA</td>
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<td>P FAM-TGGTGCTGCTGCTGTTGCCC-BHQ1</td>
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F, forward primer; R, reverse primer; P, probe.
synthesized by Biosource International. Reverse transcription (RT) reactions used 1.0 μg total RNA, oligo-dT primers, MMLV reverse transcriptase in a final volume of 20 μl and were incubated at 37°C for 60 min after a denaturation at 70°C for 10 min. Real-time PCR used 2.5 μl RT product, 0.5 μM primer, and 0.4 μM probe, and was performed using ABI Tagman Universal Master Mix in a final volume of 25 μl. The PCR mix was denatured at 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s and elongation at 60°C for 60 s. The assay was run on a SmartCycler (Sunnyvale, CA) and fluorescence changes were monitored after each cycle. The average Ct (threshold cycle) of fluorescence unit was used to analyze the mRNA levels. The TRPC6 mRNA levels were normalized by actin mRNA levels.

Quantification was calculated as follows: mRNA levels (percent of control) = \(2^{\Delta\Delta CT}\), where \(\Delta CT = CT_{TRPC} - CT_{actin}\) and \(\Delta(\Delta CT) = \Delta CT_{normal~glucose} - \Delta CT_{high~glucose}\).

**Materials.** Inactivating constructs directing the expression of short hairpin RNAs specific for human trpc6 (designated as hTRPC6-shRNA) were kind gifts from Dr. M. L. Villereal at the University of Chicago (44). The rat TRPC6 expression plasmids (pEF-BOS-TRPC6A) were obtained from Dr. D. Saffen at the Ohio State University (45). Antibodies and all chemicals were purchased from Sigma (St. Louis, MO) except TRPC1 antibody. TRPC1 antibody (1F1, mouse IgG, monoclonal) and YFP vectors were obtained from Dr. L. Tsioskas (University of Oklahoma Health Sciences Center, Oklahoma City, OK).

**Statistical analysis.** In all immunoblotting and RT-PCR experiments, at least three independent experiments were performed for each group. Data are reported as means ± SE. The one-way ANOVA plus Student-Newman-Keuls test, Student’s unpaired t-test, and Student’s paired t-test were used to analyze the differences among multiple groups, between two groups, and before and after treatment in the same group, respectively. \(P < 0.05\) was considered statistically significant. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA).

**RESULTS**

TRPC6 mediated ANG II-stimulated membrane currents and Ca\(^{2+}\) influxes in cultured MCs. TRPC6 has been known as a Ca\(^{2+}\)-conductive cation channel and regulates myogenic tone or agonist-stimulated constriction of blood vessels (5, 7, 19, 31, 32).

Fig. 1. A: cell-attached patch-clamp experiments showing single-channel activities (NPO) before (Pre-ANG II) and after (Post-ANG II) ANG II (1 μM) stimulation in mock-transfected (Mock; \(n = 5\)), hTRPC6-shRNA-transfected (hTRPC6-shRNA; \(n = 7\)), and TRPC6 expression plasmid-transfected (TRPC6 overexpression; \(n = 6\)) mesangial cells (MCs). Holding potential was 80 mV (Pipette). *Significant difference between the groups as indicated. B: percent changes of channel activities in response to ANG II stimulation in the groups as indicated in A. *Significant difference compared with Mock. C: representative Ca\(^{2+}\) imaging experiments (fura-2 fluorescence measurement) showing ANG II-stimulated Ca\(^{2+}\) responses in Mock and hTRPC6-shRNA MCs. Ca\(^{2+}\) influx in response to Ca\(^{2+}\) readmission and ionomycin are indicated by small and large dashed boxes, respectively. 

\([\text{Ca}^{2+}]_B\) represents Ca\(^{2+}\) concentration in the bathing solution. \([\text{Ca}^{2+}]_B\), application of ANG II (1 μM), and ionomycin (5 μM) are indicated by the horizontal bars at top. D: ANG II-induced Ca\(^{2+}\) influxes in Mock and hTRPC6-shRNA MCs, normalized to ionomycin-induced Ca\(^{2+}\) responses. \(n\), Number of cells analyzed. *Significant difference compared with Mock. E: Western blot showing overexpression of TRPC6 protein in human MCs by transient transfection of rat TRPC6 expression plasmids (pEF-BOS-TRPC6A). UT, untransfected cells; Mock, pSAGH empty vector)-transfected cells; rTRPC6, cells transfected with pEF-BOS-TRPC6A. F: real-time RT-PCR showing specific and efficient knockdown of TRPC6 mRNA by hTRPC6-shRNAi in human MCs. *Significant difference compared with control (Con). \(n\), Number of independent experiments.
Our previous study demonstrated that TRPC6 is endogenously expressed in human and rat MCs (36). To determine the physiological relevance of the TRPC6 channel protein in MCs, cell-attached patch-clamp experiments were performed to measure single-channel currents in response to ANG II in human MCs that were cultured in normal-glucose medium (5.6 mM) and were transiently transfected with rat trpc6 expression plasmids (pEF-BOS-TRPC6A) or hTRPC6-shRNA constructs. GFP was cotransfected at amounts of 10 times less for identifying positively transfected cells. As shown in Fig. 1, A and B, application of ANG II (1 μM) into the bath increased the channel activity in MCs of all three groups. However, the channel activation was strikingly enhanced in the cells with overexpression of TRPC6 (606 ± 76 and 229 ± 33%, TRPC6 overexpression vs. Mock, P < 0.05), but blunted in the cells with knockdown of TRPC6 (51 ± 6 and 229 ± 33%, hTRPC6-shRNA vs. Mock, P < 0.05; Fig. 1, A and B). In addition, the basal activity of the channels was significantly raised by overexpression of TRPC6 but was not affected by knockdown of TRPC6 (Fig. 1A). Overexpression of TRPC6 protein by transient transfection with pEF-BOS-TRPC6A was verified by Western blot in human MCs (Fig. 1E). The specificity and efficiency of hTRPC6-shRNA constructs were confirmed by real-time RT-PCR, which showed that the RNAi constructs significantly reduced the mRNA expression of TRPC6, but not TRPC1 or TRPC4 in MCs (Fig. 1F). These data suggest that TRPC6 channels play an important role in mediating agonist-stimulated cation influxes in MCs. However, at resting state, this channel might be relatively quiet.

To further evaluate the role of TRPC6 in ANG II-induced MC Ca\(^{2+}\) responses, we measured fura-2 fluorescence-activated [Ca\(^{2+}\)]\(_i\) in MCs with and without knockdown of TRPC6. As shown in Fig. 1C, in the 1 mM Ca\(^{2+}\) extracellular solution, ANG II evoked a rapid and striking cytosolic Ca\(^{2+}\) transient in both the mock-transfected and hTRPC6-shRNA-transfected MCs. This initial Ca\(^{2+}\) spike is composed of Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) influxes from outside the cell (15). Removal of extracellular Ca\(^{2+}\) reduced the [Ca\(^{2+}\)]\(_i\) to a level lower than baseline. Readmission of Ca\(^{2+}\) resulted in an increase in [Ca\(^{2+}\)], which is known to be attributed to Ca\(^{2+}\) influxes (22). This Ca\(^{2+}\) entry in response to Ca\(^{2+}\) add-back protocol was significantly inhibited in the MCs with knockdown of TRPC6. Since nifedipine (10 μM) was included in the bathing solution, the voltage-operated Ca\(^{2+}\) channels can be excluded from mediation of the Ca\(^{2+}\) influxes (40). To minimize the influence of other factors such as cell volume on the results, 5 μM ionomycin was applied at the end of our experiments to maximize Ca\(^{2+}\) influxes and the ANG II-induced Ca\(^{2+}\) entry (indicated by a small dashed box in Fig. 1C) was normalized to the ionomycin-induced Ca\(^{2+}\) responses (indicated by a large dashed box in Fig. 1C) in each group of cells. As shown in Fig. 1D, this ratio is significantly lower in the cells with TRPC6 knockdown compared with the mock control. These results indicate that similar to vascular smooth muscle cells, TRPC6 constitutes an important channel mediating vasoconstrictor-stimulated Ca\(^{2+}\) influxes in MCs.

**Ca\(^{2+}\)** influxes of MCs in response to ANG II were impaired by high glucose. It has been known that Ca\(^{2+}\) signaling in MCs is impaired by high glucose, whereas the underlying mechanism is still unclear (29, 42). We confirmed this observation in our experimental settings. We evaluated fura-2 fluorescence-activated [Ca\(^{2+}\)]\(_i\) in MCs cultured in normal glucose (5.6 mM) and high glucose (30 mM) using the same protocol as described in Fig. 1C. In accordance with knockdown of TRPC6, high-glucose treatment markedly attenuated the ANG II-stimulated Ca\(^{2+}\) entry (dashed boxes in Fig. 2, A and B). Averaged data from eight cells of each group showed a significant difference in the agonist-induced Ca\(^{2+}\) influxes (Fig. 2C).

![Fig. 2. Fura-2 fluorescence indicated ANG II-stimulated Ca\(^{2+}\) entry in MCs cultured in 5.6 mM glucose (A) and 30 mM glucose (B) for 7 days. Ca\(^{2+}\) entry is reflected by a rise in the ratio of 340/380 nm fluorescences upon switching bathing solution from Ca\(^{2+}\)-free to 1 mM Ca\(^{2+}\) (indicated by the dashed boxes in A and B). [Ca\(^{2+}\)]\(_i\) represents Ca\(^{2+}\) concentration in bathing solution, indicated by the lower horizontal bar at top. Application of ANG II (1 μM) is indicated by the upper horizontal bar at top. NG, 5.6 mM glucose; HG, 30 mM glucose. C: summary data showing the ANG II-stimulated Ca\(^{2+}\) influxes in 5.6 mM (NG) and 30 mM (HG) glucose-cultured MCs, indicated by the dashed boxes in A and B, respectively. *Significant difference compared with NG group. n, Number of cells analyzed.](http://ajprenal.physiology.org/)

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These results strongly suggest that the vasoconstrictor-induced Ca\(^{2+}\) response in MCs is impaired by high glucose.

**High glucose downregulated expression of the TRPC6 protein in cultured MCs.** To determine whether TRPC6 contributes to the high glucose-induced impairment of Ca\(^{2+}\) signaling in MCs and whether TRPC6 is involved in diabetes-associated glomerular dysfunction, we cultured MCs in high-glucose medium to mimic diabetic milieu and performed a Western blot for TRPC6 protein. We found that the expression of the TRPC6 protein was remarkably decreased by high-glucose treatment. This inhibitory effect of high glucose was time dependent with a significant inhibition as early as 2 days after incubation and the maximal effect was seen on day 7 (Fig. 3, A and B). Thereafter, TRPC6 protein was sustained at a significantly lower level for the entire observation period of 28 days. Furthermore, glucose also displayed a dose-dependent effect on TRPC6 expression (Fig. 3, C and D). At 10 mM glucose for 7 days, the amount of TRPC6 protein had a tendency to decrease. Significant reduction of TRPC6 protein occurred at 20 mM concentration of glucose with a further decrease at 30 mM for the same time period of incubation (7 days). The high glucose-induced reduction of TRPC6 protein expression seems to be MC specific because no difference in TRPC6 protein expression was observed in conditionally immortalized mouse podocytes between normal (5 mM)- and high-glucose (25 mM) treatments (Fig. 3E).

Our previous study demonstrated that human MCs also express TRPC1, TRPC3, and TRPC4 (36). To determine whether the high-glucose effect is specific for TRPC6, we measured the protein expression levels of TRPC1 and TRPC3 in MCs cultured in 5 and 30 mM glucose for 7 days. As shown in Fig. 4, A and B, high glucose did not affect the expression of TRPC1 or TRPC3, but TRPC6 only.

Furthermore, to determine whether the decrease in TRPC6 protein is a specific effect of glucose treatment, we recultured the MCs, which had been exposed to high glucose for 7 days, in 5 mM glucose medium for an additional 3 days. Under this condition, the high glucose-induced reduction of TRPC6 expression was nearly restored (Fig. 4C).

Taken together, these biochemical results strongly suggest that high glucose specifically downregulated TRPC6 protein expression in cultured MCs.

**TRPC6 protein expression was downregulated in the glomeruli of diabetic rats.** In the in vitro effect of high glucose on TRPC6 protein expression in cultured MCs was further examined in intact animals. First, immunohistochemistry was performed in rat kidney sections to confirm the expression of TRPC6 protein in glomerular MCs. Glomeruli were easily distinguished by their characteristic circular morphological aspect bordered by the peripheral lumen. Glomerular MCs were identified with positive staining with antibody against desmin. Although Möller et al. (27) found that TRPC6 is primarily expressed in glomerular podocytes, we clearly detected the existence of TRPC6 protein in glomerular MCs (Fig. 5), which is consistent with our previous observation (36). No staining was detected in the samples treated with control immunoglobulins (data not shown). Not surprisingly, TRPC6 staining was also found in the cells without desmin staining.

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Fig. 3. Western blot showing time- and dose-dependent effects of high-glucose treatment on TRPC6 protein expression in cultured MCs. A: TRPC6 protein expression in 5.6 mM (NG) and 30 mM (HG) glucose-treated MCs over various time periods. L, ladder of protein marker; D, days of HG incubation. β-Actin was used as a loading control. B: quantification of TRPC6 protein expression from 4 independent experiments indicated in A. The levels of TRPC6 expression were expressed as the percentages of optical densities of TRPC6 immunoblots to actin immunoblots. *Significant difference compared with day 0. C: influence of glucose concentrations on TRPC6 protein expression. Human MCs were incubated with different concentrations of glucose for 7 days. β-Actin was used as a loading control. D: summary data from 3 independent experiments indicated in C. The levels of TRPC6 expression were expressed as the percentages of optical densities of TRPC6 immunoblots to β-actin immunoblots. *Significant difference compared with 5.6 mM glucose. In all HG experiments, an appropriate concentration of α-mannitol was included into the culture medium for osmotic control. E: effect of high glucose on TRPC6 protein expression in cultured mouse podocytes. Conditionally immortalized mouse podocytes were grown on type I collagen at 33°C in the presence of 20 U/ml interferon-γ in RPMI 1640 media containing 5 mM glucose, 10% FBS, and antibiotics. To induce differentiation, podocytes were maintained at 37°C without interferon-γ for 7–10 days. The cells were serum starved for 24 h before the 3-day treatment by high glucose. NG, normal glucose (5 mM glucose + 20 mM α-mannitol); HG, high glucose (25 mM). β-Tubulin was used as a loading control.
staining, suggesting that TRPC6 also resides in other types of glomerular cells, such as podocytes (27).

We then examined whether diabetes changed the expression level of the TRPC6 protein in glomeruli. Since MCs constitute one-third of the glomerular cell population (26), the changes in the TRPC6 protein in MCs are expected to be detectable at the level of the entire glomeruli. Glomeruli were isolated from the kidneys of normal and STZ-induced diabetic rats. STZ-induced diabetes was demonstrated by slow increase in body weight, sustained elevation of blood glucose, and high urine output following STZ injection (Table 2). Figure 6C shows the purity of isolated glomeruli which is estimated to be >99%. Crude proteins were extracted from the normal and diabetic glomeruli and, subsequently, were loaded for Western blot. In agreement with the results from the MCs cultured in high glucose, the amount of the TRPC6 protein was dramatically reduced in diabetic glomeruli. However, the TRPC1 expression level did not have a discernible change (Fig. 6, A and B). Since MC is a major type of glomerular cell and high glucose did not affect TRPC6 protein expression in glomerular podocytes (Fig. 3E), we inferred that the reduction of TRPC6 protein in diabetic glomeruli can most likely be attributed to the diseased MCs.

Fig. 5. Immunohistochemistry showing expression of TRPC6 protein in a normal rat kidney section. Glomerular MCs were labeled with desmin. TRPC6 was stained with green fluorescence and desmin was stained with red fluorescence. Right (Merge): yellow signals indicate colocalization of TRPC6 protein and glomerular MCs.
Because high glucose inhibited the responses of MCs to agonists that stimulate Ca\(^{2+}\) entry, we determined whether the expression of TRPC6, which is involved in agonist-stimulated Ca\(^{2+}\) entry, was affected by high glucose. In the present study, we used an in vitro model of MCs in which high glucose was added to the culture medium for 24 h and then replaced with low-glucose medium. We quantified the expression of TRPC6 by Western blot analysis and real-time RT-PCR. The results showed that high glucose inhibited the expression of TRPC6 mRNA in MCs. However, the expression of TRPC1 and TRPC4 mRNA was not affected by high glucose. This indicates that high glucose specifically downregulated the expression of TRPC6 mRNA.

We further confirmed the decrease in TRPC6 mRNA by Western blot analysis. The results showed that high glucose significantly reduced the expression of TRPC6 protein. The decrease in TRPC6 protein expression was consistent with the decrease in TRPC6 mRNA expression. In addition, we found that high glucose inhibited the Ca\(^{2+}\) entry responses in MCs. The results suggest that the downregulation of TRPC6 by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs.

To determine whether the decrease in TRPC6 expression by high glucose is a cause of impaired Ca\(^{2+}\) entry, we performed experiments to confirm that the impaired Ca\(^{2+}\) entry responses were rescued by increasing the expression of TRPC6. We found that the expression of TRPC6 mRNA and protein in MCs was significantly restored by increasing the expression of TRPC6 using a lentiviral vector expressing TRPC6. The results suggest that the decrease in TRPC6 expression by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs.

These findings suggest that the downregulation of TRPC6 by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs. The results also suggest that the decrease in TRPC6 expression by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs. The results also suggest that the downregulation of TRPC6 by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs. The results also suggest that the downregulation of TRPC6 by high glucose is a key event in the impaired Ca\(^{2+}\) entry responses in MCs.
**Fig. 7.** Influence of high glucose on TRPC6 mRNA expression. The mRNA level of TRPC6 was determined by real-time RT-PCR and normalized by β-actin mRNA levels. A: time-dependent change in TRPC6 mRNA in response to 30 mM glucose treatment. *Significant difference compared with day 0. B: expression of TRPC6 mRNA in MCs cultured in 5.6 mM (NG) for 12 days, 30 mM (HG) for 12 days, and 30 mM for 12 days followed by 5.6 mM for 3 days (HG→NG). *Significant difference compared with NG. #Significant difference compared with HG.

A and B: n indicates the number of independent experiments.

smooth muscle cells, MCs possess similar contractile phenotype and biophysical properties as those exhibited by vascular smooth muscle cells. Thus it is not surprising that TRPC6 participates in the contractile function of MCs and deficiency of this channel protein leads to impairment of agonist-induced mesangial contraction that subsequently results in supranormal glomerular filtration rate. Our patch-clamp experiments revealed that knockdown of endogenous TRPC6 in MCs reduced the ANG II-induced channel activity by ~77% (Fig. 1B), implying that TRPC6 protein might be the channel or a critical component of the channel complexes mediating the ANG II-stimulated membrane response. These findings suggest that deficiency of the TRPC6 protein might be an underlying mechanism for the hyperfiltration seen in the early stages of diabetes. Interestingly, knockdown of TRPC6 did not affect the basal channel activity of MCs (Fig. 1A), implying that the TRPC6 channel might not be important in maintaining tonic mesangial tone at the resting state. Since human MCs possess multiple isoforms of TRPC channel proteins (36), we inferred that the resting tone of MCs might be the function of those TRPC isoforms or could be mediated by other types of ion channels in the plasma membrane. Indeed, we recently found that the biological knockdown of TRPC1 using an RNAi approach or functional blockade of TRPC1 channel using a specific TRPC1 antibody significantly reduced basal membrane currents in cultured MCs (8).

Recently, the physiological and pathophysiological relevance of TRPC6 in kidney glomeruli has drawn intense attention from many investigators. In a recent study from Dr. Reiser’s group (32), TRPC6 was detected and characterized as a component of the slit diaphragm multiprotein complex of glomerular podocytes, suggesting that it functions as a critical regulator of normal renal function. This speculation was validated by two discoveries that gain-in-function mutations in the TRPC6 gene in glomerular podocytes cause hereditary familial focal segmental glomerulosclerosis (43) and that upregulation of TRPC6 in podocytes results in acquired forms of proteinuric kidney disease (27). Using immunohistochemistry, we found that the TRPC6 protein also existed in human and rat glomerular MCs (Fig. 5) (36). Moreover, the results from the present study display a link between high glucose or hyperglycemia and deficiency of TRPC6 protein expression in glomerular MCs, suggesting a potential implication of TRPC6 in the development of DN. We believe that the high glucose-induced downregulation is specific for TRPC6 because TRPC1 and TRPC3 protein expression levels were not affected by high glucose (Fig. 3, A and B) and the TRPC1 protein did not have any significant change in the diabetic glomeruli compared with the controls (Fig. 6A). Also, TRPC6 downregulation is a specific effect of high glucose because the reduction of the TRPC6 protein showed a clear dose dependence on the concentration of glucose (Fig. 3C), and replacement of high glucose with normal glucose in culture medium could restore the decreased TRPC6 expression at both protein and messenger levels (Fig. 4, B and C).

The mechanism for the high glucose-induced downregulation of TRPC6 protein is unknown from the present study. Several downstream signaling pathways, such as advanced glycation end products, protein kinase C, and ANG II, have been implicated in hyperglycemia-associated progression of DN (35). Recently, oxidative stress has been proposed to be an important downstream mediator of hyperglycemia as well as a critical pathogenic factor in the development of DN (11, 17, 21, 34, 35). In MCs, oxidants can activate most of the known pathways that have been implicated in diabetes, including protein kinase C (14), mitogen-activated protein kinases, TGF-β1 (12, 18), and fibronectin (11, 18). It would be interesting and important to investigate whether there is a cause-effect relationship between overproduction of reactive oxygen species and downregulation of TRPC6 protein in the future. As a support, our data indicate that the high glucose-induced reduction of the TRPC6 protein is due to a decrease in TRPC6 mRNA level (Fig. 7) and oxidative damage to DNA in diabetes mellitus has been reported (4).

In summary, our findings from the present study imply that TRPC6 is an important channel mediating agonist-stimulated mesangial contraction, and this channel protein is significantly reduced in diabetes. The importance of these findings is to provide a possible molecular mechanism for glomerular hyperfiltration, a characteristic of diabetes mellitus at the early stage, and thereby provide a new clue for therapeutic targets to slow down the progression of DN.

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