Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:869-878

Accepted: October 20, 2010

TGF-β1 Induces Podocyte Injury Through Smad3-ERK-NF-κB Pathway and Fyn-dependent TRPC6 phosphorylation

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Key Words
Podocyte • TGF- β1 • TRPC6

Abstract
TGF-β1 plays an important role on podocyte injury and glomerular diseases, while the underlying molecular mechanisms are still elusive. Here, the potential role of the ion channel TRPC6 and the proximal signaling was explored in TGF-β1-treated mouse podocyte. Our results showed that TGF-β1 significantly increased podocyte apoptosis and induced obvious disorganization of actin filaments in a time-dependent pattern. In TGF-β1-treated podocyte, TRPC6 protein, especially the phosphorylated TRPC6, and the cytosolic free Ca^{2+} level upregulated, which was evidently inhibited by the specific knockdown of TRPC6. TRPC6 knockdown also alleviated TGF-β1-induced podocyte apoptosis. Moreover, the Src kinase Fyn increased obviously in TGF-β1-treated podocyte, displaying increment of the active form pY418 and reduction of the inactive form pY530. Immunoprecipitation assay revealed that Fyn interacts with TRPC6 in podocyte. Notably, Fyn knockdown blocked TRPC6 phosphorylation and intracellular Ca^{2+} increment following TGF-β1 stimulation, but not affect the expression of TRPC6 protein. In addition, Western blot showed that TGF-β1 induced significant activation of p-Smad3, p-ERK and RelA/p65. Importantly, obvious translocation of ERK and RelA/p65 to nuclei was observed in TGF-β1-treated podocyte, which was reduced by ERK inhibitor U0126. Both U0126 and NF-κB inhibitor PDTC obviously inhibited the increment of TRPC6 protein and the flux of cytosolic free Ca^{2+} induced by TGF-β1. Together, we provide evidences that TGF-β1 induces podocyte damage by upregulating TRPC6 protein most possibly through Smad3-ERK-NF-κB pathway, in which Fyn-dependent tyrosine phosphorylation of TRPC6 might exert a crucial role on the activation of its channel function.

Introduction

Transforming growth factor beta1 (TGF-β1), one of important cytokines in renal tissues, plays a critical role in the pathogenesis of glomerulosclerosis [1]. Indeed,

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strong expression of TGF-β1 is observed in some experimental [2, 3] and human kidney diseases [4, 5]. Some studies also showed that local production of TGF-β1 has diverse effects on glomerular cells, such as stimulating expansion of extracellular matrix and promoting podocyte injury, including cell morphology change, detachment from glomerular basement membrane, and apoptosis [6, 7]. It was reported that TGF-β1 reduces podocyte adhesion through α3β1 integrin downregulation and thus induces podocyte apoptosis [8]. In chronic glomerular diseases, TGF-β1, which is secreted by mesangial cells and stored in extracellular matrix as latent complexes, seems to be transported to podocyte surface and then activated [9]. On podocytes, TGF-β1 may bind to its receptor and activate its downstream signalling pathway including Smad, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and phosphatidyl inositol-3-kinase (PI3K) to modulate the expression of its target genes [4, 10-12]. NF-kappa B (NF-κB) family of transcription factors regulates the expression of numerous genes that play a central role in the inflammatory response during human and experimental kidney injury [13]. In mammalian cells, the family of NF-κB consists of five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and cRel. The hallmark of NF-κB family is the presence in all members of a Rel-homology domain at their NH2-teminus, responsible for DNA binding, dimerization and association with the I-κB inhibitory proteins [14]. It was reported that persistent NF-κB activation was observed in podocytes in a mouse model of HIV-associated nephropathy, implying NF-κB plays a crucial role in podocyte damage [15]. Nevertheless, the underlying mechanisms by which TGF-β1 induces podocyte injury have not been clearly defined.

Recently, the canonical ion chqnnel transient receptor potential C6 (TRPC6) was reported to be mutated in a subset of autosomal dominant focal segmental glomerulosclerosis patients. Importantly, TRPC6 is also localized to podocyte [16, 17], which makes TRPC6 as the first podocyte ion channel that is related to the pathogenesis of proteinuria. TRP channels as multiple transmembrane proteins are supposed to mediate the release of cytosolic compartmentalized calcium [18]. TRPC6 can be activated either through diacylglycerol (DAG) via phospholipase C stimulation or directly through mechanical stimuli such as membrane stretch [19]. Notably, it has been reported that TRPC6 is also a target of the Src kinase Fyn, which can enhance TRPC6 channel conductivity [20]. Some TRPC6 mutants have been shown to increase the cytosolic calcium signals [16, 21], but the mechanisms that lead to glomerular podocyte damage are incompletely elucidated. Here, we explored the potential role of TRPC6 and the potential proximal signalling pathway on TGF-β1-induced podocyte apoptosis to further understand the underlying mechanism by which TGF-β1 promotes podocyte injury and the development of glomerulosclerosis.

Materials and Methods

Antibodies

The following primary antibodies were used: rabbit polyclonal antibody against mouse TRPC6 (Chemicon), mouse anti-phosphotyrosine clone 4G10 antibody (Millipore), mouse monoclonal antibody against GAPDH (Chemicon), mouse monoclonal antibody against phospho-ERK1/2 (Santa Cruz), rabbit anti-ERK1/2 antibody (Santa Cruz), rabbit or mouse anti-Fyn polyclonal antibody (Santa Cruz), mouse anti-phospho-Fyn (pY528)/c-Src(pY530) antibody (BD Biosciences), rabbit anti-phospho-Src (pY418) antibody (Invitrogen), goat polyclonal antibody against human lamin B (Santa Cruz), rabbit anti-Smad3 antibody (Calbiochem), rabbit anti-phospho-Smad3 antibody (cell Signaling), rabbit anti-Smad7 antibody (Santa Cruz), and rabbit anti-NFκB/p65 antibody (Santa Cruz).

The secondary antibodies included: HRP-conjugated goat anti-rabbit or mouse antibody (GE Healthcare Bioscience), HRP-conjugated rabbit anti-goat antibody (Santa Cruz), and TRITC-conjugated goat anti-rabbit IgG (Invitrogen).

RNA interference

The specific small interference RNA from Ambion (siRNA-188581, sense: 5'-GGU UAU GUA CGG AUU GUG Gtt-3'; antisense: 5'-CCA CAA UCC GUA CAU AAC Ctt-3') was used to downregulate TRPC6 expression. Silence Negative Control siRNAs show no any similarities to mouse transcripts.

According to the standard molecular clone procedure and the siRNA designed guidelines of Ambion, the siRNA inserts (sense: 5'-*GAT CC*G GAT AAA GAA GCA GCG AAA TTC AAG AGA TTT CGC TGC TTC TTT ATC CTT TTT TGG AA*A*-3', antisense: 5'-*AGC TT*T TCC AAA AAA GGA TAA AGA AGC AGC GAA A TCT CTT GAA TTT CGC TGC TTC TTT ATC C*G*-3') with an underlined target sequence, a loop sequence (TTC AAG AGA) and a termination signal (6A) were cloned into pSilencer2.1U6 between BamH I and Hind III sites, immediately downstream of a human U6 promoter, to knockdown the expression of Fyn. The blank vector pSilencer2.1U6 was used as control.

Podocyte culture and treatment

Conditionally immortalized mouse podocyte cell line, a kindly gift from Prof. Peter Mundel (USA), was maintained at 33°C for proliferation in RPMI 1640 containing 10% fetal bovine serum (Gibco) and 10U/ml of recombinant mouse γ-interferon (Invitrogen), then moved to 37°C for differentiation and cultured without γ-interferon [22]. When they grew to about 80% confluence, podocytes were treated with 5 ng/ml TGF-β1

(Invitrogen) in serum-free medium for indicated times. ERK inhibitor U0126 (2 μM) and NF-κB inhibitor pyrrolidine-dithiocarbamate (PDTC, 4 μM) (Sigma) was respectively administrated 30 minutes before applying TGF-β1. To increase the efficiency of podocyte transfection, the proliferated podocytes were incubated for 8 hours with the TRPC6-siRNA, psiRNA-Fyn or pReceiver-M29 with the whole coding sequences of TRPC6 (FulenGen) plasmid according to the manufacture's protocol, then moved to 37°C and treated with 5ng/ml TGF-β1 for 24 hours.

Apoptosis assay

Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was applied to evaluate podocyte injury. Briefly, podocytes were harvested and washed two times with pre-cold phosphate-buffered saline (PBS). 1 x 10^5 cells were resuspended in 1 μg/ml FITC-Annexin V for 30min at 4°C followed by adding 5 μl of 50 μg/ml propidium iodide immediately prior to detection with flow cytometry (FACScan).

Measurement of the cytosolic free Ca^{2+} level

The cytosolic free Ca^{2+} level ($[Ca^{2+}]i$) was measured with the specific fluorescent indicator fluo-3am [23]. Briefly, 5 x 10^5 cells were resuspended in Ca^{2+}/Mg^{2+} free PBS containing 10 μM fluo-3am at 37°C for 30 minutes incubation, followed by washing three times with PBS, and then resuspended again in fresh Ca^{2+}/Mg^{2+} free PBS containing the indicated stimuli and inhibitors immediately prior to fluorescence detection using Spectrophotofluorometer (BioTek). After the basal $[Ca^{2+}]i$ was recorded, the cells were stimulated by 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Sigma), followed by 2mM $CaCl_2$ at one minute interval. The average individual cell $[Ca^{2+}]i$ was used for statistical analysis.

Immunofluorescence staining

Podocytes treated with 5ng/ml TGF-β1 were fixed with 4% paraformaldehyde, then permeabilized and blocked with 0.3% Triton X-100 and 5% bovine serum albumin. Rabbit anti-TRPC6 antibody was applied for overnight at 4°C. TRITC-conjugated goat anti rabbit IgG and the nuclei dye Hoechst were used for 45min at room temperature. TRITC-phalloidin (Sigma-Aldrich) was directly used to label the actin filaments. Finally, the coverslips were mounted and images were taken by using a confocal laser scanning microscope LSM510-META (Carl Zeiss, Germany).

Western blot and immunoprecipitation assay

Cells were lysed in the lysis buffer [1% Tritonx-100, 150 mM NaCl, 1mM EDTA, 50mM Tris-HCl (pH 7.7), and a protease inhibitor cocktail (Roche)]. Nuclear protein extraction kit (SunBio-Tech) was used to analyze NF-κB expression. Fifty microgram of total protein was loaded to run 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein was transferred to nitrocellulose membranes (GE Healthcare Bioscience). Then, the membranes were incubated for 30 minutes in 5% low-fat milk prepared with Tris-buffered saline containing 0.05% Tween-20 (TTBS). Subsequently, the membranes were incubated with the indicated primary antibodies. After rinsed three times with TTBS, the membranes were incubated with HRP-conjugated secondary antibodies for 45 minutes at room temperature, and then developed by using ECL chemiluminescence reagent (GE Healthcare Bioscience).

For immunoprecipitaion assay, Protein A or G Sepharose 4B (GE Healthcare Bioscience) was pre-saturated with 5% BSA-PBS for 4 hours at 4°C before use. Five hundred microgram of total protein in 0.5 ml of washing buffer (0.1% NP40-PBS) with proteinase inhibitors was incubated for 2 hours at 4°C with 2 μg of the indicated antibody, followed by incubation with 50 μl beads at 4°C for overnight. The beads were washed extensively with 0.1% NP40-PBS, and the precipitated proteins were resolved by 7.5-10% SDS-PAGE and analyzed by immuno-

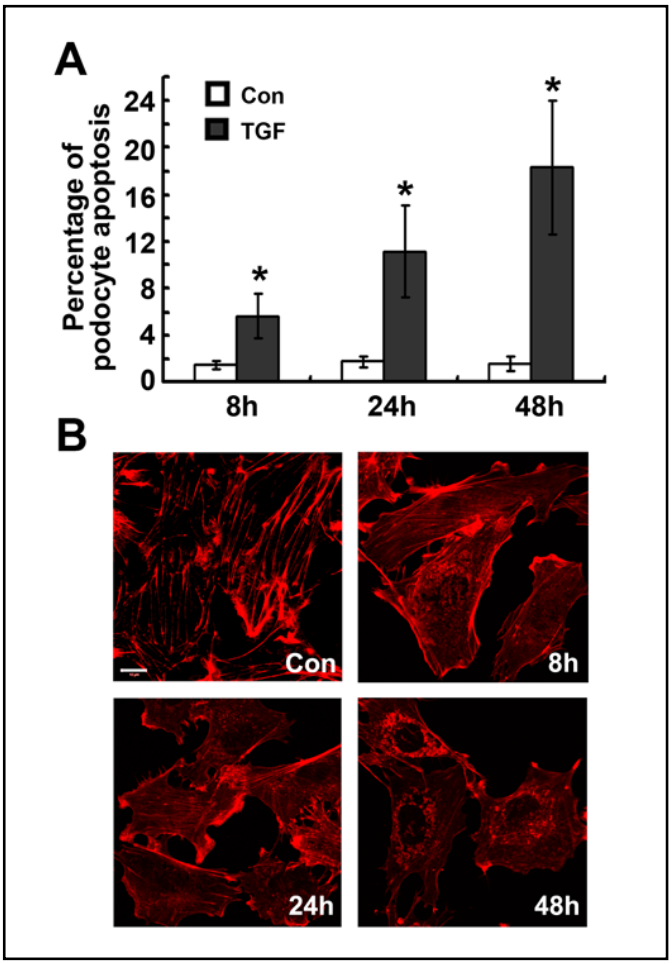

Fig. 1. Podocyte injury induced by TGF-β1. A. Apoptosis analysis of Annexin V and propidium iodide staining. As compared with control, the percentage of podocyte apoptosis increased significantly following 5ng/ml of TGF-β1 stimulation, presenting with a time-dependent manner. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con*. B. Confocal microscopy analysis of pholloidin staining. TGF-β1 induced obvious reorganization of podocyte cytoskeleton, while long intracellular bundles of actin filaments were observed in control cell. Bar = 10 μm.

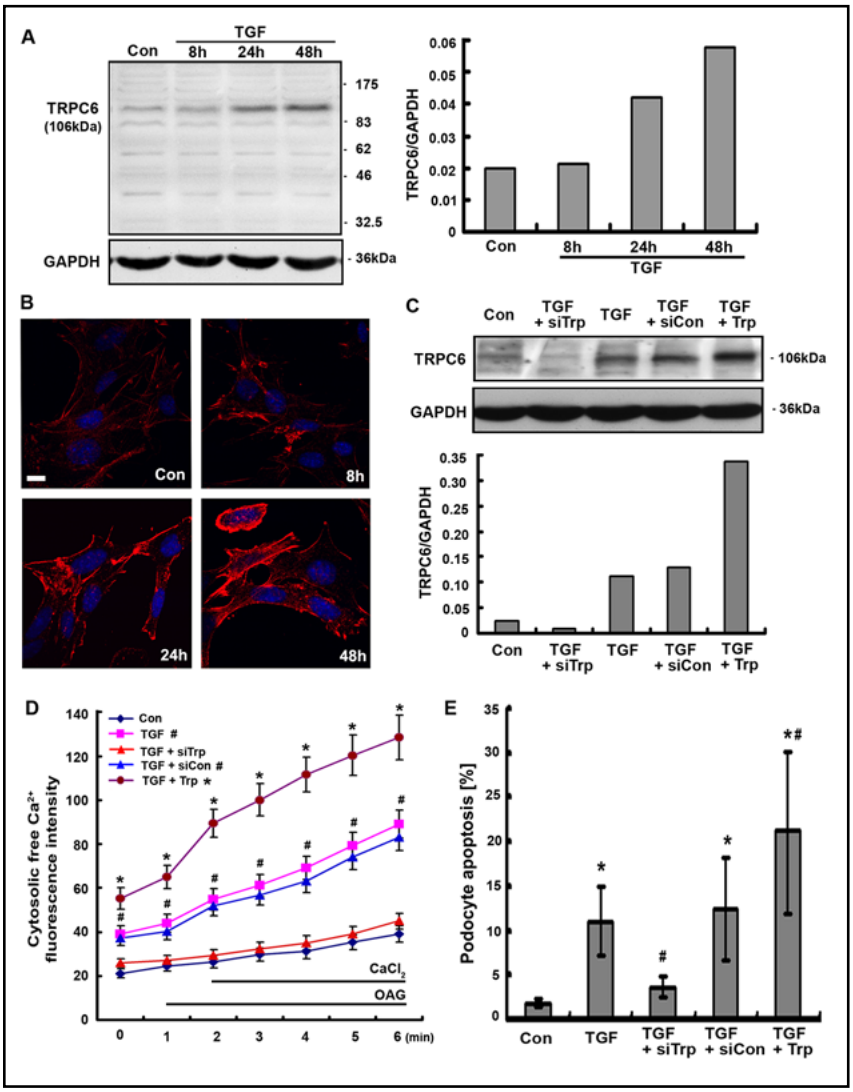

Fig. 2. TGF-β1 caused podocyte injury via upregulating TRPC6 expression. A. Western blot showed that TRPC6 increased evidently in time-dependent pattern in the cultured podocyte following TGF-β1 stimulation. Data are shown as mean from two independent experiments. B. Confocal microscopy analysis of TRPC6 staining. The abundance of TRPC6, especially on the cell membrane, increased markedly at 8h, 24h and 48h after TGF-β1 stimulation. Bar = 10 μm. C. As compared with control, TRPC6 expression was obviously inhibited by the specific siRNA targeted to TRPC6 (siTrp), not by the control siRNA (siCon). Overexpressed TRPC6 was detected in the plasmid pEGFP-Trpc6-transfected podocyte (Trp). Data are shown as mean from two independent experiments. D. The analysis of the cytosolic free Ca^{2+} level using Fluo-3AM. As compared with control, TGF-β1 increased significantly the fluorescence intensity of podocyte cytosolic free Ca^{2+}, especially in TRPC6-overexpressed cells. The knockdown of TRPC6 markedly inhibited the TGF-β1-induced increment of the cytosolic Ca^{2+}. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con* and *TGF*; #: $p < 0.05$ *vs Con*. E. Effect of TRPC6 on podocyte apoptosis. TGF-β1-induced podocyte apoptosis was obviously inhibited by the specific TRPC6 knockdown. TRPC6 overexpression notably aggravated podocyte apoptosis induced by TGF-β1. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con*; #: $p < 0.05$ *vs TGF*.

blotting. Notably, normal non-immune rabbit or mouse IgG (Alpha Diagnostics, San Antonio) was used as control.

Statistics analysis

Data were shown as mean ± SD. Statistical evaluation was performed using ONE-WAY ANOVA and nonparametric tests (Mann-Whitney U). $P < 0.05$ was considered as statistic significance.

Results

Podocyte injury induced by TGF-β1

In the preliminary experiments, we respectively used 2.5, 5.0 and 7.5ng/ml of TGF-β1 to stimulate the cultured podocyte. We found that there is a dose-dependent effect of TGF-β1 on podocyte apoptosis. In this study, we used 5.0 ng/ml TGF-β1 to induce podocyte damage. Following 5 ng/ml of TGF-β1 stimulation, podocyte injury was evaluated by podocyte apoptosis using Annexin V and PI staining, and the alterations of cytoskeleton arrangement using pholloidin staining. As compared with control, flow cytometry results showed that the percentage of podocyte apoptosis increased significantly in TGF-β1-treated podocytes, and presented with a time-dependent manner (Fig. 1A). Confocal microscopy analysis of pholloidin staining displayed that the fine and long bundles of the intracellular actin filaments were observed in control cells, whereas TGF-β1 induced obvious reorganization of cytoskeleton, presenting with the loss of the cytoplasmic cytoskeleton and the congregating around nuclei, especially at 48 hours following stimulation (Fig. 1B).

TGF-β1 induced podocyte injury via activating the channel function of TRPC6

TRPC6 protein was detected with the expected size of 105 kDa in the differentiated cultured mouse podocyte

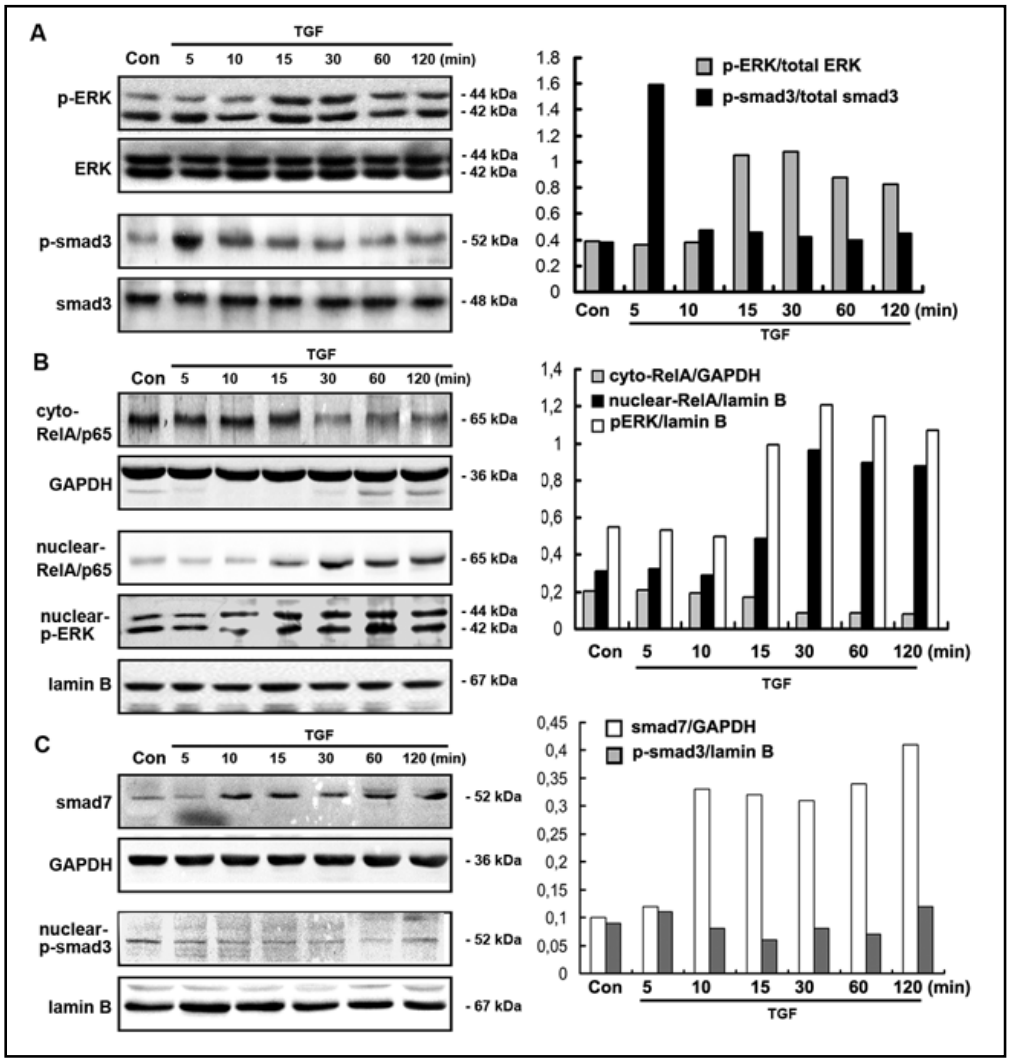

Fig. 3. TGF-β1 induced the activation of ERK and Smad3 as well as the translocation of NF-κB. A. After TGF-β1 stimulation, Western blot showed that as compared with control, ERK was obviously activated at 15min with a persistent increment to 120 min, whereas Smad3 was significantly activated at 5min. B. As compared to control, Western blot displayed that the abundance of RelA/p65 decreased in cytoplasm, while increased in nuclear extraction from 15min, and ERK expression in nuclei also increased starting at 15min following TGF-β1 stimulation. C. Expression of Smad7 protein upregulated from 10 minutes following TGF-β1 stimulation. The abundance of p-Smad3 in nuclei showed no obvious alteration in TGF-β1-treated podocytes. Data are shown as mean from two independent experiments.

cells. Western blot showed that TRPC6 expression increased markedly following TGF-β1 stimulation, presenting with a time-dependent manner (Fig. 2A). Similarly, confocal microscopy analysis showed that the abundance of TRPC6, especially on the cell membrane, increased evidently at 8h, 24h and 48h after TGF-β1 stimulation (Fig. 2B).

To evaluate the effects of ion channel protein TRPC6 on podocyte injury induced by TGF-β1, TRPC6 knockdown by RNA interference and overexpression by pEGFP-Trpc6 vector were performed in this study. As compared with control, TRPC6 expression was obviously inhibited by the specific siRNA targeted to TRPC6, and the overexpressed TRPC6 was detected in the pEGFP-Trpc6-transfected podocyte (Fig. 2C). Importantly, the cytosolic free Ca^{2+} level ($[Ca^{2+}]i$) reflecting the channel activity of TRPC6 was measured by using the fluorescence indicator Fluo-3am at the basal and the OAG/$CaCl_2$ stimulations conditions. As compared with control, TGF-β1 increased significantly the $[Ca^{2+}]i$, especially in TRPC6-overexpressed cells. The knockdown of TRPC6 markedly inhibited the increment of the $[Ca^{2+}]i$ induced by TGF-β1 (Fig. 2D). Here, the effect of TRPC6 on podocyte apoptosis was also evaluated. TRPC6 overexpression notably aggravated podocyte apoptosis induced by TGF-β1. Nevertheless, TGF-β1-induced podocyte apoptosis was obviously inhibited by the specific TRPC6 knockdown (Fig. 2E).

TGF-β1 induced the expression of TRPC6 via Smad3-ERK-NF-κB pathway

In the present study, the Smad3, ERK and NF-κB pathway was explored to elucidate the possible signaling pathway associated with TGF-β1-induced podocyte injury. Western blot showed that as compared with control, phosphor-Smad3 increased significantly at 5 minutes, followed by the obvious increment of phosphor-ERK from 15 minutes to 120min in TGF-β1 stimulated podocytes (Fig. 3A). Notably, the abundance of RelA/p65 decreased markedly in the cytoplasm compartment, while increased

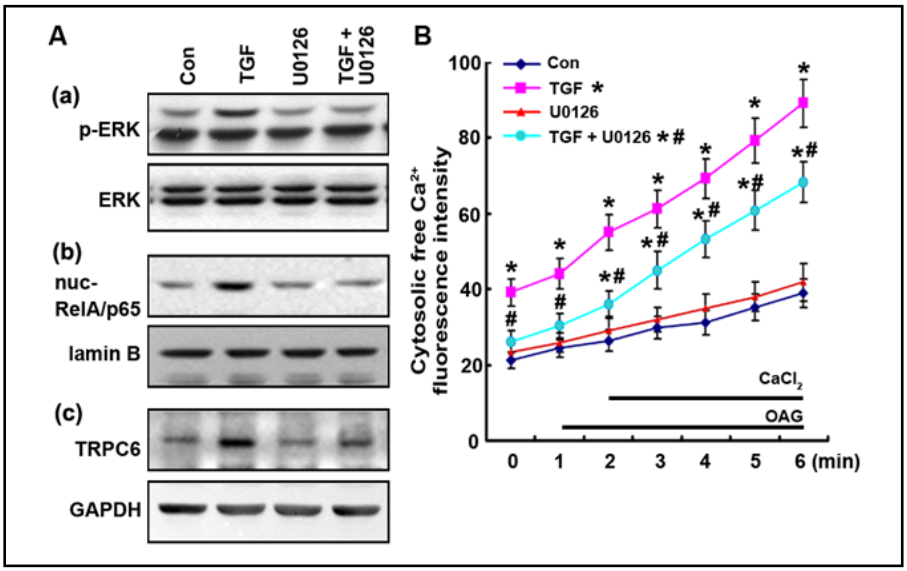

Fig. 4. Inhibitions of p-ERK blocked the translocation of NF-κB and the upregulation of TRPC6. The ERK pathway inhibitor U0126 (2 μM) was applied 30min before TGF-β1 stimulation. A. After 24 hours, Western blot showed that TGF-β1 led to the activation of ERK (a), the translocation of NF-κB (b) and the upregulation of TRPC6 (c), all of which were evidently inhibited by U0126. B. The TGF-β1-induced increment of the cytosolic free Ca^{2+} level was obviously attenuated by U0126. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con*; #: $p < 0.05$ *vs TGF*.

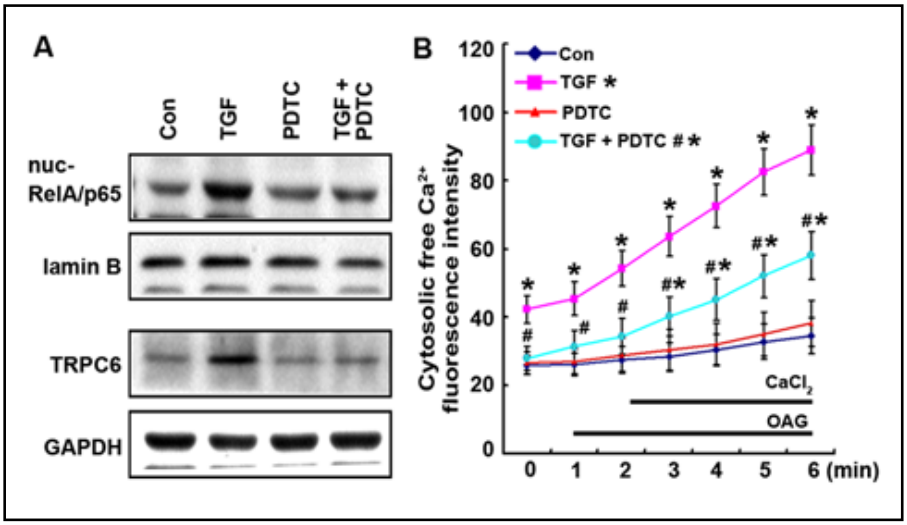

Fig. 5. Inhibitions of NF-κB blocked the upregulation of TRPC6. The NF-κB pathway inhibitor PDTC (4 μM) was applied 30min before TGF-β1 stimulation. A. After 24 hours, Western blot showed that the nuclear translocation of RelA/p65 and the upregulation of TRPC6 induced by TGF-β1 were obviously inhibited by PDTC. B. The TGF-β1-induced increment of the cytosolic free Ca^{2+} level was obviously attenuated by PDTC. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con*; #: $p < 0.05$ *vs TGF*.

obviously in the nuclear extraction from 15 minutes following TGF-β1 treatment. The translocation of ERK to nuclei was also observed starting from 15 minutes (Fig. 3B), while the abundance of p-Smad3 in nuclei showed no obvious alteration in TGF-β1-treated podocytes. As compared to control, Smad7 protein upregulated at 10min with a persistent increment to 120min following TGF-β1 stimulation (Fig. 3C).

The ERK pathway inhibitor U0126 was applied 30 minutes before TGF-β1 administration. Western blot showed that pre-treated U0126 evidently inhibited the activation of ERK and the translocation of NF-κB as well as the upregulation of TRPC6 expression induced by TGF-β1 stimulation (Fig. 4A). Importantly, the TGF-β1-induced increment of the $[Ca^{2+}]i$ was also attenuated by the pre-treatment of U0126 (Fig. 4B). The NF-κB pathway inhibitor PDTC was applied 30 minutes before TGF-β1 stimulation. Western blot showed that the nuclear translocation of RelA/p65 and the upregulation of TRPC6 expression induced by TGF-β1 were obviously inhibited by the pre-treatment of PDTC (Fig. 5A). The increment of the $[Ca^{2+}]i$ induced by TGF-β1 was obviously attenuated by the pre-treated PDTC (Fig. 5C).

Activation of Fyn induced TRPC6 phosphorylation in TGF-β1-treated podocyte

In TGF-β1-treated podocytes, Western blot results showed that total Fyn expression upregulated at 24 hours and 48 hours after stimulation (Fig. 6A). Twenty-four hours following TGF-β1 stimulation, immunoprecipitation assay revealed that TGF-β1 led to the increment of Fyn active form (pY418), and the reduction of Fyn inactive form (pY530). Interestingly, the interaction between Fyn and TRPC6 increased obviously in TGF-β1-treated

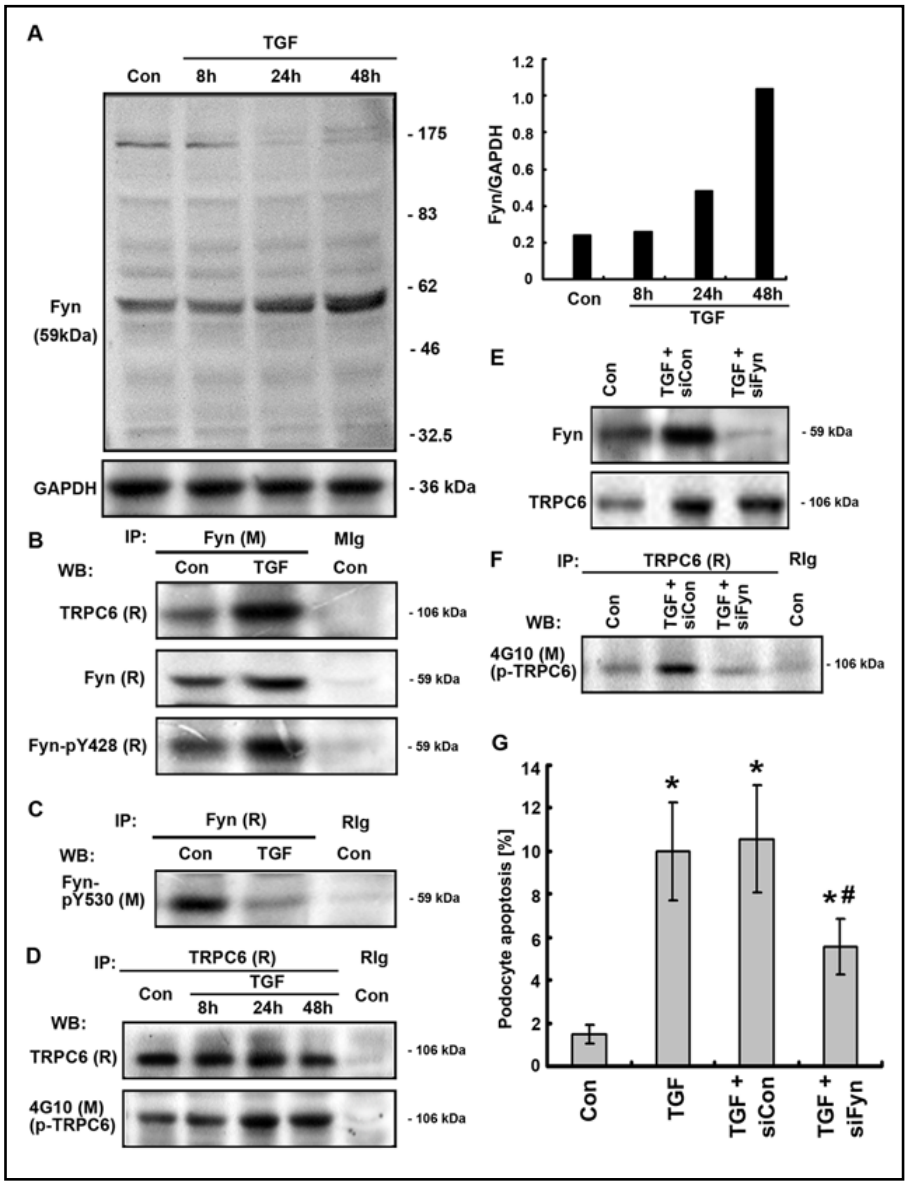

Fig. 6. Activation of Fyn induced TRPC6 phosphorylation in TGF-β1-treated podocyte. A. Western blot showed that total Fyn upregulated at 24h and 48h after TGF-β1 stimulation. Data are shown as mean from two independent experiments. B. Twenty-four hours after TGF-β1 stimulation, immunoprecipitation assay showed that TGF-β1 induced the activation (pY418) of Fyn (lower panel), and that the interaction between Fyn and TRPC6 increased obviously (upper panel). C. The inactive form (pY530) of Fyn decreased evidently at 24h after TGF-β1 stimulation. D. Immunoprecipitation of TRPC6 followed by immunoblotting of eluates with anti-phospho-tyrosine antibody (4G10). Note that for a similar protein level of TRPC6, phospho-TRPC6 is significantly increased in a time-dependent manner after TGF-β1 stimulation. E. Western blot showed that the upregulation of Fyn following TGF-β1 stimulation was effectively inhibited by the specific knockdown of Fyn (upper panel). As compared with control, Fyn knockdown showed no effects on TRPC6 expression in TGF-β1-treated podocytes (lower panel). F. Immunoprecipitation assay displayed that Fyn knockdown inhibited the phosphorylation of TRPC6 in TGF-β1-treated podocytes. G. Fyn knockdown significantly reduced TGF-β1-induced podocyte apoptosis. Data are represented with mean ± SD. n=4. *: $p < 0.05$ *vs Con*; #: $p < 0.05$ *vs TGF and TGF + siCon.*

podocytes as compared with control (Fig. 6B, 6C). Immunoprecipitation of TRPC6 followed by immunoblotting of eluates with anti-phosphor-tyrosine antibody displayed that for a similar protein level of TRPC6, phosphorylated TRPC6 was significantly increased in a time-dependent manner after TGF-β1 stimulation (Fig. 6D).

The effects of Fyn activation on TRPC6 phosphorylation was further explored by knockdown of Fyn. Western blot showed that Fyn upregulation following TGF-β1 stimulation was effectively inhibited by the specific knockdown of Fyn. As compared with control, Fyn knockdown showed no effects on TRPC6 expression in TGF-β1-treated podocytes (Fig. 6E). Nevertheless, immunoprecipitation assay displayed that Fyn knockdown inhibited the phosphorylation of TRPC6 in TGF-β1-treated podocytes (Fig. 6F). In addition, TGF-β1-induced podocyte apoptosis was significantly reduced by the specific knockdown of Fyn (Fig. 6G).

Discussion

In the present study, our results demonstrated that the application of TGF-β1 obviously caused podocyte injury, presenting with significant apoptosis and disorganization of actin filaments in a time-dependent pattern. In some human and experimental primary glomerular diseases, where podocyte is the target of injury, expression of TGF-β1 protein increased in these podocytes

that cover the cellular lesions of FSGS [2-5]. In addition, podocytes undergo apoptosis at early stages in the course of progressive glomerulosclerosis in TGF-β1 transgenic mice [24, 25]. Here, our findings directly displayed that local production of TGF-β1 may be related to the progression of podocyte damage. In the cultured murine podocytes, it was also reported that TGF-β1 induces podocyte apoptosis [26]. Interestingly, the ion channel protein TRPC6 was evidently induced in TGF-β1-treated podocyte, implying that TRPC6 might be involved to TGF-β1-induced podocyte injury. In podocytes, it has been reported that the cytosolic free Ca^{2+} level is tightly regulated by TRPC6, which can be activated by the exogenous application of OAG. The enhanced cytosolic Ca^{2+} activates the Ca^{2+}-dependent phosphatase calcineurin linked to the induction of apoptosis [17, 27]. Therefore, we investigated the effects of TGF-β1 on TRPC6 channel activity in podocyte. We found that TGF-β1 indeed caused the increment of the cytosolic free Ca^{2+} level. Importantly, our RNAi assay showed that the specific knockdown of TRPC6 not only inhibited the upregulation of cytosolic free Ca^{2+}, but also alleviated podocyte apoptosis in TGF-β1-treated podocyte. Therefore, strong evidences have been provided that TRPC6 protein is associated with podocyte damage induced by TGF-β1.

In this study, immunoprecipitation assay firstly showed that the Src kinase Fyn binds and interacts with TRPC6 in the cultured mouse podocytes. In COS-7 cells when coexpressed with Fyn, it was reported that Fyn interacts with TRPC6, and TRPC6 is tyrosine-phosphorylated. The physical association of TRPC6 with Fyn was also demonstrated in the mammalian brain [20]. Western blot revealed that expression of Fyn increased obviously in TGF-β1-treated podocytes, displaying increment of the active form pY418 and reduction of the inactive form pY530. Tyrosine phosphorylation of TRPC6 was also found increment in TGF-β1-treated podocytes. Notably, we found that the specific knockdown of Fyn significantly blocked the phosphorylation of TRPC6 and the increment of cytosolic free Ca^{2+} following TGF-β1 stimulation, but not affect the expression of TRPC6 protein. Using single channel recording, it was showed that Fyn modulates TRPC6 channel activity via tyrosine phosphorylation [20]. Our results reasonably proposed that the Src kinase Fyn-dependent phosphorylation of TRPC6 might be responsible for the activation of its channel function, which play a critical role in TGF-β1-induced podocyte apoptosis, although it should be further studied how TGF-β1 caused the activation of Fyn in podocyte damage.

To further understand the molecular mechanisms of TGF-β1-induced podocyte injury, we explored the possible signaling transduction pathway. Western blot showed that TGF-β1 caused significant activation of p-Smad3 and p-ERK as well as RelA/p65. Some studies showed that TGF-β1 induces activation of both Smad2 and ERK1/2 [11]. Similarly, TGF-β1 activates ERK and p-38 MAPK, which is required for the induction of apoptosis by TGF-β1 in podocytes [28]. Activation of the TGF-β1-Smad2/3 signaling pathway was also observed in focal segmental glomerulosclerosis [4, 12, 26].

In this study, activation of Smad3 was observed at 5min, while p-ERK1/2 increased obviously at 15 minutes following TGF-β1 stimulation. Interestingly, Western blot from nuclei extraction showed that expression of p-ERK, not Smad3, obviously increased in nuclei starting at 15min in TGF-β1-treated podocyte, consistent well with the nuclear translocation of RelA/p65, suggesting that activation of ERK might promote the translocation of RelA/p65 into nuclei. These results implied that the cascade reaction of Smad3-ERK-RelA/p65 might play an important role in TGF-β1-induced podocyte injury. Nevertheless, the results from Böttinger's team showed that TGF-β had no significant effect on NF-κB/p65 activities, but it was in the transfected podocyte with a NF-κB/p65-responsive luciferase report gene construct, only implying that TGF-β had no influence on NF-κB activation at transcriptional level. They did not explore the expression/activation alterations of the endogenous NF-κB/p65 protein in TGF-β-stimulated podocyte apoptosis [26]. In addition, mounting evidence showed that NF-κB/p65 is a cell survival factor preventing apoptosis induced by TGF-β, especially in B-lymphocytes, hepatocytes, epithelial cells and central nervous systems [29-31]. Moreover, Lallemand et al. reported that the stable overexpressed Smad7 increased TGF-β or TNF-α-mediated apoptosis through decreasing the activity of NF-κB transcription factor in MDCK cells [32]. However, among the NF-κB proteins, only RelA, RelB and c-Rel have a COOH-terminus transactivation domain, so that they can function as transcriptional activators [33]. Here, the translocation of RelA/p65 to nuclei was obviously observed in TGF-β1-treated podocyte, which was attenuated by the application of ERK inhibitor U0126. Importantly, both U0126 and NF-κB inhibitor PDTC significantly inhibited the increment of TRPC6 protein and the flux of cytosolic free Ca^{2+} caused by TGF-β1. These findings prompt us to propose that the translocation of RelA/p65 to nuclei might be responsible for the

increment of the ion channel TRPC6 protein, although the direct evidence should be further investigated. There were some evidences that NF-κB could regulate the expression of the TRPC family. Takahashi Y et al. found that angiotension II could upregulate TRPC1 expression through the activation of NF-κB in vascular smooth muscle cells [34], while Zhang H et al. showed that angiotension II increases the expression and channel activities of TRPC6 protein via NF-κB activation in cultured mouse podocyte [35]. Our results strongly suggested that TGF-β1 leads to induction of TRPC6 protein at least partially through Smad3-ERK-NF-κB axis.

In addition, our preliminary result displayed that the abundance of Smad7 protein increased in a time-depenendent manner in TGF-β1-induced podocyte apoptosis. It was also found that TGF-β1 induces the increment of Smad7 at mRNA level in cultured podocyte, and that the overexpressed-Smad7 has an additive effects on TGF-β1-induced podocyte apoptosis through inhibiting the NF-κB transcriptional activities [26]. Now, it is well accepted that TGF-β regulates fibrosis positively by receptor-associated Smads such as Smad2 and Smad3, but negatively by an inhibitory Smad, called Smad7. Lan et al. has shown that gene transfer of Smad7 is able to inhibit renal fibrosis through the blockade of Smad2/3 activation in several experimental models of chronic kidney diseases [36]. Smad7 also prevents Smad2/3 from being phosphorylated by the TGF-β type I receptor, resulting in inhibition of Smad2/3 and Smad4 complex formation and subsequent nuclear accumulation of this complex [37]. Therefore, the increment of Smad7 in this study might play a positive role on inhibiting Smad3 activation induced by TGF-β stimulation in podocyte. The detailed studies should be needed to determine the regulatory relationships and molecular determinants among the complex signaling transduction network, especially Smad3, Smad7 and NF-κB in the course of podocyte injury. The specific knockdown assay seems to be a useful tool to seperate the role of Smad3 and Smad7 in TGF-β1-induced podocyte damage.

Collectively, these data provide a support that TGF-β1 induces podocyte damage by upregulating TRPC6 protein most possibly through Smad3-ERK-NF-κB pathway, in which Fyn-dependent tyrosine phosphorylation of TRPC6 might exert a crucial role on the activation of its channel function.

Acknowledgements

This work was supported by a grant from the Research Foundation of Emphasis Specialty of Kunshan awarded to J.Y.

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