Regulation of STIM1, store-operated Ca\textsuperscript{2+} influx, and nitric oxide generation by retinoic acid in rat mesangial cells

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Zhang W, Meng H, Z-H Li, Shu Z, Ma X, B-X Zhang. Regulation of STIM1, store-operated Ca\textsuperscript{2+} influx, and nitric oxide generation by retinoic acid in rat mesangial cells. Am J Physiol Renal Physiol 292: F1054–F1064, 2007. First published November 7, 2006; doi:10.1152/ajprenal.00286.2006.—It has been shown that store-operated Ca\textsuperscript{2+} influx (SOC) plays critical roles in the activation of endothelial nitric oxide (NO) synthase (eNOS) and generation of NO in endothelial cells. Recent studies indicate stromal interaction molecule 1 (STIM1) is the molecule responsible for SOC activation following Ca\textsuperscript{2+} depletion in the ER. Retinoic acids (RA) have beneficial effects in the treatment of renal diseases. The mechanism of the RA action is still largely unknown. In the current study, we used primary cultured rat mesangial cells to examine the effect of RA on SOC and STIM1. In these cells, BK caused concentration-dependent [Ca\textsuperscript{2+}] mobilization. Treatment of the cells with RA, while it had no effect on the initial peak, reduced the plateau phase of BK-mediated [Ca\textsuperscript{2+}], response, indicating the inhibition of SOC by RA. The level of STIM1 protein but not mRNA in RA-treated cells was significantly reduced. RA treatment did not affect TGF-β-mediated gradual Ca\textsuperscript{2+} influx which occurred by superoxide anion-mediated mechanism, indicating RA treatment specifically inhibited SOC in mesangial cells.

RT-PCR and Western blot analysis demonstrated that eNOS was expressed in rat mesangial cells grown in media containing 11 and 30 but not 5.5 mM glucose. Downregulation of STIM1 protein and BK-induced SOC by RA treatment or STIM1 dsRNA were associated with abolished NO production. The 26S proteasome inhibitor lactacystin blocked the RA-mediated downregulation of BK-induced SOC, suggesting that ubiquitin-proteasome pathway may be involved in RA-mediated STIM1 protein downregulation in rat mesangial cells. Our data suggest that glucose-induced eNOS expression and NO production in mesangial cells may contribute to hyperfiltration in diabetes and RA may exert beneficial effects by downregulation of STIM1 and SOC in mesangial cells.

diabetes; protein downregulation; glomerular hyperfiltration; proteasome; lactacystin; eNOS

Diabetes is the leading cause of renal disease in humans with 30% of type 1 and 2 diabetic patients developing diabetic nephropathy and end-stage renal disease (2). The initial phase of diabetic nephropathy is characterized by an increase of glomerular filtration rate (GFR) or glomerular hyperfiltration, which may gradually progress to end-stage renal failure (13, 25, 42). In the diabetic patients who are destined to develop clinically significant diabetic nephropathy, the GFR is usually elevated by 20–40% with no renal histological alterations at the time of initial diagnosis (32). Glomerular hyperfiltration occurring in early diabetes in the absence of histological changes in the kidney indicates that abnormality of carbohydrate/lipid metabolism and/or altered renal hemodynamics in the diabetic state may be the factor(s) responsible for diabetic hyperfiltration (32). Animal studies demonstrate that elevated NO production by endothelial nitric oxide (NO) synthases (eNOS) or neuronal NO synthase (nNOS) is causally linked with diabetic renal hyperfiltration (15, 45–47). Shear stress and agonist-induced intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) mobilization plays an important role in the regulation of eNOS and nNOS activity. It has been shown that [Ca\textsuperscript{2+}]i mobilization, especially store-operated Ca\textsuperscript{2+} influx (SOC), is required for agonist-mediated eNOS activation and NO production in vascular endothelial cells (16, 19). Although multiple molecules, particularly the transient receptor potential (TRP) family of ion channels, are implicated in SOC, the molecular identity of SOC remains a mystery. Recently, by screening of hundreds to thousands of genes using RNA interference (RNAi), stromal interaction molecule 1 (STIM1) and 2 (STIM2) have been identified by two independent groups to play critical roles in the regulation of SOC in a number of cell types (20, 33). Evidence indicates that STIM1 may function as a Ca\textsuperscript{2+} sensor in the ER, i.e., STIM1 is initially located in the ER membrane while ER Ca\textsuperscript{2+} store is full and translocated to the plasma membrane to activate SOC when ER Ca\textsuperscript{2+} store is depleted (53).

Retinoic acid (RA) and other retinoids have beneficial effects in the treatment of diabetic and nondiabetic nephropathy (5, 11, 35, 49). In the anti-Thy1.1 nephropathy rat model, RA treatment has been shown to limit glomerular cell proliferation and renal damage by reduction of renal TGF-β1 and TGF receptor II expression (26). In a diabetic rat model, treatment with RA caused a drop in the urinary excretion of protein and albumin, although the effect did not reach significance (11). RA regulates the expression of multiple genes by binding and subsequent activation of RA receptors (RAR) α, β, and γ and/or retinoid X receptors (RXR) α, β, and γ (39). The binding of RA or other retinoids causes the dissociation or release of corepressors and recruitment of coactivators to prompt and facilitate gene transcription (39). It is speculated that the therapeutic effect of RA in nondiabetic renal disease animal models may be linked to downregulation of genes related to inflammation, cell proliferation, and fibrosis (28). The mechanism of RA protective effect on diabetic nephropathy is currently not known.

Mesangial cell tone plays an important role in the regulation of renal hemodynamics. Mesangial cells are also shown to be

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major contributors to glomerular mesangial matrix expansion and capillary basement membrane thickening with increased expression of the extracellular matrix (EM) components collagen IV, fibronectin, and laminin observed in advanced diabetic nephropathy (50). We previously showed that the effect of TGF-β on EM is mediated by a sustained Ca\(^{2+}\) influx and activation of calcineurin (10) in rat mesangial cells. Bradykinin (BK) has been shown to stimulate mesangial cell proliferation and EM accumulation (7, 43). Diabetes and high glucose in vitro diminish BK-stimulated [Ca\(^{2+}\)]\(i\), mobilization and contraction in mesangial cells (3, 29). In the current study, we examined the effect of RA on STIM1 expression, SOC, and NO production in rat mesangial cells. Our results suggest that eNOS expression induced by high glucose may be responsible for glomerular hyperfiltration and RA may exert beneficial effect on hyperfiltration in diabetic nephropathy by regulation of SOC.

**EXPERIMENTAL PROCEDURES**

**Materials.** Fura-2 AM, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA), mouse anti-α-tubulin monoclonal antibody, DMEM, RPMI 1640, and EDTA were purchased from Invitrogen (Carlsbad, CA). Mouse anti-STIM1 monoclonal antibody was from BD Bioscience. All other chemicals were from Sigma.

**Cell culture.** Rat mesangial cells were provided by Dr. H. E. Abboud (Department of Medicine, University of Texas Health Science Center San Antonio) and were originally cultured from glomeruli isolated by differential sieving as previously described (36). For experiments in the present studies, rat mesangial cells were cultured in RPMI 1640 medium containing 2,000 mg/l (11 mM) glucose unless otherwise specified, supplemented with 14% fetal bovine serum, and penicillin (50 U/ml)/streptomycin (50 μg/ml) at 37°C in a humidified 5% CO\(_2\) atmosphere incubator. Mesangial cells were harvested with trypsin (0.05%)-EDTA (0.02%) when reached confluence (usually 48-h culture following passage).

**Measurement of intracellular calcium.** Rat mesangial cells suspended in HING buffer containing 140 mM NaCl, 5 mM KCl, 1 mM Ca\(_{Cl2}\), 1 mM MgCl\(_2\), 1.2 mM KH\(_2 PO_4\), 10 mM glucose, 10 mM HEPES, pH 7.4, were labeled with fura-2 AM (1 μM) at 37°C for 30 min with agitation at 50 rpm. Loaded cells were washed and resuspended in HING and [Ca\(^{2+}\)]\(i\), mobilization in populations of mesangial cells was measured in a fluorometer (QM-6, Photon Technology International) using a cuvette with the temperature stabilized at 37°C through a connected water bath. The ratio of fluorescence excited at 340 and 380 nm with emission of 510 nm was recorded and used to index the change of [Ca\(^{2+}\)]\(i\), as previously reported (52).

For experiments involving measurement of [Ca\(^{2+}\)]\(i\) in Ca\(^{2+}\)-free medium, following fura-2 loading the cells were washed and resuspended in HING buffer containing 100 μM EGTA, instead of 1 mM Ca\(^{2+}\), and immediately used the same procedure as described above to measure [Ca\(^{2+}\)]\(j\).

**Simultaneous measurement of [Ca\(^{2+}\)]\(i\) and NO production.** Rat mesangial cells suspended in HING buffer were double-labeled with the [Ca\(^{2+}\)]\(i\) indicator fura-2 AM (1 μM) and NO indicator DAF-FM DA (0.5 nM) at 37°C for 30 min in the dark in the presence of 100 μM t-arginine with agitation at 50 rpm. Double-labeled cells were washed and resuspended in HING buffer containing 100 μM t-arginine. Alterations of [Ca\(^{2+}\)]\(i\) and cellular NO production were simultaneously measured using the multi-dye function of the QM-6 fluorometer. The ratio of fluorescence excited at 340 and 380 nm with emission of 510 nm was recorded and used to index the change of [Ca\(^{2+}\)]\(i\), and fluorescence excited at 488 nm with emission wavelength of 515 nm was used to detect NO production inside the cells. The validity of the use of DAF-FM fluorescence to index the production of cellular NO was confirmed with the specific NOS inhibitor NG\(^{-}\)-monomethyl-l-arginine (l-NNAME; 1 mM). The presence of l-NNAME blocked increase in DAF-2 fluorescence and had no effect on fura-2 fluorescence ratio.

**Measurement of STIM1 and eNOS mRNA.** The cellular mRNA levels of STIM1 and eNOS genes were measured in primary cultured rat mesangial cells by RT-PCR as previously described (54). Total RNA was isolated from rat mesangial cells grown to confluence in RPMI 1640 medium containing different concentrations of glucose as specified in 100-mm dishes, using the SV total RNA isolation system following the manufacturer’s instruction (Promega, Madison, WI). For RT-PCR, 2 μg of total RNA were digested with DNase I to eliminate any DNA contamination and then used to synthesize the first-strand cDNA using cDNA synthesis kit (Promega). PCR amplification of the cDNA of STIM1 and eNOS was used to measure STIM1 and eNOS mRNAs. Parallel amplification of 18S rRNA under the same condition was used as internal control. The PCR condition was 94°C, 5 min and 28 cycles of 94°C, 1 min; 56°C, 1 min; 72°C, 1 min, with a final extension of 10 min at 72°C. PCR products were separated on 1% agarose gel and analyzed. The accession number and amplified fragment size of each gene and the specific primer pairs used were: STIM1, XM_341896, 544 bp; 5'-AGGCTGCAGAGCATGTGCCG-3' and 5'-GAAACCTCTTCTCGCCTGCTG-3'; 18S rRNA, M11188, 728 bp, 5'-TAGAGTGGAAATTTTGAGCC-3' and 5'-GACTTATACGCAACGAGCTT-3'; eNOS, NM_021838, 715 bp, 5'-TACTACCTCAGCTCCTCC-3' and 5'-CTGGGTTGCAGGATGCC-3'.

Additionally, the effect of RA on STIM1 mRNA levels was also measured with real-time RT-PCR using 18S rRNA as an internal standard. Real-time PCR was carried out using an Applied Biosystems 7500 Real-Time PCR System and 96-well MicroAmp optical plates. PCR conditions were 2 min 50°C, 10 min 95°C, 40 cycles consisting of 15 s 95°C, and 1 min 60°C. The Taqman primers of human STIM1 and 18S rRNA (Cat. nos. 4351372 and 4319413E, respectively), which have the same sequences as rat, were purchased from Applied Biosystem ( Foster City, CA). Each sample was running in duplicate. Standard curves for 18S rRNA and STIM1 were performed in each experiment. The relative STIM1 mRNA levels of vehicle- and RA-treated cells were calculated using the 2\(^{-ΔΔCt}\) protocol (21).

**Western blot analysis of STIM1 and eNOS proteins.** Rat mesangial cells grown to confluence (48 h after passage) in 100-mm plates in RPMI 1640 media with different concentrations of glucose (5.5, 11, or 30 mM) as indicated were resuspended in ice-cold RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 10 mM HEPES, pH 7.4, 1.0% Triton X-100, 1 mM Na\(_3\)VO\(_4\), 1 mM NaF, pH 7.4, and 10 μg/ml pepstatin, 1 mM Na\(_3\)VO\(_4\), 1 mM NaF, and 10 μg/ml leupeptin, 1 μg/ml phenstatin, 1 mM Na\(_3\)VO\(_4\), 1 mM NaF, pH 7.4, and kept on ice for 30 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C and protein concentrations in the supernatant were determined by the BCA assay (37) using bovine serum albumin as standard and following the instruction of the company (Pierce, Rockford, IL). Proteins (50 μg) were separated by 8% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with blocking buffer (TBS, containing 0.1% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature, followed by anti-GOK mice monoclonal antibody (1:250 dilution, BD Bioscience) or rabbit anti-eNOS polyclonal antibody (1:1,000 dilution, Cayman Chemical) overnight at 4°C. α-Tubulin was used to control protein loading. The membranes were then washed and incubated with secondary antibody (1:5,000 dilution, Amersham Life Science) and STIM1 and eNOS proteins were detected by ECL following the instruction of company (Pierce).

**Downregulation of STIM1 with RNAi.** The annealed Stealth siRNA duplexes were purchased from Invitrogen. Rat mesangial cells were transfected with 10 nM of the equal combination of three siRNA duplexes (A–C below) using Lipofectamine RNAiMAX in 100-mm dishes according to the manufacturer’s instructions (Invitrogen). Sequences of the three sets of STIM1 siRNA duplexes used were: A:
antisense 5'-UAGAGUACGGUUCUGGAUAGGC-3'; sense 5'-GCCUAUAUCCAGAACCGUUACUCUA-3'; B: antisense 5'-AUAAGAAGCUAUUCUCUGCAAGCC-3'; sense 5'-GGCUGAGGAAUGCCUCUAUU-3'; C: antisense 5'-UUCGGUAAGACUCUUCAGAAGUCGCC-3'; sense 5'-GGCGACUUCUGAA-GAGUCUACCGAA-3'.

Control cells were treated in parallel with a mixture of scrambled siRNA. Cells were harvested at 48-h posttransfection. Total RNA was isolated from cell lysates by SV total RNA isolation system (Promega). The mRNA level of STIM1 was checked with RT-PCR to ensure the downregulation of STIM1 gene expression by RNAi.

Statistical analysis. Summarized results from multiple experiments were presented as means ± SE. Differences between vehicle- and RA-treated groups were tested by Student's t-test. Significant differences were defined as P < 0.05.

RESULTS

BK is a vasoactive peptide involved in the regulation of renal hemodynamics. The effect of BK is mediated by the B2 bradykinin receptor, which belongs to G protein-coupled receptor superfamily and plays important roles in pathogenesis of diabetic nephropathy (14). As shown in Fig. 1, in suspensions of fura-2 AM-loaded rat mesangial cells BK (10^{-11}-10^{-7} M) induced a concentration-dependent increase of [Ca^{2+}]. At lower concentrations (<10^{-10} M), BK caused a gradual and sustained increase in [Ca^{2+}], similar to the response of TGF-β in these cells as we previously showed (10). At concentrations ≥10^{-9} M, BK evoked a rapid rise of [Ca^{2+}], followed by an elevated plateau phase, which is presumably reflected by the activation of capacitative or SOC. Maximal [Ca^{2+}] responses were observed at 10^{-7} M BK and this concentration was used in the subsequent experiments of this study.

Pretreatment of the rat mesangial cells with RA (50 μM) for 48 h during culture significantly inhibited the plateau phases (Fig. 2D) of the [Ca^{2+}], signal, whereas it had no effect on the initial peak (Fig. 2C) of the BK (100 nM) responses, suggesting that RA treatment selectively blocks SOC in mesangial cells. Experiments performed in Ca^{2+}-free HNG further supported the block of SOC in RA-treated mesangial cells (Fig. 3). In the absence of extracellular Ca^{2+}, BK (100 nM) caused a transient increase of [Ca^{2+}], followed by rapid decay to baseline, which reflected IP3-mediated Ca^{2+} release from the ER and ER Ca^{2+} store depletion. Subsequent addition of Ca^{2+} (2 mM) in the medium triggered SOC-mediated Ca^{2+} influx (Fig.
Pretreatment of the cells with RA during culture had no effect on BK-induced transient increase of \([Ca^{2+}]_i\), but largely inhibited SOC-mediated \(Ca^{2+}\) influx following \(Ca^{2+}\) repletion in the medium (Fig. 3, B and C). The remaining \(Ca^{2+}\) influx in RA-treated cells observed after medium \(Ca^{2+}\) replenishment is likely to be mediated by non-SOC \(Ca^{2+}\) influx, which is also observed in control cells briefly exposed to \(Ca^{2+}\)-free HNG without BK stimulation (Fig. 3, A and C). Since brief exposure to \(Ca^{2+}\) free HNG does not deplete ER \(Ca^{2+}\) store, and thus these \(Ca^{2+}\) influx belongs to non-SOC. These experiments indicate that RA selectively inhibits SOC in rat mesangial cells.

Since STIM1 proteins have been shown to be a potential ER \(Ca^{2+}\) sensor required for SOC, we investigated the effect of RA treatment on the protein levels of STIM1. Immunoblotting analysis of the STIM1 proteins indicated that the expression of STIM1 proteins in RA-treated (50 \(\mu\)M, 48 h) mesangial cells was decreased (Fig. 4A; \(*P < 0.05\) control vs. RA, \(n = 14\)). However, measurement of STIM1 mRNA with RT-PCR (Fig. 4B and C) showed that the expression of STIM1 mRNA was not significantly altered by RA treatment.
and real-time RT-PCR (Fig. 4C) demonstrated that RA treatment had no effect on STIM1 mRNA levels in mesangial cells (Fig. 4C, P = 0.46 control vs. RA, n = 3), indicating that reduced STIM1 proteins in RA-treated cells were not resulted from a reduction in STIM1 mRNA. Further experiments demonstrated that the effect of RA treatment on STIM1 protein and BK-induced activation of SOC was dependent on the concentrations of RA and time of incubation (Fig. 5). As shown in Fig. 5, A and C, both the BK-induced SOC as indexed by the amplitude of the plateau phase of BK-mediated \([\text{Ca}^{2+}]_i\) response and STIM1 proteins were reduced by treatment with increasing concentrations of RA. The time dependence of concurrent RA effects on BK-induced SOC and STIM1 was demonstrated in Fig. 5, B and D.

It has been previously shown that TGF-\(\beta\) caused a gradual and sustained \([\text{Ca}^{2+}]_i\) influx, which leads to activation of calcineurin and subsequent downstream signaling pathways in rat mesangial cells (10, 23). Both TGF-\(\beta\) and BK have been demonstrated to play critical roles in the pathogenesis and progression of diabetic nephropathy (14, 22, 43, 55). It is interesting to compare the effect of RA on the \([\text{Ca}^{2+}]_i\) responses to BK and TGF-\(\beta\), which may provide insight into the mechanisms of pathogenesis of the disease. As shown in Fig. 6 (\(B\) compared with \(A\)), RA (50 \(\mu\)M, 48 h) treatment led to reduced plateau phase of BK-mediated \([\text{Ca}^{2+}]_i\), response and inhibition of SOC. In contrast, the same treatment had no effect on TGF-\(\beta\)-mediated \([\text{Ca}^{2+}]_i\) response (Fig. 6, C and D). In vehicle (control)- or RA-treated (50 \(\mu\)M, 48 h) mesangial cells, TGF-\(\beta\) (10 ng/ml) induced similar gradual and sustained \([\text{Ca}^{2+}]_i\) responses while the plateau phase of BK-mediated \([\text{Ca}^{2+}]_i\) responses is abolished. These results indicate that RA specifically affects BK-induced SOC but not TGF-\(\beta\)-mediated \([\text{Ca}^{2+}]_i\) influx in rat mesangial cells, and thus inhibition of BK-induced SOC by RA is pathway specific and is not a result of a global downregulation of \([\text{Ca}^{2+}]_i\) transport pathways in rat mesangial cells.

SOC-mediated \([\text{Ca}^{2+}]_i\) influx may regulate NO, which is a potential contributor to diabetic nephropathy. In endothelial
cells, Ca\(^{2+}\) influx via SOC has been shown to be required for Ca\(^{2+}\)-dependent eNOS activation (16, 19). We next examined whether SOC activation also led to NO generations in mesangial cells. We took the advantage that RA treatment selectively downregulated STIM1 and SOC in rat mesangial cells (Figs. 2–5), which should result in the block of eNOS activation and NO production by BK. As shown in Fig. 7, in fura-2 and DAF-FM doublelabeled rat mesangial cells, BK (100 nM) induced simultaneous [Ca\(^{2+}\)]\(_i\) mobilization (solid traces) and NO generation (dashed traces; Fig. 7A). Treatment of the cells with RA (50 \(\mu\)M, 48 h) reduced the plateau phase of the BK-mediated [Ca\(^{2+}\)]\(_i\) response and blocked NO production (Fig. 7B). Inclusion of \(N^G\)-monomethyl-L-arginine (1 mM), a general NOS inhibitor, completely blocked BK-induced increase in DAF-FM fluorescence, e.g., NO generation, while it had no effect on BK-mediated [Ca\(^{2+}\)]\(_i\) response (Fig. 7C), indicating the specificity of DAF-FM to NO. The dependence of BK-induced NO production on SOC was further demonstrated in the experiments shown in Fig. 7D. In the absence of extracellular Ca\(^{2+}\), BK caused a transient increase of [Ca\(^{2+}\)]\(_i\) without a sustained plateau phase and NO generation. Subsequent addition of Ca\(^{2+}\) (2 mM) in the medium triggered SOC, which was accompanied by NO production (Fig. 7D). Taken together, these experiments suggested that NO production in mesangial cells was activated by BK-mediated [Ca\(^{2+}\)]\(_i\) mobilization and downregulation of STIM1 protein expression and SOC by RA was associated with eliminated NO generation in mesangial cells.

The role of STIM1 in BK-induced SOC and NO production was further supported by the data from STIM1 RNAi experiments (Fig. 8). As indicated by the results of RT-PCR (Fig. 8A), treatment of mesangial cells with STIM1 dsRNA largely reduced the STIM1 mRNA levels. Simultaneous measurement of BK-induced [Ca\(^{2+}\)]\(_i\) mobilization (solid traces) and NO production (dashed traces) demonstrated that STIM1 RNAi abolished the elevated plateau of [Ca\(^{2+}\)]\(_i\) response and NO production (Fig. 8, C compared with B). To ensure the inhibition of NO production by STIM1 dsRNA did not result from an off-target inhibition of eNOS expression, we measured and found no difference in the eNOS mRNA levels in control and STIM1 dsRNA-treated mesangial cells (data not shown).

Among the three isoforms of NOS, eNOS and nNOS are known to be regulated by Ca\(^{2+}\). It is currently unclear which isoforms of the NOS are expressed in rat mesangial cells. We next determined the expression of eNOS and nNOS in mesangial cells. RT-PCR and Western blot analysis indicated that eNOS mRNA and protein were expressed in rat mesangial cells, and the levels of eNOS mRNA and protein were depen-

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**Fig. 7.** BK-induced [Ca\(^{2+}\)]\(_i\) mobilization and nitric oxide (NO) production. Mesangial cells were double labeled with fura-2 AM (1 \(\mu\)M) and DAF-2 DA (0.5 \(nM\)) and BK (100 nM)-induced [Ca\(^{2+}\)]\(_i\) mobilization (solid traces) and NO production (dashed traces) were measured as described in EXPERIMENTAL PROCEDURES. A: BK (100 nM) responses in control cells. B: BK (100 nM) responses in the presence of l-NAME (1 mM). C: BK (100 nM) responses in cells treated with RA (50 \(\mu\)M, 48 h). D: BK (100nM)-induced [Ca\(^{2+}\)]\(_i\) mobilization and NO production in control cells in the absence of extracellular Ca\(^{2+}\) and following Ca\(^{2+}\) repletion in the media as indicated. The arrows in A–C indicated the addition of BK. In D, the arrows indicated sequential additions of BK and 2 mM Ca\(^{2+}\).
dent on the concentrations of glucose in the RPMI 1640 media during culture (Fig. 9, A and B). When the cells were cultured in RPMI 1640 media containing 11 or 30 mM glucose, the expression of eNOS mRNAs and proteins was detected by RT-PCR and Western blot analysis, respectively (Fig. 9). In cells cultured in RPMI1640 media containing 5.5 mM (1,000 mg/l) glucose, eNOS mRNA and protein were barely detectable with RT-PCR and Western blot analysis (Fig. 9). Regardless of the glucose levels during culture, nNOS mRNA was not detected with RT-PCR (Fig. 9A). Additionally, treatment of mesangial cells grown in 11 mM glucose media with RA (50 μM, 48 h) had no effect on the eNOS protein levels (Fig. 9C).

It is well established that ubiquitin-proteasome pathway plays important roles in posttranslational protein modification and degradation, which contributes to the accurate maintenance of dynamic protein levels responding to altered physiological conditions (30). Containing of the PEST region (Fig. 10, top) may target STIM1 to the degradation by proteasomes. As shown in Fig. 10, A and B, treatment of rat mesangial cells with RA (50 μM, 48 h) reduced the plateau phase, i.e., the SOC of BK-induced [Ca²⁺] responses. However, in the presence of lactacystin (10 μM) the same RA treatment showed no effect on the plateau of BK-mediated [Ca²⁺] responses (Fig. 10, A-D), indicating that proteasome pathways were involved in the RA inhibition of BK-mediated SOC activation. On the other hand, treatment of the cells with lactacystin, RA, or combination of the two reagents had no effect on the gradual [Ca²⁺] responses activated by TGF-β (Fig. 10, E-H).

DISCUSSION

Elevated NO production by neuronal and/or endothelial nitric oxide synthase (nNOS/eNOS) but not inducible NOS (iNOS) has been shown to be a major factor responsible for the pathogenesis of glomerular hyperfiltration in early diabetic nephropathy (15, 45–47). Increased evidence from animal models indicates that the BK B₂ receptor is critically involved in diabetic nephropathy (14). B₂ receptors are detected in multiple cell types in the kidney, including mesangial and endothelial cells (38). Disruption of B₂ receptor expression markedly enhances nephropathy in diabetic mice (14). Although it has been the focus of intensive investigations, the effect of hyperglycemia on eNOS expression and NO production in renal cells remains inconclusive (15). In vitro experiments using high glucose (~30 mM) in cultured cells to assess the effect of diabetes on NO production generally suggested a
regulation of renal NO production (15). Most in vivo evidence suggests increased eNOS expression and/or NO production in the renal cortex in diabetes, which may be responsible for the glomerular hyperfiltration and hypertrophy observed in early diabetes (15). Under normal physiological conditions, eNOS is detected in the afferent/efferent vascular and glomerular endothelial and epithelial cells but not mesangial cells in human and animal kidneys (1, 9). Based on in vitro experiments with cultured endothelial cells, it is unlikely that the eNOS in vascular or glomerular endothelial cells is responsible for the excess NO production and glomerular hyperfiltration in early diabetes since hyperglycemia reduced the production of NO in these cells (12, 41, 44). Even though previous evidence supported that NO production by eNOS/nNOS may cause hyperfiltration in early diabetes, the exact cell types in the kidney from which the elevated NO generation lead to hyperfiltration are still not defined. Our present study demonstrates that rat mesangial cells cultured under moderate (11 mM) and high (30 mM) but not normal (5.5 mM) glucose expressed eNOS mRNA and protein (Fig. 9). Furthermore, the expressed eNOS in mesangial cells is activated by BK-mediated [Ca\(^{2+}\)], mobilization, e.g., SOC (Fig. 7). Specific downregulation of STIM1 with RNAi, while it had no effect on the eNOS protein levels, eliminated SOC and NO generation following BK stimulation (Fig. 8). These results suggest that in response to increased glucose levels, eNOS expression may be induced and NO production may be activated by Ca\(^{2+}\)-dependent mechanisms in rat mesangial cells, which could become a potential NO generation site under hyperglycemic conditions. Our results therefore provide novel evidence that diabetic glomerular hyperfiltration may be caused by eNOS-mediated NO production in mesangial cells.

Protein level and activity of eNOS are two factors among others to affect NO production in mesangial cells. The Ca\(^{2+}\)-calmodulin-dependent activation of eNOS is mediated by SOC in endothelial cells (16, 19). As we demonstrated in the current study, SOC also plays a critical role in the activation of BK-induced NO production in rat mesangial cells (Figs. 7 and 8) and is an essential component of BK-mediated [Ca\(^{2+}\)], mobilization (Figs. 1 and 2). STIM1 and STIM2 have been identified to play critical roles in the regulation of SOC in a number of cell types (20, 33). Evidence indicates that STIM1 may function as a Ca\(^{2+}\) sensor in the ER, i.e., STIM1 is initially located in the ER membrane as monomers while the ER Ca\(^{2+}\) store is full and translocated to the plasma membrane to activate SOC when the ER Ca\(^{2+}\) store is depleted or physically coupled to the SOC channel on the plasma membrane (40, 53). We demonstrated that STIM1 mRNAs and proteins were expressed in rat mesangial cells (Fig. 4). RA as well as STIM1 RNAi treatment concurrently inhibited the expression of STIM1 and BK-induced SOC (Figs. 2, 3, 4, and 8), suggesting that STIM1 is involved in agonist-mediated activation of SOC in mesangial cells. These data suggest that STIM1 may be a mediator for regulation of eNOS in mesangial cells and can serve as a potential therapeutic target for diabetic nephropathy.

In mesangial cells, [Ca\(^{2+}\)], response may be induced by BK and other G protein-coupled receptor agonists, such as endothelin-1 (ET-1), as well as TGF-β, PDGF, and other growth factors in mesangial cells (8, 10, 27). High glucose (~30 mM) has been shown to inhibit ET-1- but not PDGF-mediated [Ca\(^{2+}\)], response (8, 27). The reduced [Ca\(^{2+}\)], response of ET-1 in high glucose was linked to PKC-dependent inactivation of PLC\(\beta\)3 (8). The effects of high glucose on the ER Ca\(^{2+}\) stores and SOC are still inconclusive (8, 27). Whether high glucose affects BK-induced [Ca\(^{2+}\)], signaling and how the effects contribute to RA regulation of STIM1 expression, SOC, and NO generation is currently unclear and requires further studies.

The therapeutic effect of RA in anti-glomerular basement membrane-induced nephritis seems linked to the downregulation of several genes related to inflammation, cell proliferation, and fibrosis (28). In a streptozotocin-induced diabetic rat ne-

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**Fig. 9. Detection of eNOS expression.**

A: eNOS mRNA levels in rat mesangial cells grown in RPMI 1640 containing 5.5, 11, and 30 mM glucose, respectively, were analyzed with RT-PCR as described in EXPERIMENTAL PROCEDURES. B and C: Western blot analysis of eNOS protein in cells grown in RPMI 1640 containing different concentrations of glucose as indicated (B), and the effect of RA on eNOS protein (C).
RA treatment may alter glucose metabolism of mesangial cells, and which may lead to downregulation of STIM1 protein, inhibition of SOC, and reduced NO production. It is also possible that RA downregulates STIM1 proteins without alteration in glucose/lipid metabolism in mesangial cells. Together with previous studies (4, 17, 18, 24, 48), our present observations suggest that RA may exert beneficial effects in diabetic renal disease treatment by lowering blood glucose and inhibiting NO production through downregulation of STIM1 and inhibition of SOC in mesangial cells.

Analysis of the proposed promoter region of STIM1 gene [the 1.8-kb fragment upstream the 5′-end of the gene (34)] indicates that the STIM1 promoter does not contain the consensus motif for potential RAR/RXR or RXR/RXR binding. Further search of a 2-kb region upstream the promoter, which could be the site for a potential enhancer, also does not show the presence of any potential RAR/RXR binding sites. Thus our data that STIM1 mRNA level was not altered by RA treatment was consistent with the lack of RA-regulatory elements in STIM1 gene.

RA and other retinoids exert prodifferentiation effects during embryonic development and antiproliferation effect in various cancer cells by selective degradation of cell cycle-specific proteins has been shown to occur via the ubiquitin-proteasome process (51). The proteasome activity as measured by the proteasome substrate sLLVY-MCA is significantly decreased in the liver and kidney homogenates of diabetic rat compared with control (31). Preincubation of mesangial cells with lactacystin prevented the inhibitory effect of RA on BK-induced SOC but had no effect on TGF-β (10 ng/ml; E-F) mediated Ca2⁺ influx (Fig. 9), suggesting that the 26S proteasome pathways may be involved in the downregulation of STIM1 protein and SOC. Although the composition, function, and the regulation of 26S proteasome in the kidney are virtually unknown, the results of our experiments provide evidence that RA downregulation of STIM1 protein and SOC is abrogated by blockage of proteasome in rat mesangial cells.

In summary, our data suggest that inhibition of BK-induced SOC and NO production by RA treatment is associated with downregulation of STIM1 protein, and which may lead to hyperfiltration in diabetic nephropathy.
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