The impact of translationally controlled tumor protein on the podocyte hypertrophy under diabetic conditions

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The impact of translationally controlled tumor protein on the podocyte hypertrophy under diabetic conditions

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ABSTRACT

The impact of translationally controlled tumor protein on the podocyte hypertrophy under diabetic conditions

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Background: Hypertrophic mechanism requires cell cycle arrest at the G_1/S interphase, which is principally mediated by inhibitors of cyclin-dependent kinase (CDK), namely CDK-inhibitors (CKIs). In addition to CKIs, recent studies have shown that activation of mRNA translation regulating signaling pathways such as mammalian target of rapamycin (mTOR) is also implicated in cellular hypertrophy in various diseases. Translationally controlled tumor

protein (TCTP) is highly conserved in eukaryotic cells and has been suggested to be involved in various intracellular processes including cell proliferation and growth by regulating mRNA translation pathway. Since glomerular cells hypertrophy is a characteristic finding in diabetic nephropathy, there is a possibility that TCTP may play an important role in the pathogenesis of diabetic nephropathy. However, the functional role of TCTP on cellular hypertrophy under diabetic conditions has never been explored. In this study, I examined not only the changes in TCTP expression in high glucose-stimulated podocytes and in experimental diabetic glomeruli but also the impact of TCTP on podocyte hypertrophy under diabetic conditions.

Methods: *In vitro*, immortalized mouse podocytes ether with or without transfection of TCTP shRNA expressing lentivirus were serum restricted for 24 hr, after which the medium was changed to RPMI medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+10^{-6} M angiotensin II (NG+ANG II), 30 mM glucose (HG), or HG+10^{-7} M L-158,809 (HG+ARB). *In vivo*, 32 C57BL/6 mice were injected either with diluent (n=16, C) or streptozotocin intraperitoneally for 5 consecutive days (n=16, DM). Eight mice from each group were treated with lentivirus containing TCTP shRNA (LV-shTCTP). Real-time PCR for TCTP mRNA expression and Western blotting for TCTP, phospho-4EBP1, 4EBP1, phospho-p70S6K, p70S6K, p27,

p21 or β-actin protein expression were performed with cell lysates and sieved glomeruli. Double immunofluorescence (IF) staining with synaptopodin and TCTP was also performed with renal tissue. Podocyte hypertrophy was assessed by measurement of cellular protein/cell counts and by flow cytometry, and glomerular volume by morphometry.

Results: Compared to NG cells, TCTP mRNA and protein expression were significantly increased in podocytes exposed to HG for 48 hrs, and this increase in TCTP expression was abrogated by ARB treatment. Similarly, ANG II induced TCTP mRNA and protein expression in cultured podocytes. Glomerular TCTP expression was also significantly higher in DM compared with C mice. Double IF staining for TCTP and synaptopodin revealed that podocytes were the main cells responsible for the increase in TCTP protein expression under diabetic conditions. TCTP inhibition using LV-shTCTP ameliorated the increase in phospho-4EBP1, phospho-p70S6K, and p27 protein expression in high glucose-stimulated podocytes and in diabetic glomeruli along with reduced podocyte and glomerular size.

Conclusion: I demonstrate for the first time that TCTP expression is increased in podocytes under diabetic conditions and that inhibition of TCTP attenuates the activation of mTOR target molecules, 4EBP1 and p70S6K, and CKIs expression, along with reduced podocyte and glomerular size. These

findings suggest that TCTP may play an important role in the process of podocyte hypertrophy under diabetic conditions via regulating mRNA translation and inducing cell cycle arrest at the G_1/S interphase.

Key words: translationally controlled tumor protein, diabetic nephropathy, podocyte, hypertrophy

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I. INTRODUCTION

Diabetic nephropathy, the leading cause of end-stage renal disease in many countries, is characterized by renal hypertrophy in the early course of the disease[1], which is mainly due to glomerular and tubular hypertrophy, accumulation of extracellular matrix, and hemodynamic alterations[2]. Among these, glomerular hypertrophy is partly contributed by glomerular cells

hypertrophy, and recent studies have suggested that interruption of glomerular hypertrophy may ameliorate the chronic changes of diabetic nephropathy[3], suggesting that understanding the pathogenetic mechanism of cellular hypertrophy under diabetic conditions is important and can be applied to the prevention and treatment of diabetic nephropathy[4]. While the diabetic milieu *per se*, hemodynamic changes, and local growth factors such as angiotensin II (ANG II) are considered to be mediators in the pathogenesis of glomerular cells hypertrophy[5], the underlying pathways mediating these processes are not well understood.

Cell culture experiments using mesangial cells and podocytes under high glucose conditions and *in vivo* studies in various models of diabetes suggest the hypertrophic mechanism requires the combined effect of mitogen-induced entry into the cell cycle and subsequent arrest at the G_1/S interphase[5-8]. Numerous studies have demonstrated this cell cycle arrest is principally mediated by inhibitors of cyclin-dependent kinase (CDK), namely CDK-inhibitors (CKIs), which are classified into two groups according to their structural homology: the INK family (p15, p16, p18, and p19) and the Cip/Kip family (p21, p27, and p57)[9,10].

In addition to CKIs, activation of mRNA translation regulating signaling pathways including phosphatidylinositol-3 (PI3) kinase/Akt/mammalian

target of rapamycin (mTOR), the AMP-activated protein kinase (AMPK)/tuberous sclerosis complex (TSC) 1/TSC 2/Ras homologue enriched in brain (Rheb), and the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway have been found to contribute to glomerular hypertrophy in animal model of diabetes[11-16]. These pathways are closely linked with one another and eventually promote the initiation and elongation phase of mRNA translation, resulting in increased cell size[14-16]. Among these pathways, mTOR and its downstream effectors such as eukaryotic elongation factor 4E binding protein 1 (4EBP1) and p70 S6 ribosomal protein kinase (p70S6K) have shown to play a pivotal role in the hypertrophic process under diabetic conditions[14-17].

Since MacDonald *et al*[18] first identified translationally controlled tumor protein (TCTP) as a stimulus of histamine release from human basophils, it has been believed to play an important role in the chronic allergic disease process. Recently, however, many studies have demonstrated that TCTP has multifunctional intracellular functions, including cell proliferation, growth, survival, and stress responses[19], in addition to its original histamine releasing function. Even though the precise mechanisms of TCTP on the regulation of cell growth and proliferation remain elusive, recent investigations have revealed that TCTP directly interact with eukaryotic elongation factor (eEF) 1A, subsequently rendering more efficient elongation phase of mRNA

translation[20]. Moreover, TCTP is reported to activate PI3 kinase/Akt and Ras/Raf/ERK pathway and to directly regulate Rheb, which is considered a upstream regulator of mTOR/S6K/4EBP signaling, suggesting that TCTP may not only play a key physiological role in cell survival but also induce cellular or organ hypertrophy through the phosphorylation of the effectors of mRNA translation such as 4EBP1 and p70S6K[21-23].

Since glomerular cell hypertrophy is a characteristic finding in diabetic nephropathy, there is a possibility that TCTP may play an important role in the pathogenesis of cellular hypertrophy through the activation of mRNA translation. However, the functional role of TCTP under diabetic conditions has never been explored. In this study, I examined not only the changes of TCTP expression in podocytes under diabetic conditions but also the impact of TCTP on podocyte hypertrophy. In addition, the signaling pathway regulating podocyte hypertrophy was also investigated.

II. MATERIALS AND METHODS

1. Generation of TCTP shRNA expressing lentivirus

The cDNA sequence of the mouse TCTP (GenBank accession number, NM009429) was examined using computer software to select appropriate siRNA target sites (http://jura.wi.mit.edu/bioc/siRNAext/home.php). Three pairs of oligonucleotides corresponding to TCTP cDNA positions 34~54 bp, 178~198 bp, and 447~467 bp were designed. A Blast search of these sequences confirmed their specificity to TCTP only. In addition, a scrambled sequence with no near match to any known sequence was designed as control. Each oligonucleotide was composed of a 21-nucleotide sense sequence, a loop, a 21-nucleotide antisense sequence, five thymidines as well as overhang sequences CGA at the 5′ and TTT at the 3′ for cloning purposes (Table. 1). The pairs of corresponding designed oligonucleotides were annealed and inserted between the *BstBI* and *BbsI* sites of the pCMV-U6 plasmid (Gift from Dr. Pavel Osten, Feinberg School of Medicine, IL, USA)[24]. Positive clones were identified by PCR using the sense primer (5'-CCC GCT AGC ATC CGA CGC CGC CAT CTC TA-3') and the antisense primer (5'-CCA CCG CAT CCC CAG CAT GCC -3') under the following conditions: 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at

72°C for 45 sec. Initial heating at 94°C for 9 min and final extension at 72°C for 7 min were performed for all PCRs. The PCR products were analyzed by electrophoresis in a 2.0% agarose gel, and the positive samples were sequenced, and named as pCMV-U6-TCTP shRNA-1, -2, -3, and -scrambled.

The U6-TCTP shRNA cassettes were recloned into lentiviral transfer vector FUGW (Gift from by Dr. Pavel Osten)[25] using *NheI*/*BstBI*, and as a result, a vector FUG-TCTP shRNA was created.

TCTP construct was also generated by PCR from mouse genomic DNA with primers 5'-A ACC GCT AGA TCT ATG ATC ATC TAC CGG GAC ATA-3' and 5'-AGG CCG GTC GAC TTA ACA TTT CTC CAT CTC TAA GCC-3', and cloned into pEGFP-C1 (BD Biosciences Clontech, Franklin Lakes, NJ, USA) using *BglII*/*SalI*, resulting in a vector pEGFP-C1-TCTP.

Lentiviruses were produced as previously described[26]. Human embryonic kidney (HEK) 293FT cells (Invitrogen, Carlsbad, CA, USA) were transfected by using the calcium phosphate method with the expression and two helpers, packaging plasmid pCMV Δ8.9 and vesicular stomatitis virus G protein plasmids at 1, 7.5, and 5.5 μg of DNA per 100 mm plate. After 48 hr, the supernatants of four plates were pooled, spun at 780 g for 5 min, and filtered at a 0.45 μm pore size. Next, a centrifugation at 83,000 g for 1.5 hr was performed, and the pellet was resuspended in 100 μl of phosphate-buffered

saline (PBS). Titers of lentivirus were determined by transfecting HEK 293T cell with a dilution series of viral suspension, and the lentivirus with a titer of 4×10^8 transfection units/ml was stored at -80°C.

Table 1. Sequences of TCTP shRNAs and TCTP shRNA-scrambled

		Sense	Loop	Antisense
TCTP shRNA-1				
Forward :	TTT	GAGCTGTTCTCCGACATCTAC	GTGAAGCCACAGATG	GTAGATGTCGGAGAACAGCTC
Reward:	CGAAAAA	GAGCTGTTCTCCGACATCTAC	CATCTGTGGCTTCAC	GTAGATGTCGGAGAACAGCT
TCTP shRNA-2				
Forward:	TTT	GAAGGTACCGAAAGCACAGTA	GTGAAGCCACAGATG	TACTGTGCTTTCGGTACCTTC
Reward:	CGAAAAA	GAAGGTACCGAAAGCACAGTA	CATCTGTGGCTTCAC	TACTGTGCTTTCGGTACCTT
TCTP shRNA-3				
Forward:	TTT	GGACTACCGTGAAGATGGTGT	GTGAAGCCACAGATG	ACACCATCTTCACGGTAGTCC
Reward:	CGAAAAA	GGACTACCGTGAAGATGGTGT	CATCTGTGGCTTCAC	ACACCATCTTCACGGTAGTCC
TCTP shRNA-scrambled				
Forward:	TTT	GCGTATGAGTAGTGCGCAGAT	GTGAAGCCACAGATG	ATCTGCGCACTACTCATACGC
Reward:	CGAAAAA	GCGTATGAGTAGTGCGCAGAT	CATCTGTGGCTTCAC	ATCTGCGCACTACTCATACGC

2. Podocyte culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (University of Miami Miller School of Medicine, Miami, Florida, USA) and were cultured as previously described[27]. Briefly, frozen podocytes were first grown under permissive conditions at 33°C in RPMI 1640 media containing 10% fetal bovine serum, 50 U/ml γ-interferon and 100 U/ml of penicillin/ streptomycin in collagen coated flasks, and the γ-interferon tapered down to 10 U/ml in successive passages. Cells were then trypsinized and subcultured in six-well plates without γ-interferon (non-permissive conditions) and allowed to differentiate at 37°C with media changed on alternate days. Differentiation of podocytes grown for 10 days at 37°C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by RT-PCR and Western blotting (data not shown). In case of lentiviral transfection to podocytes, 1 ml of the lentivirus suspension was added to the wells after the confirmation of differentiation.

Lentiviral transfected or non-transfected podocytes were serum restricted for 24 hr, after which the medium was changed to RPMI medium containing normal glucose (NG) (5.6 mM glucose), NG+24.4 mmol mannitol, NG+10^{-6} M/L ANG II (NG+ANG II), high glucose (HG) (30 mM glucose), HG+10^{-7} M/L L-158,809, a selective ANG II type I receptor blocker (HG+ARB). After

48 hr, cells were harvested for either RNA or protein.

3. Animals

All animal studies were conducted using approved protocols. C57BL/6 mice weighing 18~22 g were injected either with diluent [n=16, Control (C)] or with 55 mg/kg/day streptozotocin (STZ) intraperitoneally for 5 consecutive days [n=16, Diabetes (DM)]. Diabetes was confirmed by tail vein blood glucose levels above 300 mg/dl on the third day after the last STZ injection. After the confirmation of diabetes, eight mice from each group were treated with lentivirus vector containing TCTP shRNA at a dose of 4×10^8 transfection units on day 0 and 7. Mice were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 6-week study period.

Body weights were checked biweekly, and kidney weights were measured at the time of sacrifice. Serum glucose and 24-hr urinary albumin were measured weekly and at the time of sacrifice, respectively. Blood glucose was measured by glucometer and 24-hr urinary albumin excretion by enzyme-linked immunosorbent assay (ELISA) (Albuwell M, Exocell, Inc., Philadelphia, PA, USA).

4. Glomerular isolation

Glomeruli were isolated by sieving technique as previously described[28]. Purity of the glomerular preparation was greater than 98% as determined by light microscopy.

5. Total RNA isolation

Podocytes RNA from each plate was extracted as previously described[29]. Briefly, after cell scraping with 800 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA), the mixture was vortexed and stored for 5 minutes at room temperature. Next, 160 μl of chloroform was added and the mixture was shaken vigorously for 30 sec. After 3 min, the mixture was centrifuged at 12,000 g for 15 min at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 μl isopropanol and pelleted with centrifugation at 12,000 g for 30 min at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using Speed Vac, and dissolved in DEPC-treated distilled water. Total RNA from sieved glomeruli was extracted similarly. Podocyte and glomerular RNA yield and quality were assessed based on spectrophotometric measurements at the wavelength of 260 and 280 nm.

6. Reverse transcription

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from cultured podocytes and sieved glomeruli were reversely transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 hr followed by inactivation of the enzyme at 99°C for 5 min.

7. Real-time PCR

The primers used for TCTP and 18s amplifications were as follows: TCTP sense 5'- GCT AGG ACA GTT ATA AGC TCA GGC -3', antisense 5'- TCC TGGTGT TGT ATG GAT GG -3'; and 18s sense, 5'-AGT CCC TGC CCT TTG TAC ACA-3', antisense 5'-GAT CCG AGG GCC TCA CTA AAC-3'. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the PCR was performed with a total volume of 20 μl in each well, containing 10 μl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA corresponding to 25 ng of RNA,

and 5 pmol sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the TCTP gene normalized to the 18s gene. The PCR conditions were as follows: for TCTP, 38 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min; and for 18s, 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. An initial heating at 95°C for 9 min and a final extension at 72°C for 7 min were performed for all PCRs. After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta CT}$. The results are given as relative expression normalized to the 18s gene and expressed in arbitrary units. Signals from C glomeruli and NG cells were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band on agarose gels.

8. Western blot analysis

Podocytes harvested from plates and sieved glomeruli were lysed in

sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol). Lysate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant was stored at -70°C until all mice were sacrificed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 50 μg proteins were treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in 8% or 12% acrylamide denaturing SDS polyacrylamide gel. Proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A (1 × PBS, 0.1% Tween-20, and 8% non-fat milk) for 1 hr at room temperature, followed by an overnight incubation at 4°C in a 1:1000 dilution of monoclonal antibody to TCTP (MBL International, Inc., Woburn, MA, USA), polyclonal antibodies to phospho-4EBP1 (Thr37/46), 4EBP1, phospho-p70S6K (Thr389), p70S6K (Cell Signaling, Inc., Beverly, MA, USA), p27, p21, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was then washed once for 15 min and twice for 5 min in 1 × PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-mouse IgG (Amersham Life Science,

Inc.). The washes were repeated, and the membrane was developed with chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

9. Immunohistochemistry

Slices of kidney for Immunohistochemical staining were fixed in 10% neutral buffered formalin, processed in the standard manner, and 4 μm sections of paraffin embedded tissues were utilized. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 min using a Black and Decker vegetable steamer. For TCTP staining, the primary monoclonal anti-TCTP antibody (MBL International, Inc.) was diluted in 1:200 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added for 20 min, and the slides were then washed and incubated with a tertiary rabbit-PAP complex for 20 min. DAB was added for 2 min and the slides were counterstained with hematolxylin. A semi-quantitative score for measuring the intensity of TCTP staining within glomeruli was determined by

examining thirty glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA) as previously described[30]. Briefly, the degree of staining was semi-quantitated on a scale of 0~4+ and a staining score was obtained by multiplying the intensity of staining by the percentage of glomeruli staining for that intensity; these numbers were then added for each experimental animal to give the staining score.

$\sum$ (intensity of staining) × (% of glomeruli with that intensity) = staining score

10. Immunofluorescence

Slices of kidney for immunofluorescence staining were snap-frozen in optimal cutting temperature (OCT) solution and 4 μm sections of tissues were utilized. Slides were fixed in acetone for 10 min at 4°C, air dried for 10 min at room temperature, and blocked with 10% donkey serum for 20 min at room temperature. For TCTP staining, the monoclonal antibody to TCTP (MBL International, Inc.) was diluted in 1:200 with antibody diluent (DAKO, Glostrup, Denmark) and was applied for 3 hr at room temperature. After washing, Cy3 (red)-conjugated anti-mouse IgG antibody (Research Diagnostics, Inc., Flanders, NJ, USA) was added for 60 min. For

synaptopodin double staining, a 1:200 dilution of polyclonal anti-synaptopodin antibody (Santa Cruz Biotechnology, Inc.) was applied, followed by Cy2 (green)-conjugated anti-goat IgG antibody. A semi-quantitative score for measuring intensity of TCTP was determined as aforementioned.

11. Assessment of hypertrophy

Hypertrophy of cultured podocytes was assessed by measurement of cellular protein/cell counts and by flow cytometry. After seeding podocytes on 100 mm dishes and serum restriction for 24 hr, medium was changed as above, and after 48 hr the cells were harvested with 0.05% trypsin and 0.25 mM/l ethylenediaminetetraacetic acid (EDTA), pelleted at 1,500 g for 5 min, and resuspended in PBS. Aliquots of podocytes were used for cell counts using a hemocytometer, and remaining cells were lysed in 0.5 M NaOH and total protein content was measured by using a modified Lowry method. To determine the cell size directly, cells were harvested by trypsinization after 48 hr of treatment as above, fixed with 75% methanol, washed, and incubated with 100 μg/ml RNase and 10 μg/ml propidium iodide in PBS for 1 hr at 37°C. Samples were analyzed by forward light scattering on a FACScan flow cytometer (BD Biosciences). Data were computer analyzed with Cell Quest

Pro software (BD Biosciences).

Glomerular hypertrophy was determined by measuring glomerular volume (V_G) as previously described[31,32]. Briefly, the surface areas of 20 glomeruli cut at the vascular pole on periodic acid-Schiff-stained tissue sections were traced along the outline of the capillary loops using a computer-assisted color image analyzer Image-Pro Ver. 2.0 (Media Cybernetics, Silver Spring, MD, USA). V_G was calculated using the equation: $V_G=\beta/k \times (Area)^{3/2}$, where $\beta=1.38$ is the shape coefficient for spheres, and $k=1.1$ is the size distribution coefficient.

12. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed by the Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Cultured podocytes studies

A. Efficacy of TCTP knockdown by shRNA expressing lentivirus

To test the silencing efficiency of shRNA, COS7 cells (American Type Culture Collection, Manassas, VA, USA) were cultured until reaching a confluency of 70% and co-transfected with pEGFP-C1-TCTP (4 μg) and pCMV-U6-TCTP shRNA-1, -2, -3, or -scrambled (4 μg) using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. Two days after co-transfection, cells were examined microscopically for EGFP expression (Olympus IX51, Olympus, Japan). The knockdown efficacy of TCTP protein by shRNA was further confirmed by Western blot analysis. As seen in Figure 1A, there was a significant reduction of green fluorescent signals in cells transfected with vectors containing pCMV-U6-TCTP shRNA-1 and -3 compared to the scrambled vector. Western blot analysis further confirmed that TCTP-EGFP fusion protein levels were significantly decreased in COS7 cells transfected with these vectors (Fig 1B). After then, cultured podocytes were incubated with lentiviruses produced with these expression cassettes for 7 days. Lentivirus expressing TCTP shRNA-1 (LV-shTCTP1) inhibited the

protein expression of TCTP more efficiently compared to lentivirus expressing TCTP shRNA-3 (LV-shTCTP3) (Fig. 1C). Based on these results, all subsequent *in vitro* and *in vivo* experiments were performed with lentivirus expressing TCTP shRNA-1 (LV-shTCTP1).

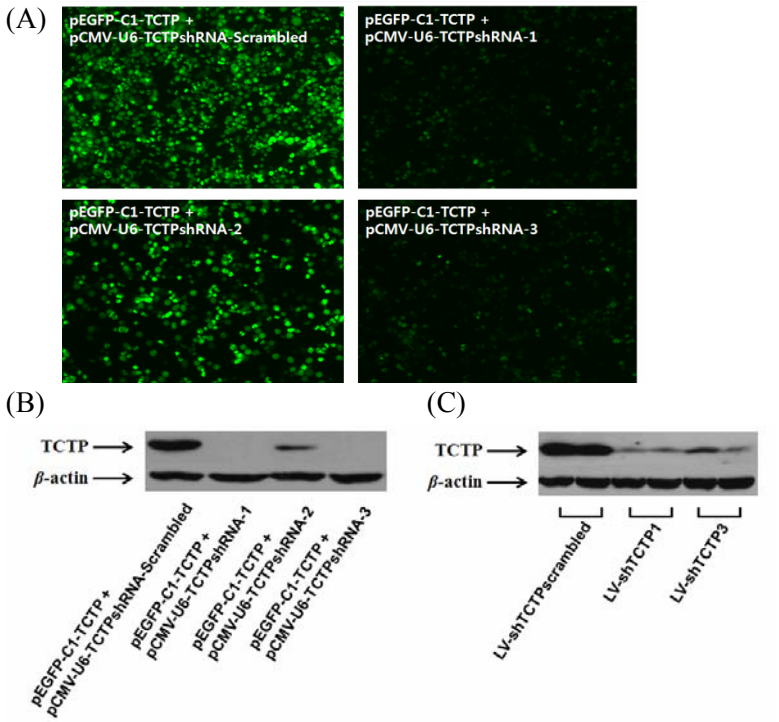

Figure 1. Efficiency of RNA interference for TCTP. (A) Fluorescent microscopic findings of COS7 cells 2 days after co-transfection with pEGFP-C1-TCTP and pCMV-U6-TCTP shRNA-1, -2, -3, or -scrambled. There was a significant reduction of green fluorescent signals in cells transfected with vectors containing pCMV-U6-TCTP shRNA-1 and -3 compared to the scrambled vector. (B) A representative Western blot of TCTP with COS7 cells co-transfected with pEGFP-C1-TCTP and pCMV-U6-TCTP shRNA-1, -2, -3, or -scrambled (representative of five blots). TCTP-EGFP fusion protein levels were significantly decreased in COS7 cells transfected with vectors containing pCMV-U6-TCTP shRNA-1 and -3 compared to the scrambled vector. (C) A representative Western blot of TCTP with podocytes incubated with lentiviruses expressing TCTP shRNA-1 (LV-shTCTP1) and shRNA-3 (LV-shTCTP3) (representative of five blots). LV-shTCTP1 inhibited the protein expression of TCTP more efficiently compared to LV-shTCTP3.

B. TCTP mRNA and protein expression in cultured podocytes

TCTP mRNA expression assessed by real-time PCR was significantly increased in HG- and ANG II-stimulated podocytes. Compared to NG cells, there were 1.5- and 1.8-folds increases in the ratio of TCTP mRNA/18s rRNA in cultured podocytes exposed to HG and ANG II, respectively ($p<0.05$) (Fig. 2A). TCTP protein expression, assessed by Western blot, showed a similar pattern to its mRNA expression. HG and ANG II significantly increased TCTP protein expression in cultured podocytes and L-158,809 significantly abrogated HG-induced TCTP protein expression (Fig. 2B). On the other hand, mannitol had no effect on TCTP mRNA and protein expression (data not shown).

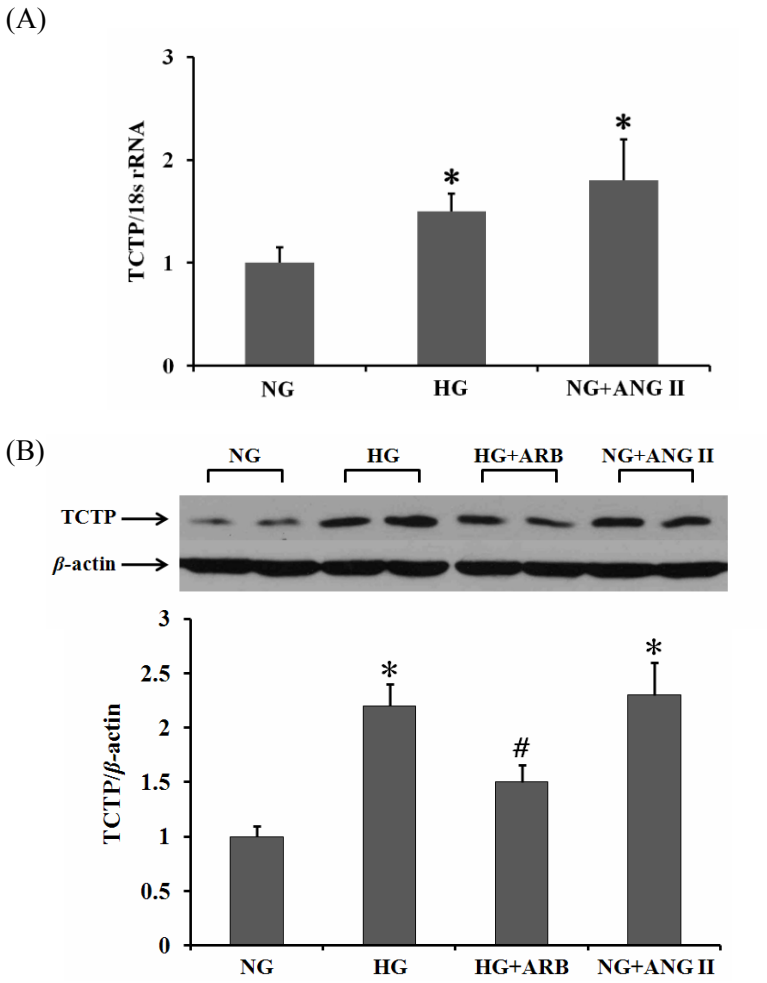

Figure 2. TCTP mRNA and protein expression in cultured podocytes. (A) The ratios of TCTP mRNA/18s rRNA were significantly increased in cultured podocytes exposed to HG and ANG II by 1.5- and 1.8-folds compared to NG cells. (B) A representative Western blot of TCTP protein in cultured podocytes exposed to NG, HG, HG+L-158,809 (ARB), and NG+ANG II (representative of four blots). HG and ANG II significantly increased TCTP protein expression in cultured podocytes and L-158,809 significantly abrogated HG-induced TCTP protein expression. *; $p<0.05$ *vs*. NG group, #; $p<0.05$ *vs*. HG group.

C. Effect of TCTP inhibition on HG-induced 4EBP1 and p70S6K activation

HG significantly increased phospho-4EBP1 and phospho-p70S6K protein expression in cultured podocytes ($p<0.01$), and these increases in 4EBP1 and p70S6K activation were significantly ameliorated by LV-shTCTP1 by 54.8% ($p<0.05$) and 79.0% ($p<0.01$), respectively. In contrast, there were no differences in 4EBP1 and p70S6K protein expression among the groups. On the other hand, LV-shTCTPscrambled had no effect on the expression of TCTP, phospho-4EBP1, 4EBP1, phospho-p70S6K, and p70S6K protein expression in cultured podocytes (Fig. 3).

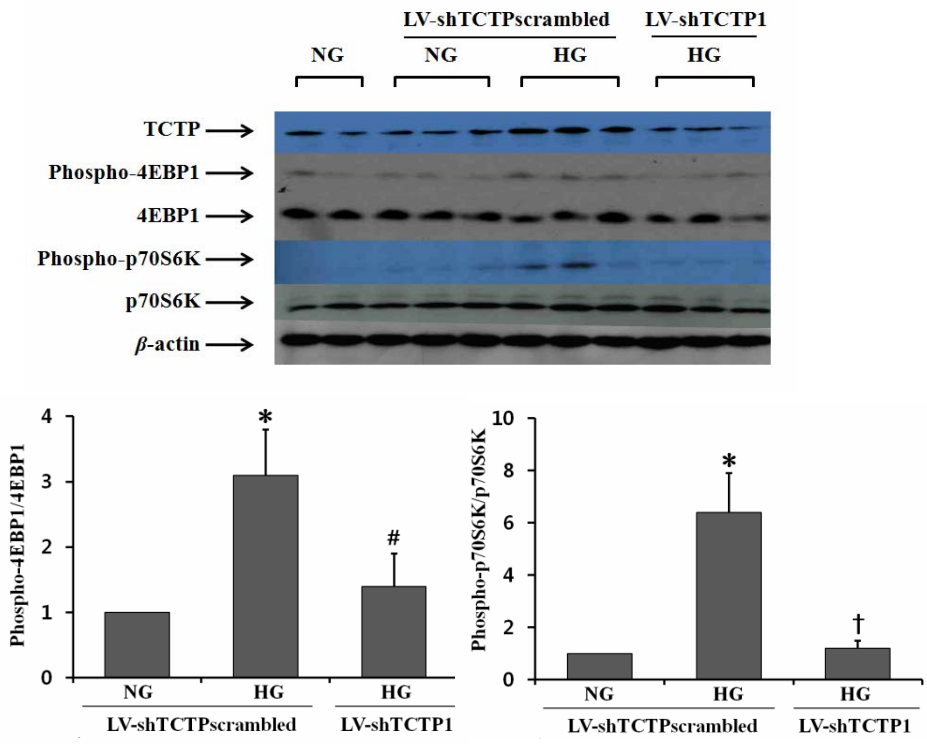

Figure 3. . A representative Western blot of TCTP, phospho-4EBP1, 4EBP1, phospho-p70S6K, and p70S6K in cultured podocytes (representative of four blots). phospho-4EBP1 and phospho-p70S6K protein expression were significantly increased in HG-stimulated podocytes, and these increases in 4EBP1 and p70S6K activation were significantly ameliorated by LV-shTCTP1. In contrast, there were no differences in 4EBP1 and p70S6K protein expression among the groups. On the other hand, LV-shTCTPscrambled had no effect on the expression of TCTP, phospho-4EBP1, 4EBP1, phospho-p70S6K, and p70S6K protein expression in cultured podocytes. *; $p<0.01$ *vs*. NG with LV-shTCTPscrambled group, #; $p<0.05$ *vs*. HG with LV-shTCTPscrambled group, †; $p<0.01$ *vs*. HG with LV-shTCTPscrambled group.

D. Effect of TCTP inhibition on HG-induced p27 and p21 protein expression

The protein expression of p27 was significantly increased by 1.8-fold in HG-stimulated podocytes transfected with LV-shTCTPscrambled compared to corresponding NG cells ($p<0.05$), and this increase in p27 protein expression in cultured podocytes exposed to HG was significantly attenuated by LV-shTCTP1 by 66.7% ($p<0.05$). The protein expression of p21 showed a similar pattern to p27 protein expression, but did not reach statistical significance (Fig. 4).

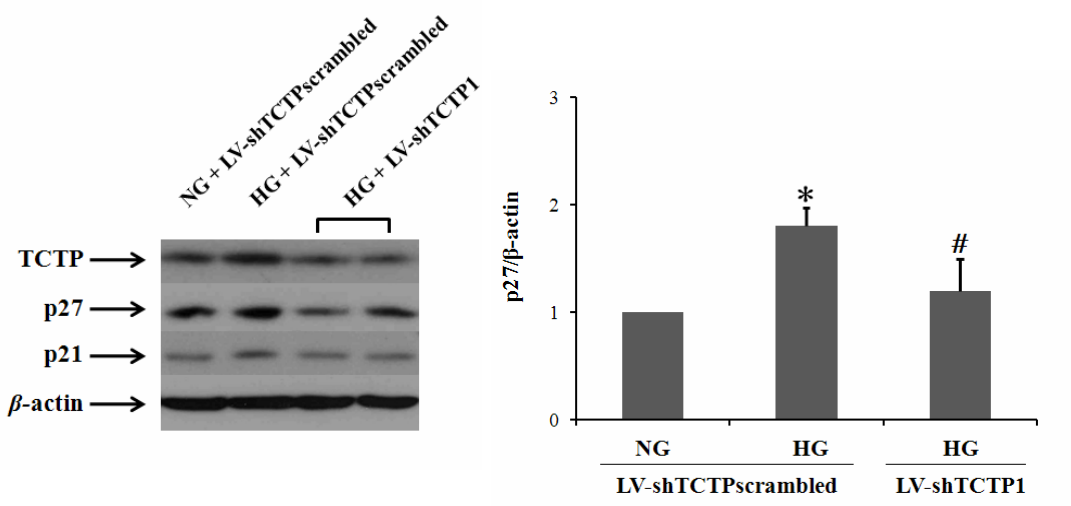

Figure 4. A representative Western blot of TCTP, p27, and p21 in cultured podocytes transfected with LV-shTCTPscrambled and LV-shTCTP1. (representative of four blots). The protein expression of p27 was significantly increased by 1.8-fold in HG-stimulated podocytes transfected with LV-shTCTPscrambled compared to corresponding NG cells, and this increase in p27 protein expression in cultured podocytes exposed to HG was significantly attenuated by LV-shTCTP1 by 66.7%. The protein expression of p21 showed a similar pattern to p27 protein expression, but did not reach statistical significance. *; $p<0.05$ *vs*. NG with LV-shTCTPscrambled group, #; $p<0.05$ *vs*. HG with LV-shTCTPscrambled group.

E. Effect of TCTP on podocyte hypertrophy

Cellular protein content was significantly higher in HG-stimulated podocytes transfected with LV-shTCTPscrambled compared to corresponding NG cell with no significant change in cell numbers, suggesting cellular hypertrophy. A 1.7-fold increase in the ratio of protein/cell numbers was observed in podocytes exposed to HG with LV-shTCTPscrambled transfection, and the increment in the ratio of protein/cell numbers by HG was significantly inhibited by LV-shTCTP1 by 75.0% ($p<0.05$) (Fig. 5A). In contrast, LV-shTCTPscrambled had no effect on the ratio of protein/cell numbers in cultured podocytes exposed to NG.

Cellular hypertrophy was also confirmed by FACScan flow cytometer (Fig. 5B). The relative cell size, determined by mean forward light scattering, was 25% larger in HG-stimulated podocytes transfected with LV-shTCTPscrambled compared to corresponding NG cells, and LV-shTCTP1 significantly abrogated the increase in cell size of HG podocytes by 85% ($p<0.05$).

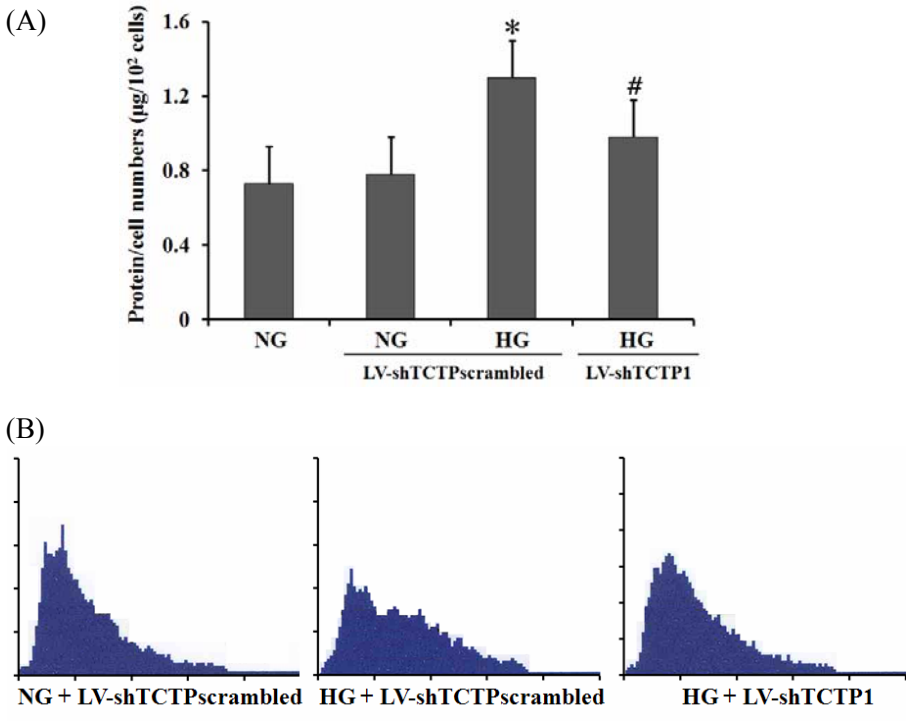

Figure 5. Assessments of podocyte hypertrophy. (A) Protein/cell numbers of podocytes. A 1.7-fold increase in the ratio of protein/cell numbers was observed in podocytes exposed to HG with LV-shTCTPscrambled transfection, and the increment in the ratio of protein/cell numbers by HG was significantly inhibited by LV-shTCTP1. In contrast, LV-shTCTPscrambled had no effect on the ratio of protein/cell numbers in cultured podocytes exposed to NG. *; $p<0.05$ *vs*. NG and NG with LV-shTCTPscrambled groups, #; $p<0.05$ *vs*. HG with LV-shTCTPscrambled group. (B) A representative FACScan of podocytes exposed to NG+LV-shTCTPscrambled, HG+LV-shTCTPscrambled, and HG+LV-shTCTP1. The relative cell size, determined by mean forward light scattering, was 25% larger in HG-stimulated podocytes transfected with LV-shTCTPscrambled compared to corresponding NG cells, and LV-shTCTP1 significantly abrogated the increase in cell size of HG podocytes by 85.0%.

2. Animal studies

A. Animal data

All animals gained weight over the 6-week experimental period, but weight gain was highest in C mice ($p<0.01$). The ratio of kidney weight to body weight in DM mice (1.63±0.04%) was significantly higher than those in C (1.13±0.03%) ($p<0.01$) and in DM+LV-shTCTP1 mice (1.27±0.04%) ($p<0.05$). The mean blood glucose levels of C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 mice were 123.2±6.3 mg/dl, 121.1±6.8 mg/dl, 462.7±41.5 mg/dl, and 475.1±36.2 mg/dl, respectively ($p<0.01$). Compared to the C group (4.07±0.96 μg/day), 24-hr urinary albumin excretion was significantly higher in DM mice (14.25±3.19 μg/day) ($p<0.01$), and LV-shTCTP1 treatment partly reversed the increase in albuminuria in DM mice (8.16±1.80 μg/day) ($p<0.05$). The serum levels of BUN and creatinine were comparable among the four groups.

B. Glomerular TCTP protein expression

Glomerular TCTP protein expression assessed by Western blot was significantly higher in DM compared to C mice ($p<0.005$), and LV-shTCTP1 treatment significantly ameliorated the increase in TCTP protein expression in diabetic glomeruli ($p<0.01$). Densitometric quantitation revealed 7.8-fold increase in TCTP protein expression in DM versus C mice, and LV-shTCTP1 treatment blocked the increase by 53.8% (Fig. 6).

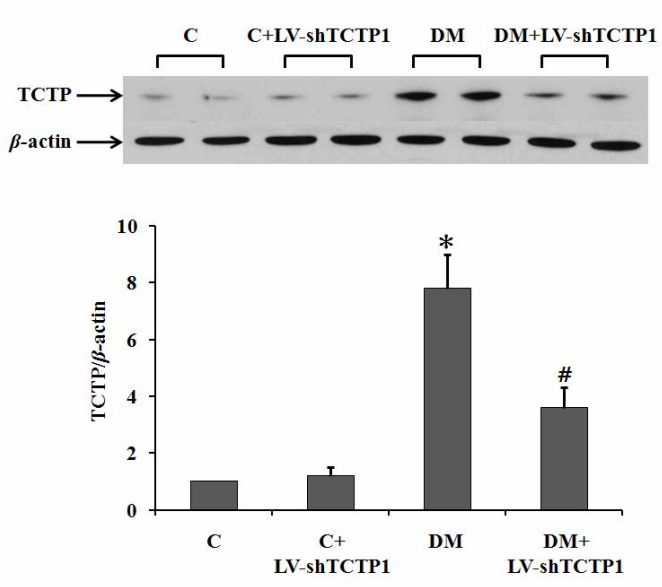

Figure 6. A representative Western blot of TCTP in C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 glomeruli (representative of four blots). Glomerular TCTP protein expression was significantly higher in DM compared to C mice, and LV-shTCTP1 treatment significantly ameliorated the increase in TCTP protein expression in diabetic glomeruli. Densitometric quantitation revealed 7.8-fold increase in TCTP protein expression in DM versus C mice, and LV-shTCTP1 treatment blocked the increase by 53.8%. *; $p<0.005$ *vs*. C and C+LV-shTCTP1 groups, #; $p<0.01$ *vs*. DM group.

C. Immunohistochemical staining for glomerular TCTP

Immunohistochemical staining for glomerular TCTP confirmed the Western blot findings. The staining for TCTP within glomeruli was significantly stronger in DM compared to C mice, and LV-shTCTP1 treatment significantly attenuated the increase in TCTP protein expression in DM glomeruli ($p<0.05$). The mean semi-quantitative staining scores for glomerular TCTP were significantly higher in DM (32.1±5.6) compared to C (10.0±2.4) and DM+LV-shTCTP1 mice (16.6±3.4) ($p<0.05$) (Fig. 7).

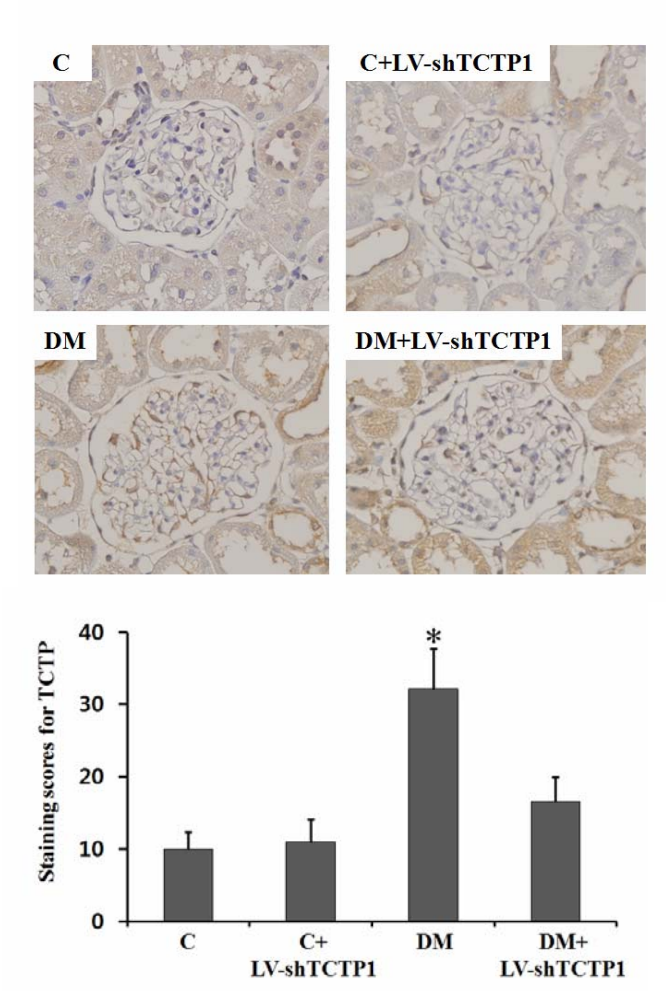

Figure 7. Immunohistochemical staining for TCTP in C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 mice. Glomerular TCTP staining was significantly stronger in DM compared to C mice, and LV-shTCTP1 treatment significantly attenuated the increase in TCTP protein expression in DM glomeruli. IHC score for TCTP within the glomeruli was significantly higher in DM mice compared to C mice, and LV-shTCTP1 treatment significantly inhibited this increase in DM mice. *; $p<0.05$ *vs.* Other groups.

D. Localization of TCTP protein expression

Double immunofluorescence staining for TCTP and synaptopodin revealed that podocytes were the main cells responsible for the increase in TCTP protein under diabetic conditions (Fig. 8).

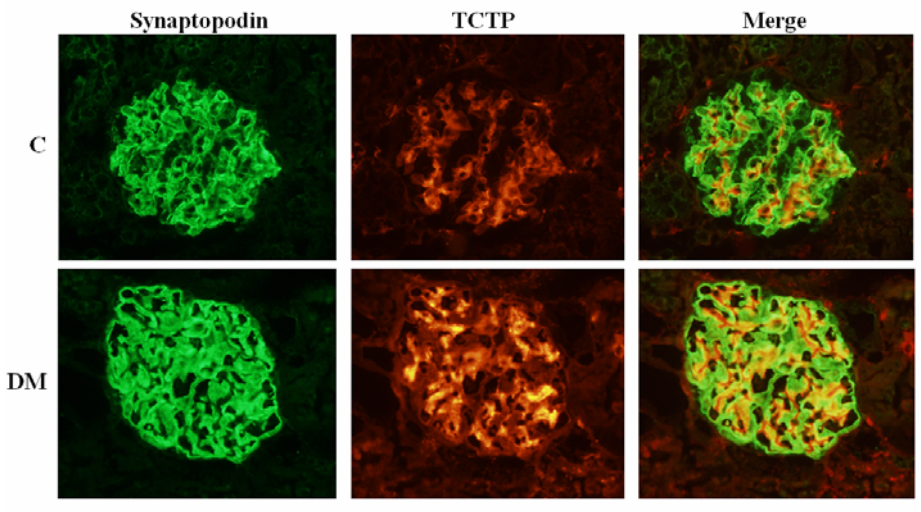

Figure 8. Double immunofluorescence staining for TCTP (red) and synaptopodin (green) in C and DM mice. Compared to C, immunofluorescence staining for TCTP was increased in DM glomeruli, and double immunofluorescence staining revealed that the increase in TCTP protein expression was mainly attributed to its increase in podocytes (× 400).

E. Effect of TCTP inhibition on 4EBP1 and p70S6K activation

Figure 9 shows a representative Western blot of equal amounts of protein from the lysates of sieved C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 glomeruli at 6-week after streptozotocin injection. Phospho-4EBP1 and phospho-p70S6K protein expression were significantly increased in DM glomeruli ($p<0.01$), and the administration of LV-shTCTP1 significantly abrogated these increases in 4EBP1 and p70S6K activation in DM glomeruli by 45.8% and 43.1%, respectively ($p<0.05$). In contrast, there were no differences in 4EBP1 and p70S6K protein expression among the four groups (Fig. 9).

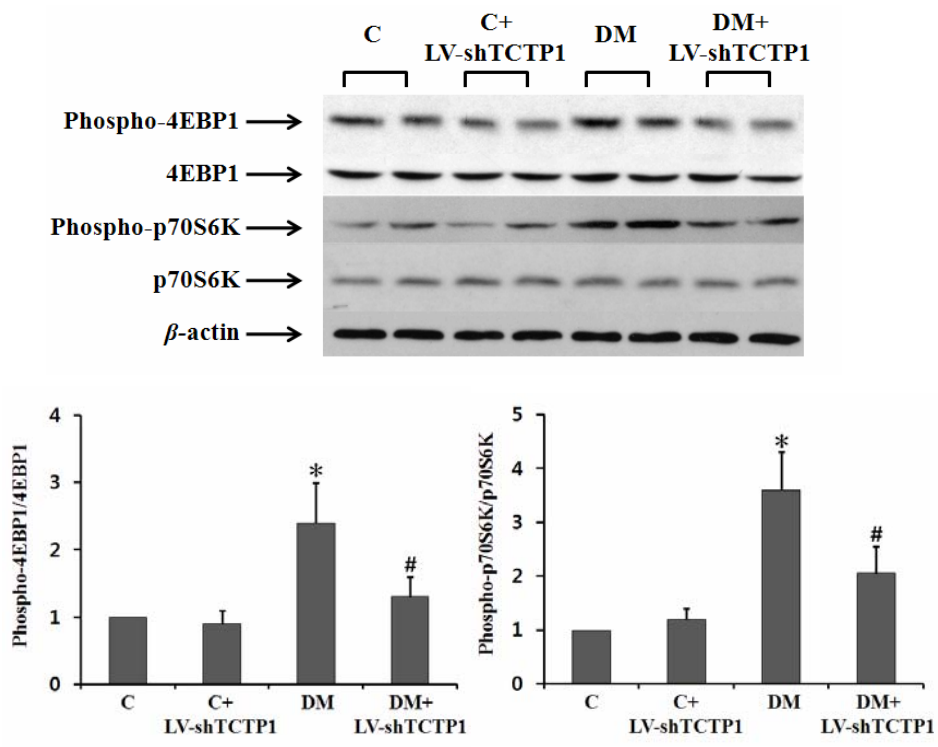

Figure 9. A representative Western blot of glomerular phospho-4EBP1, 4EBP1, phospho-p70S6K, and p70S6K in C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 mice (representative of four blots). Phospho-4EBP1 and phospho-p70S6K protein expression were significantly increased in DM glomeruli, and the administration of LV-shTCTP1 significantly abrogated these increases in 4EBP1 and p70S6K activation in DM glomeruli by 45.8% and 43.1% respectively. In contrast, there were no differences in 4EBP1 and p70S6K protein expression among the four groups. *; $p<0.01$ *vs.* C and C+LV-shTCTP1 groups, #; $p<0.05$ *vs.* DM group.

F. Effect of TCTP inhibition on glomerular p27 and p21 protein expression

Glomerular p27 and p21 protein expression assessed by Western blot were significantly higher in DM compared to C mice, and the administration of LV-shTCTP1 significantly ameliorated these increases in p27 and p21 protein expression. Densitometric quantitation revealed 4.3- and 2.3-folds increases in glomerular p27 and p21 protein expression in DM versus C mice, and LV-shTCTP1 treatment blocked these increases by 41.9% and 38.1%, respectively ($p<0.05$).

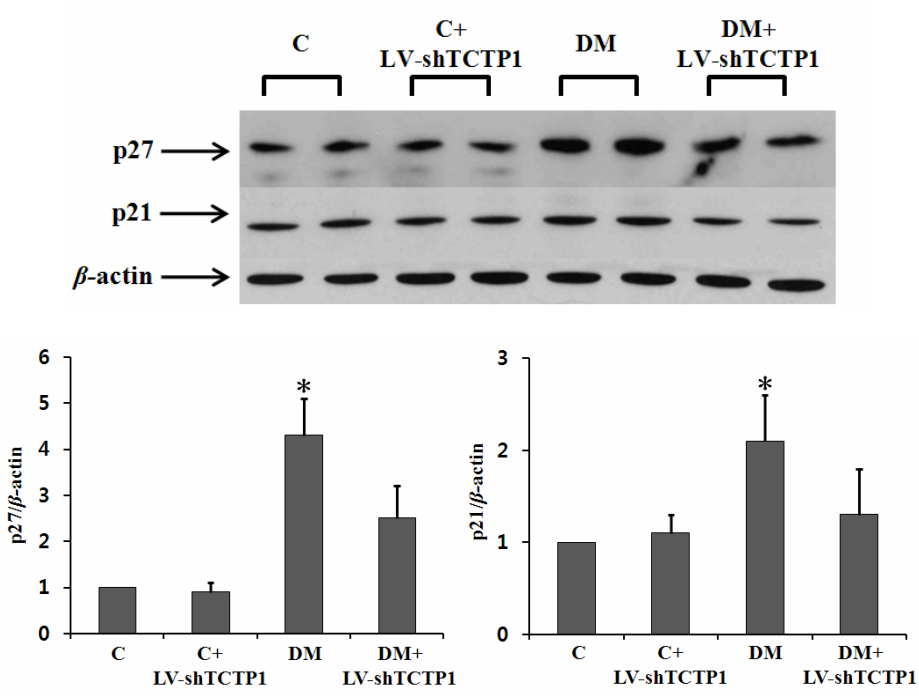

Figure 10. A representative Western blot of glomerular p27 and p21 in C, C+LV-shTCTP1, DM, and DM+LV-shTCTP1 mice (representative of four blots). Glomerular p27 and p21 protein expression were significantly higher in DM compared to C mice, and the administration of LV-shTCTP1 significantly ameliorated these increases in p27 and p21 protein expression. Densitometric quantitation revealed 4.3- and 2.3-folds increases in glomerular p27 and p21 protein expression in DM versus C mice, and LV-shTCTP1 treatment blocked these increases by 41.9% and 38.1%, respectively. *; $p<0.05$ *vs.* Other groups.

G. Effect of TCTP inhibition on glomerular hypertrophy

The mean glomerular volume in DM ($1.94\pm0.11 \times 10^5$ μm^3) was 31.1% larger than in C mice ($1.34\pm0.13 \times 10^5$ μm^3) at 6-week after DM induction, and LV-shTCTP1 administration significantly attenuated glomerular hypertrophy in DM mice ($1.65\pm0.16 \times 10^5$ μm^3) ($p<0.05$).

IV. DISCUSSION

Previous studies have demonstrated TCTP plays an important role in cellular or organ hypertrophy[22,33,34], but its functional significance in podocyte hypertrophy under diabetic conditions has never been explored. This study demonstrates for the first time that TCTP expression is increased in high glucose-stimulated podocytes and in experimental diabetic glomeruli. In addition, the results of the present study showing that inhibition of TCTP abrogates podocyte and glomerular hypertrophy via inhibiting phosphorylation of 4EBP1 and p70S6K, two important mTOR target molecules[35], and CKIs expression suggest that TCTP is involved in the process of podocyte hypertrophy in diabetic nephropathy.

Even though TCTP was originally identified as a growth-related protein in a mouse ascitic tumor and in mouse erythroleukemia[36,37], it was demonstrated as a protein triggering histamine in basophils and has been called 'IgE-dependent histamine release factor'[18]. However, accumulating evidences have shown that TCTP is widely expressed in various organisms and that its sequence is highly conserved among species, suggesting that TCTP may play a role in many essential cellular functions[38,39]. TCTP is known to be regulated at both the transcriptional and translational levels in response to a wide range

of extracellular stimuli and cellular conditions[19,38,40]. Growth signals, numerous cytokines, and various stress conditions including oxidative stress, heat shock, and endoplasmic reticulum stress have been found to induce TCTP expression[41-46]. In addition, the levels of TCTP expression are reported to be high in actively proliferating cells such as malignant cells, and to be reduced when tumor cells undergo reversion[47]. In this study, TCTP mRNA and protein expression are increased in podocytes exposed to high glucose medium and in diabetic glomeruli. Moreover, TCTP expression in cultured podocytes is also increased by ANG II, an important mediator in the pathogenesis of diabetic nephropathy through inducing extracellular matrix synthesis, oxidative stress, and hypertrophy in glomerular cells, and high glucose-induced TCTP expression is ameliorated by ARB treatment. Since local renin-angiotensin system is known to be activated within podocytes under diabetic conditions[48], I surmise that numerous cytokines, oxidative stress, and increased ANG II production under diabetic conditions may contribute to the increase in TCTP expression in podocytes though mitotically inactive.

Besides its original histamine releasing function, TCTP has been implicated in various intracellular functions. TCTP was reported to have properties of a tubulin-binding protein that was associated with microtubules

during G_1, S, G_2 and early M phase of the cell cycle, bound to the mitotic spindle at metaphase, and was detached from the spindle during metaphase-anaphase transition[49]. In addition, overexpression of TCTP resulted in cell growth retardation and increased cell size and in alterations of cell morphology[49]. Based on the fact that TCTP was directly phosphorylated in mitosis *in vivo* and by the mitotic kinase Plk *in vitro* and that overexpression of a Plk phosphorylation site-deficient mutant of TCTP disrupted the completion of mitosis, TCTP was also considered a key mitotic target of Plk for regulating anaphase progression[50]. In a recent study, checkpoint protein with FHA and RING finger domain (Chfr), which plays an important function in cell cycle progression, was demonstrated to interact and colocalize with TCTP to the mitotic spindle[51]. Moreover, Hsu *et al*[22] revealed that reducing TCTP levels by RNA interference results in decreases in cell size, cell number, and organ size, suggesting that TCTP was essential for growth and proliferation in *Drosophila*. Taken together, TCTP is believed to be important for cell growth and division. Since diabetic nephropathy is characterized by renal hypertrophy, there is a possibility that TCTP may play role in the pathogenesis of glomerular and tubular hypertrophy in diabetic nephropathy. Up to date, however, the impact of TCTP on glomerular cells hypertrophy under diabetic conditions has never been explored. The present study shows

for the first time that TCTP expression is increased in podocytes under diabetic conditions and inhibition of TCTP by shRNA attenuates glomerular hypertrophy in experimental diabetic mice and cellular hypertrophy in high glucose-stimulated podocytes, suggesting that TCTP is involved in the hypertrophic process in podocytes under diabetic conditions.

Recent studies have proven that TCTP interacts with Rheb, a small GTPase, which is a critical regulator of mTOR[52-54]. Rheb binds to FK506 binding protein 38 (FKBP38), an endogenous inhibitor of mTOR, in a GTP-dependent manner and as a result, prevents the interaction of FKBP38 with mTOR and leads to an activation of mTOR[52]. In addition, TCTP overexpression induces activation of PI3 kinase, which can in turn activate mTOR[23]. mTOR is a serine/threonine protein kinase and exists in two distinct multiprotein complex, mTORC1 and mTORC2[55]. mTORC1 is rapamycin-sensitive and is consist of mTOR, raptor, and mLST8, and regulates transcription, ribosome protein synthesis, and mRNA translation through phosphorylation of its major substrates, 4EBP1 and p70S6K[56]. In terms of the functional role of mTOR in diabetic nephropathy, recent several investigations have revealed that mTOR is involved in the pathogenesis of glomerular and tubular hypertrophy. Renal hypertrophy with increased phosphorylation of 4EBP1 and/or p70S6K in the kidneys was abrogated by rapamycin, a specific

and potent inhibitor of mTOR, in animal model of type I and type II diabetes[14-17,57]. In addition, p70S6K knockout ameliorated renal hypertrophy in diabetic mice[58], while overexpression of a constitutive active form of p70S6K resulted in increased cell size of cultured mouse proximal tubule cells[59]. These findings suggest that activation of mTOR signaling pathway causes renal hypertrophy under diabetic conditions by stimulating the initiation and elongation phase of mRNA translation through phosphorylation of p70S6K and 4EBP1. In agreement with the results of previous studies, I notice that phosphorylation of 4EBP1 and p70S6K are significantly increased in high glucose stimulated podocytes and in diabetic glomeruli, and TCTP inhibition ameliorates podocyte and glomerular hypertrophy as well as phosphorylation of these mTOR target molecules, suggesting that increased TCTP expression may contribute to podocyte hypertrophy under diabetic conditions by regulating the mRNA translation pathways.

Cell-cycle entry and progression through the cell cycle depend on the expression and activation of CDKs and their regulatory subunits, cyclins, and the kinase activity of cyclin/CDK complexes is negatively regulated by CKIs[60]. In cultured rat mesangial cells, high glucose, in the absence of other exogenous growth factors, was found to induce $p27^{Kip1}$ protein expression[61]. This high glucose-stimulated expression of $p27^{Kip1}$ involved the activation of

protein kinase C and depended partly on the induction of transforming growth factor-β[61]. In addition, $p27^{Kip1}$ antisense oligonucleotides treatment prevented glucose-induced mesangial cell hypertrophy, and resulted in a proliferative phenotype[61]. Glomerular $p27^{Kip1}$ protein expression was increased in diabetic db/db mice, a model of type 2 diabetes, and in murine streptozotocin type I diabetes[62]. $p21^{Cip1}$ protein was also enhanced in 3- and 9-day streptozotocin-induced diabetic glomeruli assessed by immunohistochemical staining[63]. In contrast to mesangial cells, mature podocytes do not actively synthesize DNA nor proliferate under normal conditions due to high levels of CKIs expression[64]. Under diabetic conditions, however, podocytes also undergo hypertrophic processes like mesangial cells, resulting in increased cell size[5,7]. Petermann *et al*[7] demonstrated that mechanical stretch, an in vitro condition of increased intraglomerular capillary pressure, reduced cell cycle progression and induced hypertrophy in cultured podocytes. Another previous study by Xu *et al*[5] showed that high glucose *per se* also induced podocyte hypertrophy, which was associated with increased $p27^{Kip1}$ expression. Taken together, CKIs is confirmed to play an important role in hypertrophy of glomerular cells under diabetic conditions.

Recently, several studies have suggested that an interaction between PI3 kinase/Akt/mTOR pathway and cyclin/CDKs/CKIs expression. High glucose

induced PI3 kinase/Akt activation, $p21^{Cip1}$ mRNA and protein expression, and cellular hypertrophy in LLC-PK1 cells, and these changes were abrogated by LY294002, a PI3 kinase inhibitor, indicating that PI3 kinase/Akt pathway were involved in $p21^{Cip1}$ expression[65]. In addition, renal expression of $p21^{Cip1}$ and $p27^{Kip1}$ in streptozotocin-induced diabetic mice were ameliorated by rapamycin[59]. Similarly, in this study, I found that the increases in $p21^{Cip1}$ and $p27^{Kip1}$ expression in high glucose-stimulated podocytes and in experimental diabetic glomeruli were attenuated by TCTP inhibition along with reduced phosphorylation of mTOR target molecules. These findings suggest that mTOR pathway and its possible upstream activator TCTP may provoke cellular hypertrophy via inducing CKIs expression under diabetic conditions. In contrast, Hong *et al*[66] have shown that activation of serum- and glucocorticoid-inducible kinase 1 (SGK1) by mTOR may promote G_1 progression through phosphorylation and cytoplasmic mislocalization of $p27^{Kip1}$. Moreover, several investigators have reported that mTOR inhibitor blocks cell cycle progression and cell proliferation through disrupting cyclin D1-CDK4 complexes, and upregulates $p27^{Kip1}$ at both mRNA and protein levels and prolongs its half-life and facilitates the formation of complexes between $p27^{Kip1}$ and CDK/cyclins[67,68]. PI3 kinase inhibitor was also shown to increase $p21^{Cip1}$ expression in phorbol 12-myristate 13-acetate-induced human

leukemia cells[69]. I assume that these absolutely opposite effects of PI3 kinase/Akt/mTOR on cell cycle progression may be largely due to the differences in cells used for experiments (normal cells versus malignant cells) and diverse experiment conditions.

Transfection using lentivirus is an exciting gene delivery tool because of its ability to efficiently transduce both dividing and non-dividing target cell populations[25]. In addition, the transfection efficiencies of synthetic siRNA oligonucleotide and plasmid-based delivery system have been known to be very low in mature podocytes. Moreover, TCTP has a relatively long half-life[70], and to some extent, its expression is controlled at the level of translation from the pre-existing mRNA[19,38]. Based on these findings, I considered transfection with lentivirus containing TCTP shRNA as an optimal method to establish long-lasting knockdown of TCTP gene and used this technique in this study. Even though the introduction of lentivirus containing TCTP shRNA in the present in vivo study does not inhibit TCTP specifically in podocytes, I presume that the abrogation of glomerular hypertrophy in diabetic mice by TCTP shRNA may be partially attributed to reduced podocyte hypertrophy.

In conclusion, I demonstrate that TCTP expression is increased in podocytes under diabetic conditions. In addition, inhibition of TCTP ameliorates the activation of mTOR target molecules, 4EBP1 and p70S6K,

and CKIs expression along with reduced podocytes hypertrophy. These findings suggest that TCTP may play an important role in the process of podocyte hypertrophy under diabetic conditions via stimulating mRNA translation and inducing cell cycle arrest at the G_1/S interphase.

V. CONCLUSION

In this study, I investigated whether TCTP expression was increased in high glucose-stimulated podocytes and in experimental diabetic glomeruli. In addition, to elucidate the functional role of TCTP in podocyte hypertrophy, I examined the changes in the expression of effectors of mRNA translation, CKIs, and podocyte hypertrophy under diabetic condition after TCTP inhibition.

1. Compared to NG cells, TCTP mRNA and protein expression were significantly increased in podocytes exposed to HG, and this increase in TCTP expression was abrogated by ARB treatment.
2. ANG II induced TCTP mRNA and protein expression in cultured podocytes.
3. The protein expression of phospho-4EBP1, phospho-p70S6K, and p27 were significantly increased in HG-stimulated podocytes compared to NG cells, and these increases were significantly ameliorated by LV-shTCTP.
4. TCTP inhibition significantly attenuated cell size in cultured podocytes cultured under HG medium.
5. Glomerular TCTP expression was significantly higher in DM compared with C mice.

6. Double immunofluorescence staining for TCTP and synaptopodin revealed that podocytes were the main cells responsible for the increase in TCTP protein expression in diabetic glomeruli.
7. Administration of LV-shTCTP significantly abrogated the increase in phospho-4EBP1, phospho-p70S6K, p27, and p21 protein expression in diabetic glomeruli along with reduced glomerular size.

In conclusion, TCTP expression is increased in podocytes under diabetic conditions and inhibition of TCTP ameliorates the activation of mTOR target molecules, 4EBP1 and p70S6K, and CKIs expression, along with reduced podocyte and glomerular size. These findings suggest that TCTP may play an important role in the process of podocyte hypertrophy under diabetic conditions via regulating mRNA translation and inducing cell cycle arrest at the G_1/S interphase.

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ABSTRACT (in Korean)

당뇨 조건하에서 translationally controlled tumor protein이 족세포의 비후에 미치는 영향

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배경: 세포 비후 과정은 세포 주기 조절 단백 중 주로 cyclin-dependent kinase 억제제 (CKIs)의 발현 증가와 밀접하게 연관되어 있다. 최근의 연구에 의하면, CKIs 이외에도 mammalian target of rapamycin (mTOR)와 같은 mRNA 전사를 조절하는 세포 신호 전달계의 활성화도 세포 비후를 유발시키는 것으로 보고되고 있다. Translationally controlled tumor protein (TCTP)은 대부분의 진핵세포에 잘 보존된 상태 (conserve)로 존재하며, mRNA 전사 조절 과정을 통하여 세포 증식 및 비후 등 다양한 세포 내 현상과 관련이 있을 것으로 생각되어 왔다. 당뇨병성 신병증의 병리학적 특징 중 하나가 사구체 비후이기 때문에, TCTP와 당뇨병성 신병증의 발생 사이에 연관이 있을 가능성이 있으나, 이에 대한 연구는 전무한 실정이다. 이에 본 연구자는 고포도당으로 자극한 족세포와 실험적 당뇨 사구체에서 TCTP의 발현 변화를 관찰하였으며, 당뇨 조건 하에서 TCTP가 족세포의 비후에 미치는 영향을 규명하고자 하였다.

방법: 생체 내 실험으로는 불멸 생쥐 족세포를 정상 포도당군 (5.6 mM), 정상 포도당+만니톨군 (24.4 mM), 정상 포도당+안지오텐신 II 군 (10^{-6} M), 고포도당군 (30 mM), 그리고 고포도당+L-158,809 처치군 (10^{-7} M)으로 나누어 배양하였으며, TCTP 발현 억제를 위하여 TCTP shRNA를 포함한 lentivirus로 전처치한 실험도 시행하였다. 생체 내 실험으로는 32마리의

C57BL/6 생쥐를 사용하였으며, 16마리는 대조군, 그리고 16마리는 streptozotocin으로 당뇨를 유발시킨 당뇨군으로 나누었고, 각 군에서 8마리씩은 TCTP shRNA를 포함한 lentivirus로 처치하였다. 배양 족세포 및 분리한 사구체 내 TCTP mRNA의 발현은 real-time PCR으로, TCTP, phospho-4EBP1, 4EBP1, phospho-p70S6K, p70S6K, p27, p21, 그리고 β-actin의 단백 발현은 Western blot을 이용하여 분석하였다. 사구체 내 TCTP 단백의 발현 위치는 synaptopodin과 TCTP을 이용한 이중 면역형광염색으로 관찰하였다. 족세포 비후는 단백량/세포 수 및 유세포 분석으로, 그리고 사구체 비후는 morphometry를 이용하여 확인하였다.

결과: TCTP mRNA 및 단백의 발현은 정상 포도당군에 비하여 고포도당에서 의미있게 증가되었으며, 고포도당군에서의 TCTP 발현 증가는 L-158,809에 의하여 의의있게 억제되었다. 안지오텐신 II 역시 배양 족세포에서 TCTP의 발현을 유의하게 증가시켰다. 사구체 내 TCTP의 발현도 대조군에 비하여 당뇨군에서 의미있게 증가되었으며, 이중 면역형광염색상 당뇨 사구체 내 TCTP의 발현 증가는 주로 족세포의 발현 증가에 기인함을 확인하였다. 생체 내 및 생체 외 실험상 TCTP shRNA를 포함한 lentivirus로 처치한 경우 당뇨 조건 하에서 phospho-4EBP1, phospho-p70S6K, 그리고 p27의 단백 발현 증가뿐만 아니라 족세포 및 사구체 비후가 유의하게 억제되었다.

결론: 당뇨 조건 하에서 족세포 내 TCTP의 발현이 증가되었으며, TCTP 억제를 통하여 당뇨 조건 하에서 세포비후와 관련된 4EBP1과 p70S6K의 활성화 및 CKIs의 발현 증가와 함께 족세포 비후가 의미있게 억제되었다. 이상의 결과를 종합해 볼 때, TCTP는 mRNA의 전사 조절과 세포 주기의 진행 억제를 통하여 당뇨 조건 하에서의 족세포 비후에 관여할 것으로 생각된다.

핵심 되는 말: translationally controlled tumor protein, 당뇨병성 신병증, 족세포, 세포비후