Wnt/ β -catenin signalling pathway mediates high glucose induced cell injury through activation of TRPC6 in podocytes

Z. Li*, J. Xu*, P. Xu*, S. Liu* and Z. Yang*

*Department of Physiology, College of Medicine, Nankai University, 300071, Tianjin, China

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Abstract

Objectives: Diabetic nephropathy is a major complication of diabetes and a frequent cause of end-stage renal disease and recent studies suggest that podocyte damage may play a role in the pathogenesis of this. At early onset of diabetic nephropathy there is podocyte drop-out, which is thought to provoke glomerular albuminuria and subsequent glomerular injury; however, the underlying molecular mechanisms of this remain poorly understood. Here we report that we tested the hypothesis that early diabetic podocyte injury is caused, at least in part, by up-regulation of transient receptor potential cation channel 6 (TRPC6), which is regulated by the canonical Wnt signalling pathway, in mouse podocytes.

Materials and methods: Mechanism of injury initiation in mouse podocytes, by high concentration of D-glucose (HG, 30 mM), was investigated by MTT, flow cytometry, real-time quantitative PCR, and western blot analysis.

Results: HG induced apoptosis and reduced viability of differentiated podocytes. It caused time-dependent up-regulation of TRPC6 and activation of the canonical Wnt signalling pathway, in mouse podocytes. In these cells, blockade of the Wnt signalling pathway by dickkopf related protein 1 (Dkk1) resulted in effective reduction of TRPC6 up-regulation and amelioration of podocyte apoptosis. Furthermore, reduction of cell viability induced by HG was attenuated by treatment with Dkk1.

Conclusion: These findings indicate that the Wnt/ β -catenin signalling pathway may potentially be

active in pathogenesis of TRPC6-mediated diabetic podocyte injury.

Introduction

Diabetic nephropathy involves serious diabetic microangiopathy complications in type 1 and type 2 diabetics. It is estimated that there will be 439 million adults with diabetics around the world by 2030 (1), and global health expenditure on diabetes is expected to total at least USD 490 billion or ID 561 billion in 2030 (2). It is generally accepted that diabetic nephropathy has become the most important cause of end-stage renal disease (ESRD) worldwide. In its early stages, podocyte number is markedly reduced, which in turn leads to injury to the integrity of the glomerular filtration barrier. Several recent studies suggest that podocyte damage may play a role in processes of diabetic nephropathy (3–8). Thus, further study of mechanisms of podocyte injury may provide a promising therapeutic modality for treatment of diabetic nephropathy, in the future.

The Wnt signalling pathway is known to be a conservative signal transduction pathway in the evolutionary process, and plays a prominent role in development of animals (9–12). Wnt signals are involved in numerous aspects of development, including proliferation, polarity and fate of cells. Wnt signals consist of at least two distinct types, the best studied ‘canonical Wnt pathway’ (or Wnt/ β -catenin pathway), and the ‘non-canonical pathway’ (or pathway that is β -catenin independent). The canonical Wnt pathway being β -catenin dependent, has central roles in histogenesis and development. Several common human diseases, including cancer, are confirmed to be associated with activity of this pathway (13,14). There are several secreted antagonists of Wnt signalling, including soluble frizzled-related protein, Wnt inhibitory factor and Dickkopf (Dkk) (13,15). Among them, Dkk is unique as it specifically inhibits the canonical Wnt signalling pathway

Correspondence: Professor Z. Yang, College of Medicine, Nankai University, Tianjin 300071, China. Tel.: +86 22 23504364; Fax: +86 22 23502554; E-mail: zhuoyang@nankai.edu.cn

by binding to the LRP5/6 component of the receptor complex (16,17). Recent studies have indicated that the canonical Wnt pathway is rapidly activated in the context of diabetic nephropathy (18,19). By using a model of adriamycin-induced nephropathy, Dai *et al.* have shown that the Wnt/ β -catenin pathway plays a critical role in podocyte injury and proteinuria (18). Conversely, inhibition of the Wnt/ β -catenin pathway by treating with paricalcitol results in amelioration of proteinuria and prevention of podocyte dysfunction, in adriamycin nephropathy (20). Nonetheless, depending on the complexity of the diabetic milieu, further mechanisms of action of downstream Wnt/ β -catenin pathway are required to be further elucidated in the pathogenesis of diabetic nephropathy.

Transient receptor potential (TRP) channels, a superfamily of cation-selective ion channels, makes up a family of non-selective Ca^{2+} -permeable cation channels widely expressed in vertebrate tissues. The TRPC subfamily (TRPC1-TRPC7) is made up of a group of calcium-permeable cation channels important for regulation of intracellular calcium signalling (21). In particular, the most extensive analyses of TRPC channels have been carried out on TRPC6 channels and TRPC6 protein expression and function have been reported in the central nervous system, kidneys, and cardiovascular system (22–24). Mutation of TRPC6 can lead to familial focal segmental glomerulosclerosis (FSGS) (25,26) and in this context TRPC6 is very important in experimental nephrology, worldwide. Down-regulation of TRPC6 induced by high glucose, has been thought to be a possible mechanism for impaired Ca^{2+} signalling by glomerular mesangial cells (MCs), seen in diabetic nephropathy (27). A further study has shown that up-regulation of TRPC6 induced by angiotensin II contributes to podocyte injury *via* a nuclear factor of activated T-cell (NFAT)-mediated positive feedback signalling pathway (28). The crucial role of TRPC6 makes it a potential therapy target of original injury in diabetic nephropathy; however, functional analysis, especially crosstalk between TRPC6 and other signal transduction pathways still needs further elucidation. One study has indicated that the Wnt/ β -catenin pathway causes human colonic epithelial cell injury through cross-talk with calcium related signalling (29). Interestingly, as non-selective Ca^{2+} -permeable cation channels, TRPC6 is reported to cause podocyte injury by incorrect modulation of calcium influx (23,30,31). Thus, these results indicate potential association of TRPC6 with canonical Wnt signalling in diabetic nephropathy. Herein, we hypothesize that TRPC6 mediates high glucose-induced cell injury through Wnt/ β -catenin signalling pathway in podocytes. The aim of this study was to

investigate effects of D-glucose (30 mM, HG) on podocyte injury and TRPC6 protein expression, and evaluate whether TRPC6 production depends on the Wnt/ β -catenin canonical signalling pathway. In addition, we have examined the potential role of Wnt/TRPC6 signalling in HG induced podocyte injury.

Materials and methods

Cell culture and drug treatment

Mouse podocytes from a conditionally immortalized cell line were cultured, as described previously (32). Cells were cultured under growth-permissive conditions at 33 °C in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (Gibco), 20 U/ml mouse recombinant interferon-gamma ($\text{IFN-}\gamma$; Sigma, St. Louis, MO, USA) and 100 U/ml penicillin plus 100 mg/ml streptomycin (Sigma) under humidified atmosphere containing 5% CO_2 . To induce differentiation, podocytes were maintained in non-permissive conditions at 37 °C in the absence of $\text{IFN-}\gamma$ for at least 2 weeks and were used for experiments. After serum starvation for 16 h, cells were treated with high concentration of D-glucose (30 mM) for a range of times, as indicated. For some experiments, podocytes were also treated with dickkopf related protein 1 (Dkk1) (R&D System, Inc., Minneapolis, MN, USA). According to instructions of products and recent studies (19), 200 ng/ml Dkk1 was chosen as an effective concentration for this study. Differentiated podocytes were divided into the following groups: control (5.6 mM), HG and HG+Dkk1 (HG+D, 200 ng/ml) groups.

Cell viability assay

Cell viability was assessed using the colorimetric reagent, 3-[4, 5- dimethylthiazol - 2-yl]-2, 5- diphenyl-tetrazolium bromide (MTT) (Sigma). Briefly, cells were seeded in 96-well plates, 1×10^4 cells in 200 μl medium per well, and cultured for 24 h for cell stabilization. Cultures were incubated with HG for 24, 48, 72 and 96 h. As osmotic control, podocytes were cultured in medium containing 5.6 mmol/l D-glucose and 30 mmol/l D-mannitol (Man). For a proportion of experiments, differentiated podocytes were divided into three groups (control, HG and HG+D), and cultured for 48 h. Then, cultures were incubated with MTT (20 μl , 5 mg/ml) for 4 h at 37 °C. Medium was removed and 150 μl DMSO was added to each well to dissolve the formazan product and finally, formazan absorbance was assessed using a microplate reader (Multiskan Mk3; Thermo Labsystems,

Helsinki, Finland). Each group of cells was set up in six parallel wells, and experiments were repeated three times. Results are presented as mean $\pm$ SD.

Annexin V/PI staining assay

To determine type and ratio of apoptotic cell death, cells were exposed to HG in the presence or absence of Dkk1, as described above. After 24 h incubation, cells were harvested, washed in phosphate-buffered saline (PBS) and stained for 15 min at room temperature, with annexin V-FITC (5 μ l) and propidium iodide (PI, 5 μ l) in binding buffer in the dark. Level of fluorescence was determined using flow cytometry (Beckman-Coulter, Fullerton, CA, USA) equipped with CellQuest software. All experiments were performed in triplicate.

Measurement of ROS

Generation of reactive oxygen species (ROS) was evaluated by fluorometric assay using intracellular oxidation of 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Cells in the logarithmic growth phase were incubated in 6-well plates for 24 h for stabilization, and then medium was replaced with fresh medium containing HG, in the presence or absence of Dkk1, as described above. Six hours later, cells were washed in PBS, and then resuspensions were stained with DCFH-DA (Genmed Scientifics Inc., Wilmington, DE, USA) for 20 min at 37 °C. Fluorescent probes were commonly used for detection of intracellular oxidants. During an intracellular oxidative burst, ROS are usually generated, leading to conversion of non-fluorescent (DCFH) probe into fluorescent molecules (DCF). Oxidation product green emission 525 nm, was detected and analysed by flow cytometry. All experiments were performed in triplicate.

Table 1. Primers used for real-time PCR

Gene name	Primer and probe sequence (5'–3')	GenBank accession number	Product length (bp)
Wnt1	F ATAGCCTCCTCCACGAACCT R GGAATTGCCACTTGCACTCT	NM_021279	175
Wnt2	F GCCCGCACACGGAGTCTGAC R ACACCCAGGCCAATGGCAG	NM_023653.5	251
Wnt5a	F GCTGCGGAGACAACATCGACT R ACTGTCCTACGGCCTGCTTCA	NM_009524.2	148
β -catenin	F ACGCCATCACGACACTGCATA R GCTTGCTCTCTTGATTGCCA	NM_001165902.1	177
TRPC6	F ACCAAGCTCCTCCCTAATGAA R AGTATTCTTTGGGGCCTTGAG	NM_013838.2	163
β -actin	F GCGGACTGTACTGAGCTGCGT R TGCTGTGCGCTTCACCGTTCC	NM_007393	217

F, forward primer; R, reverse primer.

RNA extraction and real-time quantitative PCR

Total RNA isolation and real time-PCR for detecting Wnt, β -catenin and TRPC6 mRNA expression were carried out. Briefly, total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration and purity was assessed by UV spectroscopy due to small amounts of RNA available. Aliquots of RNA extracted from cultured podocytes were run on agarose gel. Reverse transcription (RT) reactions used 1.0 μ g total RNA, oligo-dT primers, Moloney murine leukaemia virus (M-MLV) reverse transcriptase final volume of 20 μ l, and were incubated at 42 °C for 60 min after denaturation at 95 °C for 5 min. Real-time PCR used 2.0 μ l RT product, 10 μ l SYBR Green PCR mix (QIAGEN 204143; Germantown, MD, USA), and 0.5 μ M primer, and was performed in a final volume of 20 μ l. PCR mix was denatured at 95 °C for 5 min, followed by 35 cycles melting at 95 °C for 30 s, annealing at 56 °C–60 °C for 30 s and elongation at 72 °C for 30 s. Sequences of primers for β -catenin, TRPC6, β -actin and 3 different Wnts were designed according to their respective mouse DNA sequences. Primers used for RT-PCR are shown in Table 1 and experiments were performed in triplicate. Average Ct (threshold cycle) of fluorescence unit was used to analyse mRNA levels and mRNA levels were normalized to those of actin. Quantification was calculated as follows: mRNA levels (percent of control) = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = C_{T, \text{others}} - C_{T, \text{actin}}$ and $\Delta(\Delta CT) = \Delta C_{T, \text{high glucose}} - \Delta C_{T, \text{normal glucose}}$.

Western blotting

Podocytes were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate [SDS], 0.5% deoxycholic acid sodium salt [DOC]). Proteins were extracted for analy-

sis according to established protocol. Equal amount protein loadings were separated by SDS-PAGE on 8% or 10% gels and electrophoretically transferred to a nitrocellulose membrane. Non-specific binding sites

were blocked with 5% zero fat milk powder in PBS and 0.05% Tween 20, for 1 h at room temperature. Membranes were incubated with the following primary antibodies at appropriate concentrations: rabbit anti-

Figure 1. High glucose induced injury to podocytes. Podocytes were treated with high glucose (30 mM, HG) for a range of periods of time, in different experiments. (a) Exposure of podocytes to HG resulted in relatively rapid generation of ROS. Podocytes were maintained in HG for 6 h. (b) Apoptosis is a typical physiological characteristic of early cell injury. Podocytes were maintained in HG for 24 and 48 h, respectively, and HG-induced podocyte apoptosis was confirmed by annexin V-FITC/PI staining. Dot plots indicate intensity of annexin V-FITC fluorescence on the X-axis and PI fluorescence on the Y-axis. Lower left quadrant (LL): viable cells (annexin V⁻/PI⁻), upper left quadrant (UL): necrotic cells (annexin V⁻/PI⁺), upper right quadrant (UR): late apoptotic cells (annexin V⁺/PI⁺), lower right quadrant (LR): early apoptotic cells (annexin V⁺/PI⁻). Percentages of cells presented in areas of respective quadrant profiles. (c) Corresponding histogram of apoptosis. Total percentages of early apoptotic and late apoptotic cells were analysed by flow cytometry. (d) Cell viability was assessed after incubation with HG for 24, 48, 72 and 96 h. Man: D-mannitol. Results represent means of three separate experiments, and error bars represent standard errors of the mean. * $P < 0.05$ compared to the control group (5 mM). ** $P < 0.01$ versus control.

TRPC6, rabbit anti-beta-actin (KangChen, Bio-Tech, China). All experiments were performed at least in triplicate.

Image quantitation

Images were digitally scanned or collected using phase contrast microscopy and converted to threshold values using Image J software (Bethesda, Maryland, USA). Densitometry of each image was then measured using Image J software.

Statistical analysis

All data were expressed as mean $\pm$ SD. Independent samples *t*-test was applied where two groups of data were compared. Means are presented in bar charts, with T-bars referring to standard deviation. Multiple samples were statistically analysed for significance using one-way analysis of variances (one-way ANOVA). Post hoc statistics were made using the LSD test (Fisher's least significant difference *t*-test) for multiple comparisons. All statistical analyses were performed using SPSS 11.5 software (Chicago, IL, US). *P*-values less than or equal to 0.05 were considered significant. All experiments were performed at least in triplicate.

Results

High glucose induced the injury of podocytes

Injury of podocytes induced by high glucose, a model characterized by initial podocyte injury and albuminuria and subsequent renal inflammation, was determined (7,33,34). Podocytes were treated with HG for various periods of time in different experiments. Several recent studies have indicated that with stimulation of different pathogenic factors in podocytes, rapid generation of reactive oxygen species would be induced, rapid abundant generation of ROS usually leading to series of injuries. Thus, ectopic generation of ROS can be used as an early prediction of cell injury. ROS generation here was detected after 6 h exposure to HG, and as shown in Fig. 1a, HG significantly induced generation of ROS. Since apoptosis is a typical physiological characteristic of early cell injury, HG-induced podocyte apoptosis was confirmed by annexin V/PI staining assay, which showed that the apoptosis was significant after 48 h exposure to HG (Fig. 1b and c). Viability of cells was examined using the MTT assay (Fig. 1d) and results showed that HG reduced cell viability ($P < 0.05$) compared to control cells.

Figure 2. Activation of the Wnt/β-catenin pathway induced by high glucose in podocytes. (a) Podocytes incubated with HG for 24 h. RT-PCR assay showing non-activation of Wnt1 and Wnt2 compared to control. Four lanes labelled 'HG' present four independent HG treatment experiments. (b) Real-time quantitative PCR assay showing rapid increase of Wnt5a mRNA expression at 48 h by HG. (c) β-catenin mRNA expression detected by real-time quantitative PCR assay. Data presented as mean $\pm$ SD of three (b and c) or four (a) independent experiments. * $P < 0.05$ compared to control.

Activation of Wnt/ β -catenin pathway induced by high glucose in podocytes

To ascertain activation status of Wnt signalling in pathogenesis of early injury diabetic nephropathy, activation of the canonical Wnt signalling pathway was detected by real-time quantitative PCR assay. mRNA expressions of Wnt1, Wnt2 and Wnt5a were measured in common with several previous studies, and confirmed activation of these Wnts (19,35). As shown in Fig. 2b, mRNA expression of Wnt5a was induced significantly by 48 h after HG treatment. However, mRNA expression of Wnt1 and Wnt2 were not evidently changed in the HG group compared to that of controls (Fig. 2a).

As β -catenin is the principal mediator of canonical Wnt signalling, we next examined its regulation in HG-induced injury of podocytes. As shown in Fig. 2c, quantitative PCR analyses revealed a dramatic increase in podocyte β -catenin mRNA by 48 h after HG treatment. These results suggest that the canonical Wnt pathway may play a crucial role in this cell model.

Activation of TRPC6 induced by high glucose in podocytes

To assess the potential role of TRPC6 in podocyte injury, expression of TRPC6 was examined at 24 h and 48 h. As shown in Fig. 3a, expression of TRPC6 protein was examined by western blotting. TRPC6 protein expression in podocytes started to increase at 24 h, and reached its peak by 48 h (Fig. 3b). TRPC6 levels increased to $112.00 \pm 0.30\%$ and $123.91 \pm 5.72\%$ ($P < 0.05$) at 24 and 48 h respectively, compared to those of control groups, indicating that TRPC6 upregulation may play a pivotal role here.

Inhibition of the Wnt signalling pathway down-regulated high glucose-induced TRPC6 upregulation

To provide mechanistic insight into podocyte injury in HG-induced early nephropathy, the hypothesis that TRPC6 protein was a downstream mediator of Wnt/ β -catenin signalling in injured podocytes, was investigated. Cells were treated with recombinant Dkk1 protein, a natural inhibitor of canonical Wnt signalling, by its ability to bind to and block the LRP5/6 co-receptor (16). Dosage used in our experiments was selected according to previous studies (19). As shown in Fig. 4a and b, quantitative PCR analyses confirmed that Wnt/ β -catenin signalling was indeed blocked, in which mRNA expression of Wnt5a and β -catenin were effectively down-regulated by Dkk1, with sustained high ambient glucose treatment. Results showed that expression of TRPC6

Figure 3. Activation of TRPC6 induced by high glucose in podocytes. Podocytes were maintained in high glucose (30 mM) for 24 and 48 h respectively. Podocyte lysate proteins (60 μ g) were separated by SDS-PAGE, immunoblotted using anti-TRPC6 and anti-actin antibodies. (a) Western blot assay of TRPC6. (b) Corresponding histogram of TRPC6 protein expression. Data representative of three independent experiments. * $P < 0.05$ compared to control.

protein (Fig. 4c and d) was inhibited by Dkk1 compared to that of the HG group. These results suggest that Wnt/ β -catenin signalling modulated downstream TRPC6 in podocytes, which was a key mechanism in HG-induced early nephropathy.

Blockade of the Wnt signalling pathway reduced ROS generation, alleviated apoptosis and heightened cell viability

Effects of blockade of Wnt signalling on podocytes were detected. Compared to the HG group, ROS generation was significantly reduced in the HG+D group (Fig. 5a). HG-induced podocyte apoptosis was effectively alleviated by Dkk1, which was confirmed by annexin V/PI staining assay. Cell viability was examined using the MTT assay (Fig. 5b) and results showed that blockade of Wnt signalling significantly heightened cell viability in the HG+D group compared to that of HG ($P < 0.05$, Fig. 5c). Together, it appeared that TRPC6 mediated high glucose-induced cell injury through Wnt/ β -catenin signalling in these mouse podocytes.

Figure 4. Inhibition of the Wnt/β-catenin pathway down-regulated high glucose-induced TRPC6 expression. Podocytes were incubated with HG or HG+Dkk1(200 ng/ml) for 24 and 48 h respectively. (a, b) mRNA levels of Wnt5a and β-catenin first analysed to confirm effective inhibition of Wnt/β-catenin pathway. (c) TRPC6 protein level examined by western blotting. (d) Corresponding histogram of TRPC6 protein expression in western blot assay. TRPC6 level decreased from $126.79 \pm 9.182\%$ in HG group to $105.08 \pm 4.59\%$ in HG+Dkk1(HG+D) group. Values are means $\pm$ SD of four independent experiments, * $P < 0.05$ versus control, ** $P < 0.01$ versus control.

Discussion

In this study, the results demonstrate that the relationship between the Wnt/β-catenin signalling pathway and TRPC6 in high glucose, induced podocyte injury. The results show that Wnt/β-catenin signalling mediated HG-induced cell injury through activation of TRPC6. Furthermore, blockade of the canonical Wnt signalling pathway by Dkk1 inhibited ectopic expression of TRPC6 and ameliorated a series of podocyte injuries induced by HG. Our studies suggest that the hyperactive Wnt/TRPC6 signal is detrimental in development of podocyte injury. Loss of podocytes may lead to

glomerular injury and failure of the glomerular filtration barrier, which in turn triggers a cascade of events that can finally lead to end-stage renal failure. HG treatment of podocytes has been widely used as a model, which mimics the pathogenic state of podocytes in human diabetic nephropathy (7,36,37). Thus, our findings underscore that the Wnt/TRPC6 signal may represent a promising target in developing new therapeutic modalities for treatment of a variety of proteinuric kidney diseases in humans.

Several recent studies have shown that activation of the canonical Wnt signalling pathway plays an important role in mediating podocyte dysfunction. One study has

Figure 5. Blockade of Wnt signalling reduced ROS generation, alleviated apoptosis and increased cell viability. (a) Exposure of podocytes to HG and HG+D resulted in generation of ROS. Pink: control. Green: HG. Black: HG+D. Curve of HG+D group clearly was moved to the left, indicating effective reduction of ROS generation. (b) Apoptosis was confirmed by annexin V/PI staining assay at 48 h. Corresponding histogram of apoptosis showed percentage of apoptotic cells reduced from $17.31 \pm 2.53\%$ in HG group to $10.46 \pm 0.07\%$ in HG+Dkk1 (HG+D) group. (c) Cell viability was examined by MTT assay at 48 h. Results showed that blockade of Wnt signalling significantly elevated cell viability in HG+D group compared to that of HG group. Man: D-mannitol. Data are presented as mean $\pm$ SD of three independent experiments. $*P < 0.05$ compared to control.

shown that incubation of podocytes with transforming growth factor- β 1 (TGF- β 1) activated Wnt/ β -catenin signalling and stimulated expression of Wnt/ β -catenin downstream target genes, subsequently leading to podocyte injury and albuminuria (38). Two further studies from Dai *et al.* and He *et al.* have also demonstrated that blocking Wnt/ β -catenin signalling can ameliorate podocyte injury, proteinuria and renal fibrosis in adriamycin (ADR) nephropathy (19,20). It should be noted that whether or not this scenario extends to high glucose-induced early nephropathy, a model characterized by initial podocyte injury and albuminuria and subsequent renal inflammation, remains to be demonstrated. Here, we confirm that Wnt/ β -catenin signalling was effectively activated in this *in vitro* cell model. β -catenin, the common downstream mediator of Wnt/ β -catenin signalling, was activated (Fig. 2d), indicating robust activation of the canonical pathway of Wnt signalling in

this model. Nonetheless, it is important to note that of Wnts analysed in our experiment, only Wnt5a was significantly induced (Fig. 2c), which is inconsistent with previous studies (19). It is conceivable that various members of the Wnt family of proteins are selectively expressed in different circumstances. Therefore, it is not surprising that expression of Wnt1 and Wnt2 was not significantly induced.

It is important to emphasize that Wnt/ β -catenin signalling is not limited to any simple effect, but appears to have some completely opposite effects in a variety of different cell types and systems. A recent study by Lin *et al.* has reported that Wnt/ β -catenin signalling is required for protecting glomerular mesangial cells from high glucose-mediated apoptosis (39). Hence, data presented here may provide a new perspective in which to explore the function of Wnt/ β -catenin signalling in connection to different cell phenotypes of the nephron.

The pivotal role of TRPC6 in glomerular disease makes it a potential target for therapy after initial injury of diabetic nephropathy. Nevertheless, analysis of the mechanism, especially crosstalk between TRPC6 protein and the Wnt/ β -catenin signalling pathway appears to be clear, in high glucose-induced nephropathy. Several lines of evidence support this hypothesis. A number of studies have indicated that activated Wnt/ β -catenin signalling inhibited expression of nephrin protein in the injured podocytes (19,40). In addition, a further study has shown that TRPC6 co-localized and directly interacted with nephrin, and TRPC6 was up-regulated in glomeruli of 2 day old nephrin-deficient mice (26). Concordantly, it has also been revealed that nephrin inhibited activity and membrane expression of TRPC6 under normal conditions, but conversely did not inhibit surface expression of any disease-causing TRPC6 mutant – in striking contrast to wild-type TRPC6 (41). Thus, our data provide direct relationships between the Wnt/ β -catenin signal and TRPC6 (Figs 3b and 4c); blockade of Wnt/ β -catenin signalling effectively mitigated podocyte injury (Fig 5a–c). Altogether, our results present a mechanistic linkage between Wnt/ β -catenin activation, TRPC6 induction and podocyte injury, and can offer important insights into pathogenesis of many common forms of proteinuric kidney disease.

We need to point out that there is significant crosstalk between signals controlling podocyte dysfunction, inflammation and fibrosis, and we cannot exclude the possibility that other signals may participate in transduction between Wnt/ β -catenin signalling and TRPC6; further study is needed to investigate any clear signalling network between these. In that regard, several studies have indicated a potential role that nephrin may play in this Wnt/TRPC6 signalling (19,26,41). In addition, other correlative cells of the nephron, such as capillary endothelial cells and parietal epithelial cells, also play crucial roles in development of diabetic nephropathy. Further study of the Wnt/TRPC6 signal in these cell types will strengthen our understanding of the Wnt/TRPC6 relationships.

In summary, our results have provided evidence that the canonical Wnt signalling pathway modulated TRPC6 protein expression and induced a series of types of podocyte injury (Fig. 6a); high glucose-activated Wnt provoked expression of β -catenin and up-regulated TRPC6, leading to this (Fig. 6b). Findings in this study highlight the importance of TRPC6 in regulating podocyte fate and the inhibitory action of Wnt/ β -catenin signalling on TRPC6, which provides evidence of a new mechanism of podocyte injury. Thus, the Wnt/TRPC6 signal in podocytes might represent a new target for maintaining glomerular integrity. Moreover, these results

Figure 6. Proposed mechanism of Wnt/ β -catenin pathway modulating TRPC6 protein expression and induction of a series of types of cell injury. (a) Wnt/ β -catenin pathway and TRPC6 are critical to podocyte survival. (b) High glucose activated Wnt and provoked expression of β -catenin, up-regulating TRPC6 and leading to podocyte injury. Fz: Frizzled protein. LRP: low-density lipoprotein receptor-related protein. β -cat: β -catenin.

may provide a promising therapeutic modality for treatment of diabetic nephropathy in the future.

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