Abundance of TRPC6 protein in glomerular mesangial cells is decreased by ROS and PKC in diabetes

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Submitted 14 January 2011; accepted in final form 22 April 2011

Am J Physiol Cell Physiol 301: C304–C315, 2011. First published April 27, 2011; doi:10.1152/ajpcell.00014.2011.—The present study was performed to investigate the underlying mechanism, particularly the roles of reactive oxygen species (ROS) and protein kinase C (PKC), in the diabetes-induced canonical transient receptor potential 6 (TRPC6) downregulation. We found that high glucose (HG) significantly reduced TRPC6 protein expression in cultured mesangial cells (MCs). TRPC6 protein was also significantly reduced in the glomeruli but not in the heart or aorta isolated from streptozotocin-induced diabetic rats. In the cultured MCs, H2O2 suppressed TRPC6 protein expression in a dose- and time-dependent manner, which emulated the HG effect. Catalase as well as superoxide dismutase were able to prevent the inhibitory effect of HG on TRPC6. The antioxidant effect observed in cultured cells was also observed in diabetic rats treated with tempol for 2 wk, which exhibited a preservation of TRPC6 in the glomeruli. Specific knockout of Nox4, a component of NADPH oxidase, increased TRPC6 protein expression. Furthermore, the PKC activator phorbol 12-myristate 13-acetate (PMA), but not its analog 4β-phorbol 12,13-di decanoate (4β-PDD), suppressed TRPC6 expression, and this PMA effect was not affected by catalase. Moreover, Gö6976, but not LY333531, attenuated the negative effect of HG on TRPC6 expression. Gö6976 also inhibited H2O2 effect on TRPC6. Furthermore, either knockdown of TRPC6 or HG treatment significantly decreased ANG II-stimulated MC contraction, and the HG-impaired MC contraction was rescued by overexpression of TRPC6. These results suggest that hyperglycemia in diabetes downregulated TRPC6 protein expression in MCs through a NADPH oxidase Nox4-TRPC6 protein expression.

GLOMERULAR MESANGIAL CELLS (MCs) are important targets of metabolic abnormalities in diabetes and contribute to the functional and structural abnormalities of diabetic nephropathy (DN). MCs exhibit impaired contractility in diabetes and thus disrupt normal glomerular hemodynamics (36). Decreased Ca2+ influx contributes to the impaired contractile function observed in MCs (43), presumably due to altered calcium channel number and/or activity resulting in disrupted Ca2+ homeostasis.

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Canonical transient receptor potentials (TRPCs) are Ca2+-permeable cation channels that play a pivotal role in regulating Ca2+ signaling in a variety of cell types (32), including MCs (7). Previously, we showed that several TRPC isoforms including TRPC1, TRPC3, TRPC4, and TRPC6 are present in MCs (35). Of these, TRPC6 is the only isoform significantly reduced in response to high glucose (HG) in cultured MCs and in isolated glomeruli of diabetic rats (17). Since TRPC6 participates in agonist-stimulated Ca2+ influx in MCs (17), these results suggest that downregulation of TRPC6 may contribute to the impaired Ca2+ signaling detected in dysfunctional MCs associated with diabetes. However, the underlying molecular mechanism of diabetes-associated TRPC6 protein downregulation in MCs is completely unknown and, therefore, serves as the major aim of the present study.

Chronic exposure to HG increases levels of reactive oxygen species (ROS) in a variety of cell types (5, 21, 23), including MCs (14, 15). In the kidneys, specifically in the renal cortex and in MCs, NADPH oxidases are the predominant source of ROS (12, 14, 34), and the Nox4 isoform of the enzymes contributes to diabetic renal hypertrophy and extracellular matrix accumulation (4, 15). ROS modulate Ca2+ channels present in different cell types by activating various signaling cascades (19, 41). Amberg et al. (2) have shown that in arterial smooth muscle cells, increased ROS levels activate L-type voltage-operated Ca2+ channels via activation of protein kinase C (PKC). Since oxidative stress and activation of PKC are major factors contributing to DN (18, 23, 25) and the interplay between ROS and PKC signaling pathways occurs in many cell types (1, 13, 22), we tested the hypothesis that the effect of HG and diabetes on TRPC6 protein expression is mediated by a ROS and/or PKC pathway.

MATERIALS AND METHODS

Generating diabetic rats. Twenty-two male Sprague-Dawley rats (~8 wk; ~200 g body wt) were purchased from Harlan (Indianapolis, IN) and were randomly assigned to control (8 rats), diabetes (9 rats), and diabetes + 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) (5 rats) groups. 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) as described in our previous publication (17). 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-tail blood sampling. STZ-injected rats with a sustained elevation of blood glucose above 300 mg/dl were designated as diabetic rats. Twenty-four-hour urine output and urine samples were collected using metabolic cages. All of the rats had
unrestricted access to food and water and were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center. The metabolic parameters of the rats in each group are summarized in Table 1.

Application of tempol to diabetic rats. Five rats in the group of STZ-injected rats were treated with tempol. The drug was supplemented in the drinking water at 1 mmol/l for 3 days before STZ injection and continued for the entire period of the experiments (~2 wk).

Isolation of glomeruli and extraction of glomerular proteins. As described in our previous publication (17), briefly, 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). After three alternate washes and centrifugations, the pellets of glomeruli were solubilized in a lysis buffer, and the supernatants were collected for Western blot analysis.

Extraction of tissue proteins. STZ- and vehicle-injected rats were euthanized 14 days after injection and the aorta, heart, and liver were removed. The tissues were chopped on ice into small pieces with blades and were homogenized with a glass homogenizer in the lysis buffer at ~0.3 ml/100 mg tissue. The homogenates were further sonicated six times for 6 s each with 30-s intervals on ice. The tissue suspension was then centrifuged at 20,817 g for 15 min at 4°C, and the supernatant was used for Western blot analysis.

MC culture. Human MCs were purchased from Cambrex and cultured as described (17). Briefly, human MCs were cultured in DMEM (Hyclone Laboratorien, Logan, UT) supplemented with 25 mM HEPES, 4 mM glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum. The concentration of D-glucose in the culture medium is indicated in the text or figure legends. An appropriate concentration of α-mannitol or L-glucose was supplemented in the culture medium as an osmotic control. Our preliminary study showed that there was no difference in the effect on TRPC6 protein expression between α-mannitol and L-glucose. Rat MCs were isolated, characterized, and cultured as described (15).

![Fig. 1](http://ajpcell.physiology.org/)

**A** shows the effect of high glucose and diabetes on TRPC6 protein expression in mesangial cells (MCs) and in tissues. A: canonical transient receptor protein 6 (TRPC6) protein expression in human MCs exposed to either 5.6 mM D-glucose + 20 mM L-glucose (normal glucose, NG) or 25.6 mM D-glucose (high glucose, HG) for 2 days. B: TRPC6 protein expression in glomeruli freshly isolated from diabetic (Db) and nondiabetic (Non-Db) rats. C: TRPC6 protein expression in the heart, liver, and aorta from nondiabetic (Con) or diabetic (Db) rats. “L” indicates a protein ladder (MagicMark XP). For A–C, actin was used as a loading control. D: quantification of TRPC6 protein expression from experiments indicated in A. *P < 0.05 compared with NG. E: quantification of TRPC6 protein expression from experiments indicated in B (exclusion of Db + Insulin) and C. *P < 0.05 compared with the corresponding Non-Db group. In D and E, n indicates the number of independent experiments.

### Table 1. Diabetic effects on metabolic parameters in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-Db</th>
<th>Db</th>
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<tr>
<td>n</td>
<td>8</td>
<td>9</td>
<td>5</td>
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<tr>
<td>BG, mg/dl</td>
<td>77 ± 3</td>
<td>425 ± 14†</td>
<td>403 ± 24*</td>
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<td>BW, g</td>
<td>348 ± 18</td>
<td>255 ± 8*</td>
<td>275 ± 12*</td>
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<td>2.8 ± 0.05</td>
<td>2.7 ± 0.2</td>
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<tr>
<td>KW/BW</td>
<td>0.77 ± 0.02</td>
<td>1.10 ± 0.04†</td>
<td>0.99 ± 0.04†</td>
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Values are means ± SE; n, number of experiments; Non-Db, nondiabetic controls; Db, diabetic rats; Db + Tempol, diabetic rats treated with tempol; BG, blood glucose; BW, body weight; KW, kidney weight (both kidneys); KW/BW, ratio of kidney weight to body weight. *P < 0.05, †P < 0.01, both were compared with Non-Db group.

*Fig. 1.* Western blot showing the effects of high glucose and diabetes on TRPC6 protein expression in mesangial cells (MCs) and in tissues. A: canonical transient receptor protein 6 (TRPC6) protein expression in human MCs exposed to either 5.6 mM D-glucose + 20 mM L-glucose (normal glucose, NG) or 25.6 mM D-glucose (high glucose, HG) for 2 days. B: TRPC6 protein expression in glomeruli freshly isolated from diabetic (Db) and nondiabetic (Non-Db) rats. C: TRPC6 protein expression in the heart, liver, and aorta from nondiabetic (Con) or diabetic (Db) rats. “L” indicates a protein ladder (MagicMark XP). For A–C, actin was used as a loading control. D: quantification of TRPC6 protein expression from experiments indicated in A. *P < 0.05 compared with NG. E: quantification of TRPC6 protein expression from experiments indicated in B (exclusion of Db + Insulin) and C. *P < 0.05 compared with the corresponding Non-Db group. In D and E, n indicates the number of independent experiments.
Knockdown of Nox4 in rat MCs. A SMARTpool consisting of four short small interfering RNA (siRNA) duplexes specific for rat Nox4 was obtained from Dharmacon. The SMARTpool of siRNA for Nox4 was transfected at 400 nM in a double transfection using X-tremeGENE (Roche Applied Science). Cells were plated in antibiotic-free media to obtain 40% confluency on the day of transfection; 100 nM scrambled control (nontargeting siRNA obtained from Dharmacon) or specific Nox4 siRNA were added to the cells. Twenty-four hours later, the medium was aspirated, and fresh medium minus antibiotics was added to the cells. The transfection was repeated, and 24 h later the cells were harvested (for a total of 48 h posttransfection) for Western blot analysis.

Rat MC transfection with Nox4. A replication-defective adenoviral vector encoding wild-type Nox4 (Ad-Nox4) was kindly provided by Dr. Barry Goldstein (Merck Research Laboratories, Rahway, NJ) and was amplified in human embryonic kidney (HEK)293 cells. Adenoviral vectors expressing green fluorescence protein (Ad-GFP) was used as a control for virus infection. Infection of cultured rat MCs was carried out for 48 h.

Western blot analyses. As described in our previous publication (35), protein extracts (40–50 μg) were fractionated by 10% SDS-PAGE, transferred to PVDF membranes, and probed with primary antibodies that were visualized with Super Signal West Femto (for TRPC6, actin, Nox4, p-ERK, p-JNK, p-p38, and p-STAT3) or Pico (for actin) luminol/enhancer solution (Pierce Biotechnology, Rockford, IL). The specific protein bands were visualized and captured using an AlphaEase FC Imaging System (Alpha Innotech, San Leandro, CA). TRPC6 protein was quantified by normalization of the optical density of TRPC6 bands to that of actin bands on the same blot using AlphaEase FC software.

Measurement of urinary F2-isoprostane levels. The levels of F2-isoprostanes were determined using gas chromatography/mass spectrometry as described by Morrow and Roberts (29) and by Ran et al. (33). Urine was added to HPLC (pH 3.0) water and mixed by vortexing. After centrifugation (2,500 g, 3 min, 4°C), the F2-isoprostanes were extracted from the clear supernatants with a C18 Sep-Pak column and a silica Sep-Pak column. The F2-isoprostanes were then converted to pentafluorobenzyl esters and subjected to thin-layer chromatography. The F2-isoprostanes were further converted to trimethylsilyl ether derivatives, and the F2-isoprostanes levels were quantified by gas chromatography/mass spectrometry. An internal standard 8-iso-PGF2α-d4 (Cayman Chemical, Ann Arbor, MI) was added to the samples at the beginning of extraction to correct for yield of the extraction process. The amount of F2-isoprostanes was expressed as nanograms of 8-iso prostaglandin F2α per milligram of urine creatinine.

MC contraction assay. As described by Du et al. (7) with modifications, human MCs were plated on a 22 × 22 mm coverslip and were transiently transfected with either green fluorescent protein (GFP) + hTRPC6-shRNA or TRPC6-EGFP constructs using GenJet (SignaGen, Gaithersburg, MD). To maintain the contractile phenotype of MCs, on the following day the cells transfected with GFP + hTRPC6-shRNA were cultured in 0.5% FBS medium with normal glucose (5.6 mM, NG). However, the cells transfected with TRPC6-EGFP were treated with 5.0% FBS medium with HG (25 mM). Contraction assays were conducted 2 days after transfection. Both hTRPC6-shRNA and TRPC6-EGFP constructs have been characterized in our previous publications (16, 17).

For the contraction assay, the coverslip with MCs was mounted onto a perfusion chamber placed on a Leica Confocal Laser Scanning fluorescence microscope (Zeiss LSM410). Cells were bathed in physiological saline solution supplemented with 10% glucose. Green cells were targeted as positively transfected cells and the neighboring nongreen cells in the same field served as controls. MC contraction was determined by the changes in the planar surface area in response to 100 nM ANG II. To this end, the images of the selected MCs were captured before, 3, and 30 min after application of ANG II. The
perimeter of each individual cell with a clearly defined border was outlined, and the planar surface area was calculated using ImageJ software (NIH, Bethesda, MD).

Evaluation of intracellular ROS levels by electron paramagnetic resonance spectroscopy. The oxidation of the cyclic hydroxylamine 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) from Alexis Biochem (Lausen, Switzerland) was used to measure the intracellular ROS levels in MCs by electron paramagnetic resonance (EPR) spectroscopy (20). CMH itself is a hydroxylamine that is silent to EPR. The reaction of CMH with ROS results in formation of a nitroxide radical that is detectable by EPR. CMH (100 μM) was added to MC culture under various treatments 30 min before the end of the treatments. After treatments, cells were washed with cold PBS, collected, and stored at −80°C until measurements were taken. When EPR measurements were conducted, cells were drawn into Teflon tubing (Zeus Industries, Raritan, NJ), folded four times, and inserted into a quartz tube. The EPR spectra were obtained with a Bruker EleXsys 540 x-band EPR spectrometer (Billerica, MA) operating at 9.03 GHz and 100 kHz field modulation at room temperature. Settings for the spectrometer were the following: magnetic field, 352 mT; scan range, 8 mT; microwave power, 5 mW; modulation amplitude, and 0.1 mT; time constant, 0.16 s. The EPR spectra were collected, stored, and manipulated using the Bruker Software Xepr (Billerica, MA).

Materials. Actin primary antibodies and all chemicals were purchased from Sigma-Aldrich (Sigma, St. Louis, MO) except LY333531, which was purchased from Axxora (San Diego, CA). TRPC6 antibody was purchased from Alexis Biochem (Lausen, Switzerland) and TRPC6-EGFP construct was generated in our laboratory (16). Statistical analysis. Data were reported as means ± SE. One-way ANOVA plus Student-Newman-Keuls test and Student’s unpaired t-test were used to analyze the differences among multiple groups and between two groups, respectively. *P < 0.05 was considered statistically significant. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

TRPC6 protein expression was reduced in HG-treated MCs and in diabetic glomeruli. In agreement with our previous study (17), incubation of human MCs with 25.6 mM D-glucose for 2 days markedly decreased the expression level of TRPC6.

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Fig. 3. Effect of antioxidants on HG-induced TRPC6 response. A: *top shows representative TRPC6 protein expression in human MCs exposed to either 5.6 mM D-glucose + 20 mM L-glucose (NG), 25.6 mM D-glucose (HG) alone, 25.6 mM D-glucose + 250 U/ml polyethylene glycol (PEG)-catalase (HG + Cat), or 25.6 mM D-glucose + 300 U/ml PEG-superoxide dismutase (SOD) (HG + SOD) for 2 days. Actin served as a loading control. For a better view, the extra space between HG + Cat lane and HG + SOD lane on the original blot was removed and both TRPC6 and actin bands for HG + SOD were moved toward the lane of HG + Cat in parallel using Photoshop software, as indicated by the dashed lines. *Bottom shows a representative TRPC6 protein expression in human MCs exposed to either 5.6 mM D-glucose + 20 mM L-glucose (NG), 25.6 mM D-glucose (HG) alone, or 25.6 mM D-glucose + 250 U/ml PEG for 2 days. Actin served as a loading control. For a better view, the extra space between HG + Cat lane and HG + SOD lane on the original blot was removed and both TRPC6 and actin bands for HG + SOD were moved toward the lane of HG + Cat in parallel using Photoshop software, as indicated by the dashed lines. B: summary data from the experiments indicated in A. *P < 0.05 compared with NG, HG + Cat, and HG + SOD. n indicates the number of independent experiments. C and D show the production of the intracellular ROS determined by electron paramagnetic resonance (EPR) spectroscopy in MCs cultured in NG, HG, HG with either PEG-SOD (HG + SOD), PEG-catalase (HG + Cat), or PEG alone (HG + PEG). C: representative spectra. The peak height in the spectrum indicates the level of reactive oxygen species (ROS). D: summary data from 3 to 4 independent experiments presented in C. *P < 0.05 compared with NG. †P < 0.05 compared with HG. n indicates the number of independent experiments.
protein (Fig. 1A). Compared with the cells treated with the same concentration of L-glucose, the abundance of TRPC6 protein was reduced by ~50% in the HG-treated cells (Fig. 1D). As reported by several groups, including ours (16, 31, 39, 47), two bands corresponding to TRPC6 protein at ~100 and ~120 kDa were detected. The lower band likely represents a newly synthesized TRPC6, whereas the higher band represents a glycosylated version of TRPC6 that has been reported by Dietrich et al. (6). The negative effect of HG on TRPC6 in cell culture was reproduced in a rat model of diabetes. As shown in Fig. 1, B and E, TRPC6 protein in glomeruli isolated from STZ-diabetic rats was dramatically decreased compared with the glomeruli from nondiabetic control rats. Considering that MCs are a major type of glomerular cells and that TRPC6 protein expression in podocytes is not altered by HG (17), the reduction of TRPC6 protein in the diabetic glomeruli likely reflects the diabetic effect on TRPC6 expression in MCs. The effect of diabetes on TRPC6 was heterogeneous in terms of tissue specificity. The expression level of TRPC6 protein increased in the whole heart tissue but had no discernible change in the aorta of diabetic rats (Fig. 1, C and E). In this study, TRPC6 protein was not detectable in liver tissue from either control or diabetic rats (Fig. 1, C and E).

H$_2$O$_2$ effects on TRPC6 protein expression in MCs. Since it has been well described that the intracellular ROS level is elevated in diabetic glomeruli and in HG-treated MCs (14, 15), we examined whether ROS are implicated in diabetes-induced TRPC6 downregulation. Human MCs were cultured in the absence and presence of H$_2$O$_2$ at 1, 10, 100, or 500 µM for 6 h and TRPC6 protein was assessed. As shown in Fig. 2, A and B, H$_2$O$_2$ recapitulated the HG response, resulting in a decrease in TRPC6 protein. This inhibitory effect of ROS was dose dependent, achieving a significant level at 100 µM and a more profound decrease at 500 µM. In another set of experiments, human MCs were treated with 500 µM H$_2$O$_2$ for 0, 2, 6, 16, and 24 h, and TRPC6 protein was evaluated at different time points. As shown in Fig. 2, C and D, in the presence of 100 µM H$_2$O$_2$, TRPC6 protein was significantly reduced within 2 h with the most pronounced effect occurring after 6 h of treatment. TRPC6 protein expression slightly rebounded after 6 h of treatment but remained at a significantly lower level than in control-treated cells (Fig. 2, C and D). H$_2$O$_2$ treatment with a shorter time period (~2 h) did not cause a significant decrease in TRPC6 protein expression (data not shown).

Effects of antioxidants on the HG-induced decrease in TRPC6 protein in MCs. If ROS are a downstream mechanism for the HG-induced decrease in TRPC6 protein, antioxidants should attenuate the HG effect. MCs were cultured either in NG, HG, HG + polyethylene glycol (PEG)-catalase, or HG + PEG-superoxide dismutase (SOD) for 2 days and the abundance of TRPC6 protein was measured. When catalase or SOD was added into the HG culture, TRPC6 protein did not decrease as observed with HG alone (Fig. 3A, top, and B). PEG
alone failed to rescue TRPC6 protein in the MCs cultured in HG (Fig. 3A, bottom, and B). To confirm the effectiveness of the antioxidants, we employed a highly specific EPR assay to quantitatively measure the intracellular ROS levels in MCs with various treatments. As shown in Fig. 3, C and D, the intracellular ROS levels were significantly elevated in MCs with HG for 2 days. However, the HG response was prevented by either PEG-SOD or PEG-catalase but not PEG itself. These data in conjunction with the results from H2O2 treatment (Fig. 2) suggest that production of ROS is a downstream mechanism for HG-induced decrease in TRPC6 protein in MCs.

Effect of in vivo antioxidant treatment on the decrease in TRPC6 protein in glomeruli of diabetic rats. The role of ROS in diabetes-associated TRPC6 downregulation was further studied in STZ diabetic rats. Tempol is a stable and cell membrane-permeable SOD mimetic and has been successfully used to investigate the role of ROS in renal function in intact animals (9, 27). In the present study, one group of STZ-diabetic rats was treated with 1 mM tempol that was supplemented in the drinking water 3 days before STZ injection and continued for 2 wk. The expression level of TRPC6 protein in the glomeruli was evaluated and compared between that in the nondiabetic and diabetic rats without tempol treatment. In agreement with the data shown in Fig. 1, A–C, tempol treatment efficiently suppressed the effect of diabetes on renal cortex and MCs (4, 12, 14, 15, 34). Therefore, this enzyme could be an upstream molecule of the ROS-TRPC6 pathway. This assumption was tested in NG-cultured rat MCs with either knockdown or overexpression of Nox4. To knockdown Nox4, rat MCs were transfected with siRNA specific for rat Nox4 (siRNA-Nox4) and a scramble siRNA (Scr) served as a control. As shown in Fig. 5, A–C, a reciprocal effect was observed between Nox4 and TRPC6 protein expression. Nox4 was markedly decreased in the siRNA-Nox4-treated cells compared with the Scr siRNA or untransfected (UT) cells (Fig. 5, A and B). Correspondingly, TRPC6 was increased in the siRNA-Nox4 cells compared with the Scr and UT cells (Fig. 5, A and C). We noted that there was a slight increase in TRPC6 expression in the scramble control cells (Fig. 5, A and B). This change might be due to a slight decrease in Nox4 expression (Fig. 5, A and B), which might result from a nonspecific effect of the scramble sequence. The change in TRPC6 expression in response to Nox4 expression led to a remarkable increase in the ratio of TRPC6 to Nox4 expression in the cells transfected with

TRPC6. Measurement of urinary isoprostane, a marker of endogenous oxidative stress, confirmed a significant decrease in the diabetic rats with tempol supplementation (Fig. 4C). Tempol treatment did not improve hyperglycemia (Fig. 4D) but significantly reduced 24-h urine output in the diabetic rats (Fig. 4E).

Effect of knockdown or overexpression of Nox4 on TRPC6 protein expression in rat MCs. NADPH oxidases, particularly the Nox4 isoform, are the most prevalent sources of ROS in the renal cortex and MCs (4, 12, 14, 15, 34). Therefore, this enzyme could be an upstream molecule of the ROS-TRPC6 pathway. This assumption was tested in NG-cultured rat MCs with either knockdown or overexpression of Nox4. To knockdown Nox4, rat MCs were transfected with siRNA specific for rat Nox4 (siRNA-Nox4) and a scramble siRNA (Scr) served as a control. As shown in Fig. 5, A–C, a reciprocal effect was observed between Nox4 and TRPC6 protein expression. Nox4 was markedly decreased in the siRNA-Nox4-treated cells compared with the Scr siRNA or untransfected (UT) cells (Fig. 5, A and B). Correspondingly, TRPC6 was increased in the siRNA-Nox4 cells compared with the Scr and UT cells (Fig. 5, A and C). We noted that there was a slight increase in TRPC6 expression in the scramble control cells (Fig. 5, A and B). This change might be due to a slight decrease in Nox4 expression (Fig. 5, A and B), which might result from a nonspecific effect of the scramble sequence. The change in TRPC6 expression in response to Nox4 expression led to a remarkable increase in the ratio of TRPC6 to Nox4 expression in the cells transfected with

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**P < 0.05, ***P < 0.01 compared with both UT and Scr. **P < 0.01 compared with Con. n indicates the number of independent experiments. In all experiments, the TRPC6 antibody was polyclonal goat IgG, purchased from Santa Cruz.
siRNA-Nox4 (Fig. 5D). On the contrary, overexpression of Nox4 by infecting rat MCs with adenovirus encoding human Nox4 (Ad-Nox4) markedly reduced TRPC6 protein expression (Fig. 5, E–G) compared with the cells infected with adenovirus encoding GFP that served as a control (Con). A low level of TRPC6 protein expression in the Nox4-expressing cells resulted in a significant decrease in the ratio of TRPC6 to Nox4 expression (Fig. 5H). These results indicate that NADPH oxidase Nox4 regulates the expression level of TRPC6 protein in MCs.

**Involvement of PKC in the HG effect on TRPC6.** It is well established that enhanced PKC activity plays a critical role in the development of DN (40, 42). Therefore, we next determined whether PKC was involved in the diabetes-induced TRPC6 decrease in MCs. In human MCs cultured in NG with 5 μM phorbol 12-myristate 13-acetate (PMA; a PKC activator) for 30 min, TRPC6 protein decreased significantly (Fig. 6, A and B). The PMA effect occurred via activation of PKC, because this response was not observed in the cells treated with 5 μM 4-α-phorbol 12,13-didecanoate (4α-PDD, a non-PKC activating phorbol ester) (Fig. 6, A and B). Furthermore, in the presence of 300 nM Gö6976 (HG + Gö6976), a PKCα and β inhibitor, HG did not reduce TRPC6 expression as it did in the cells treated with 1 μM dimethyl sulfoxide (HG + DMSO), a vehicle control for Gö6976 (Fig. 6, C and D). However, treating the cells with LY333531, a selective PKCβ inhibitor, did not alter the HG effect on TRPC6 protein expression (Fig. 6, E and F). These data suggest that PKCβ is also a mediator of the HG-induced decrease in TRPC6 protein expression.

**ROS and PKC interplay in the regulation of TRPC6 expression.** Since both ROS and PKC are downstream signaling pathways for the HG effect on TRPC6 (Figs. 2, 3, and 6), we set out to determine whether the two molecules regulate...
TRPC6 protein expression through a common pathway or through separate pathways. As shown in Fig. 7, A and B, the PMA-induced TRPC6 decrease was not affected by catalase treatment. However, the same dose and time treatment of the antioxidant significantly inhibited the HG effect on TRPC6 (Fig. 7, C and D). These data suggest that ROS and PKC share a common mechanism for suppression of TRPC6 expression with PKC residing in a position downstream of ROS in this pathway.

**TRPC6 participated in ANG II-induced MC contraction.** To determine the physiological relevance of TRPC6 in MCs, we evaluated a role of TRPC6 in the ANG II-induced contractile response. In NG-cultured MCs transfected with a EGFP vector alone (serving as a control), exposure to 100 nM ANG II induced ∼13% decrease in the cell surface area, and this response was significantly inhibited by knockdown of TRPC6 using shRNA specific for human TRPC6 (∼3% decrease in the surface area) (Fig. 8, A and Ca). In agreement with the findings that HG reduced the abundance of TRPC6 (Fig. 1, A and D), the ANG II-induced contraction was significantly impaired in the HG-cultured MCs with control transfection (EGFP vector). The blunted response was significantly rescued by overexpressing TRPC6 (Fig. 8, B and Cb).

**DISCUSSION**

Recently, involvement of TRPCs in the development of diabetes mellitus has been proposed (8, 26, 44). However, the data from different groups are strikingly controversial. For instance, a study from Niehof and Boriak (30) showed that TRPC1 was downregulated in both human and rat diabetic kidneys. However, Bishara et al. (3) reported an upregulation of TRPC1 in endothelial cells in response to HG. Wuensch et al. (46) demonstrated an increase in TRPC1, TRPC3, and TRPC6 protein expression in human monocytes treated with HG (46). However, in platelets isolated from diabetic patients, TRPC1 expression was unchanged while TRPC3 was upregulated, and TRPC6 was downregulated compared with platelets from control subjects (49). Conversely, Liu et al. (26) reported that HG results in an increase in TRPC6 in platelets. These discrepancies suggest that the effect of diabetes on TRPC protein is tissue, cell type, and even species specific. The present study further supports the heterogeneous influence of diabetes on TRPCs since TRPC6 was reduced in diabetic glomeruli but increased in the diabetic heart and did not change in the diabetic aorta. The mechanism for the glomerulus-specific TRPC6 decrease by diabetes is unclear at present and further study is needed.

![Fig. 7. Western blot showing the interplay between ROS and PKC in the regulation of TRPC6 protein expression in human MCs. A: representative experiment showing TRPC6 protein expression in MCs cultured in NG without treatment (UT) or with 5 μM PMA or with 5 μM PMA + 300 U/ml PEG-catalase (PMA + Cat) for 30 min. PEG-Cat was applied into the culture medium ∼1 h before PMA and was included in the medium throughout the experiment. B: quantification of TRPC6 protein expression in experiments shown in A. C: representative Western blot showing TRPC6 protein expression in human MCs cultured in NG either without treatment (UT) or with 500 μM H₂O₂ for 6 h (H₂O₂) or with 500 μM H₂O₂ plus 300 nM Gö6976 (H₂O₂ + Gö6976) for 6 h. D: summary data from the experiments indicated in C. In A and C, actin served as a loading control. In B and D, *P < 0.05; **P < 0.01 compared with UT. n indicates the number of independent experiments.](http://ajpcell.physiology.org/doi/abs/10.1152/ajpcell.00160.2011)
Fig. 8. Changes in the planar surface area of MCs in response to ANG II. A and B: representative photomicrographs of MCs used in contraction assays. A: MCs were cultured in NG (5.6 mM d-glucose + 20 mM l-glucose) and were transfected with either enhanced green fluorescent protein (EGFP) alone or EGFP plus shRNA construct specific for human TRPC6 (EGFP + hTRPC6-shRNA). B: MCs were cultured in HG (25.6 mM d-glucose) and were transfected with either EGFP alone or EGFP tagged TRPC6 expression plasmid (TRPC6-EGFP). The green cells indicate the positively transfected MCs and were used for analysis indicated by arrows. C: summary data from the experiments presented in A (Ca) and B (Cb) showing the contractile response of MCs with different treatments, calculated as [(the surface area of MCs after ANG II/the surface area of MCs before ANG II)/the surface area of MCs before ANG II] × 100%. n indicates the number of cells analyzed in each group. *P < 0.05; **P < 0.01 compared with EGFP group.
We previously reported that TRPC6 participated in agonist-stimulated Ca\(^{2+}\) influx in glomerular MCs (17). In the present study, we further provide evidence that TRPC6 plays a role in MC contractile function. A decrease in the abundance of TRPC6 significantly blunted MC contraction in response to ANG II. Artificial expression of TRPC6 in MCs cultured in HG, where TRPC6 normally causes TRPC6 downregulation, rescues the impaired contractile response. These data suggest that a decrease in TRPC6 protein in MCs in diabetes may contribute to diabetic hyperfiltration by impairing the contractile function of MCs.

Diabetic hyperglycemia leads to an increase in a variety of second messengers including ROS and PKC (23, 25). We propose that both ROS and PKC are downstream molecules mediating the diabetes-induced decrease in TRPC6 protein expression in MCs. This conclusion is supported by several lines of experiments: 1) antioxidants or PKC inhibition prevented the HG-induced TRPC6 decrease in the cultured MCs and antioxidant treatment significantly attenuated the decrease in TRPC6 protein in diabetic glomeruli; 2) both ROS and PKC activators recapitulated the HG effect on TRPC6. Furthermore, our data suggest that the ROS responsible for the TRPC6 decrease were primarily derived from Nox4 NADPH oxidase because knockdown of Nox4 markedly increased the expression level of TRPC6 protein.

ROS refer to several species, such as superoxide anion radical, H\(_2\)O\(_2\), and hydroxyl radical. Our data suggest that both superoxide and H\(_2\)O\(_2\) are responsible for the TRPC6 response since H\(_2\)O\(_2\) itself significantly reduced TRPC6 protein expression and catalase, SOD, or tempol abolished the decrease of TRPC6 induced by HG or diabetes. SOD converts superoxide to H\(_2\)O\(_2\). Thus SOD and tempol are expected to recapitulate the H\(_2\)O\(_2\) effect, i.e., decreasing TRPC6 protein. Two possibilities may explain the protective effect of SOD and tempol on TRPC6 in this study. First, both superoxide and H\(_2\)O\(_2\) are required for inhibition of TRPC6 and loss of either one disables the effect from the other. The second explanation is that addition of SOD might not increase or may even decrease the level of H\(_2\)O\(_2\). In addition to the classical dismutation, there are two types of alternative pathways of superoxide consumption. One type is the oxidation of superoxide to O\(_2\) by metabolites such as ubiquinone, which has a lower H\(_2\)O\(_2\) yield than dismutation. The other type involves superoxide reduction to H\(_2\)O\(_2\) by ascorbate and iron-sulfur clusters of dehydratases, which consume superoxide with a higher H\(_2\)O\(_2\) yield than via dismutation (10, 11). Therefore, the final outcome of SOD may depend on the balance of various processes of superoxide consumption. In diabetic MCs, the ratio between the rate of processes without forming H\(_2\)O\(_2\) and the rate of processes with high H\(_2\)O\(_2\) yield might be low. Thus addition of SOD or tempol resulted in a lower level of H\(_2\)O\(_2\) as reported in mouse fibroblasts and V79 Chinese hamster cells (37).

PKC has 13 different isoforms with several different subfamilies present in MCs, of which PKC\(\alpha\) and PKC\(\beta\) are tightly associated with pathological changes of glomeruli (including MCs) in response to HG or in diabetes (28, 38, 42). In the present study, an inhibitor to both PKC\(\alpha\) and PKC\(\beta\) (Go6976), but not a selective PKC\(\beta\) inhibitor (LY33531), significantly attenuated the HG effect on TRPC6, suggesting that the isoform of PKC\(\alpha\) is the major downstream molecule mediating the diabetes-induced TRPC6 decrease. ROS and PKC can function through separate signaling pathways or through a common pathway with either one upstream or downstream of the other (13). Regarding the downregulation of TRPC6 expression in diabetes, the present study suggests that ROS and PKC work in tandem with PKC residing downstream of ROS. This conclusion is based on the findings that Go6976 significantly blocked the H\(_2\)O\(_2\) effect but that catalase did not inhibit the PMA effect. However, the same concentration of catalase with the same time period of treatment abolished the HG-induced TRPC6 decrease. Interestingly, Amberg et al. (2) have recently demonstrated that ROS regulate local Ca\(^{2+}\) channels in arterial smooth muscle cells via PKC\(\alpha\) activation. Other groups have published a similar mechanism whereby HG-generated ROS activate different PKC in hepatoma (45), mesothelial (23), and adipose-derived stem cells (1). In summary, the present study suggests that hyperglycemia in diabetes results in downregulation of TRPC6 expression in glomerular MCs through NADPH oxidase (Nox4)-derived ROS, which subsequently activate PKC. A diagram illustrating the major findings and conclusion is present in Fig. 9. How PKC suppresses the expression of TRPC6 is currently unknown. It could be through a direct modulation by phosphorylating specific transcription factors that bind the promoter region of the TRPC6 gene or through an indirect mechanism by phosphorylating one or more downstream molecules. It is known that PKC activates various transcription factors that are then capable of altering the expression of a variety of genes, providing a potential mechanism by which PKC regulates TRPC6 (24). Identification of the downstream mechanism of TRPC6 suppression by PKC would further elucidate the mech-

![Fig. 9. Summary diagram depicting the pathways involved in TRPC6 protein downregulation in MCs in diabetes. Upward arrows indicate an increase and downward arrows indicate a decrease.](http://ajpcell.physiology.org/)
anism by which diabetes modulates TRPC6 expression and thus should be pursued in the future.

ACKNOWLEDGMENTS

We thank Dr. Holly Van Remmen at the University of Texas Health Science Center in San Antonio for the isoprostane measurements. We also thank the O’Brien Kidney Center at the University of Texas Southwestern Medical Center for measuring plasma and urine creatinine in our studies. We thank Dr. B. J. Goldstein (Merck Research Laboratories, Rahway, NJ) for providing the adenovirus vector expressing human Nox4.

GRANTS

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant 5 ROI DK079968-01A2 (to R. Ma) and a Research Award from the American Diabetes Association (to R. Ma).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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