Original Paper

The mTORC2/Akt/NFκB Pathway-Mediated Activation of TRPC6 Participates in Adriamycin-Induced Podocyte Apoptosis

Hai-Tao Zhang^a Wei-Wei Wang^b Li-Hong Ren^a Xia-Xia Zhao^a Zhi-Hui Wang^a
De-Li Zhuang^a Yun-Nuo Bai^a

^aDepartment of Pediatrics, ^bDepartment of anesthesiology, Harbin Medical University Second Hospital, Harbin, Heilongjiang, PR China

Key Words

Podocyte apoptosis • Adriamycin (ADR) • TRPC6 • mTOR • Akt • NFκB

Abstract

Background/Aims: Although increased expression and gain function of transient receptor potential cation channel 6 (TRPC6) has been associated with the pathogenesis of some proteinuric glomerular diseases, it remains elusive how TRPC6 participates in the process of podocyte damage. **Methods:** The potential signaling responsible for TRPC6 activation was investigated using immunoblot assays in an *in vitro* podocyte injury model induced by Adriamycin (ADR). Podocyte apoptosis was measured using FITC-conjugated Annexin V and Propidium Iodide staining. The channel activity of TRPC6 was assessed using the Ca²⁺ influx assay. **Results:** Increase of TRPC6 expression was detected in ADR-treated podocytes, and TRPC6 knockdown significantly decreased ADR-induced podocytes apoptosis. Following ADR treatment, phospho-mTOR^{Ser2481} and phospho-Akt^{Ser473} was significantly increased in a time-dependent manner, whereas phospho-mTOR^{Ser2448} and phospho-p70S6K^{Thr389} showed no change. ADR-induced apoptosis was prevented by ku0063794 (a dual mTOR complexes inhibitor), not by rapamycin (a specific mTORC1 inhibitor). Furthermore, nuclear translocation of NFκB/p65 was detected in ADR-treated podocytes, which was prevented by an Akt inhibitor triciribine. Of note, NFκB inhibitor PDTC prevented ADR-induced increase of TRPC6, and decreased ADR-induced apoptosis. We found that Akt activation and NFκB nuclear translocation was significantly inhibited by knockdown of mTORC2 protein Rictor, not by mTORC1 protein Raptor. In comparison with control, the Ca²⁺ influx was significantly increased in ADR-treated podocytes, which was remarkably prevented by TRPC6 knockdown. ADR-induced increase of TRPC6 channel activity was dramatically prevented by ku0063794, but not by rapamycin. Additionally, knockdown of Rictor, not Raptor, prevented ADR-induced increase of the Ca²⁺ influx. Moreover, the application of NFκB inhibitor PDTC also prevented the Ca²⁺ influx in ADR-treated podocytes. **Conclusions:** Our findings revealed that the mTORC2/Akt/NFκB pathway-mediated activation of TRPC6 participates in ADR-induced podocyte apoptosis.

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Introduction

Proteinuria is one of the most important clinical manifestations in children nephrotic syndrome. Glomerular visceral epithelial cells, also termed as podocytes, are the terminally differentiated and highly specialized cells with a large cell body extending primary and secondary foot processes (FPs) that are linked by the slit diaphragm (SD) necessary for maintaining normal glomerular filtration function [1]. The abnormalities of podocytes, particularly SD, play a key role in the development of proteinuria. Increasing evidences demonstrated that podocyte loss due to apoptosis/death or detachment from glomerular basement membrane is involved in progressive glomerulosclerosis [2]. Additionally, damage to the glomerular filter, in particular to podocytes and SD, is of crucial importance in the pathogenesis of proteinuric diseases. Transient receptor potential cation channel 6 (TRPC6), a non-selective cation channel, has been identified as a novel podocyte SD protein [3, 4]. Mutations of TRPC6 cause increase of its channel activity and podocyte damage, leading to autosomal dominant focal segmental glomerulosclerosis (FSGS) [3]. TRPC6, ubiquitously expressed in many types of cells, can affect intracellular Ca^{2+} level through regulating influx of Ca^{2+} [4, 5], while excessive intracellular Ca^{2+} could lead to cells apoptosis [4, 6, 7]. Interestingly, increased expression of TRPC6 was found in many acquired human proteinuric glomerular diseases, including non-familial FSGS, minimal change disease and membranous nephropathy [8, 9]. Notably, the expression and channel function of TRPC6 is increased in some *in vitro* podocyte damaged model. Angiotensin II (AngII) results in podocyte apoptosis by increasing TRPC6 expression and intracellular Ca^{2+} level, which is mediated by ERK activation-induced NF κ B nuclear translocation [10, 11]. Abundance of TRPC6 is also increased through NADPH oxidase-mediated ROS signaling pathway in response to puromycin aminonucleoside-induced podocyte injury [11, 12]. In high glucose-induced podocyte damage, activation of TRPC6 is mediated by the Wnt/ β -catenin signaling pathway [13]. Nevertheless, it remains elusive how TRPC6 participates in the pathogenesis of glomerular podocyte injury.

Recently, it was reported that the inhibition of mammalian target of rapamycin (mTOR), can regulate TRPC6 expression in cultured normal podocytes [14, 15]. The serine/threonine kinase mTOR forms two distinct functional complexes, mTORC1 and mTORC2 [16]. The mTORC1 complex including at least three additional components, Raptor, mLST8, and FKBP12, is rapamycin-sensitive since the interaction of the rapamycin and FKBP12 interferes with the binding of Raptor to mTOR [17]. Nevertheless, the mTORC2 consists of mLST8, Sin1, and Rictor, and is rapamycin-insensitive since the Rictor-mTOR complex is not able to bind the FKBP12-rapamycin conjugate [17]. Recent studies revealed that physiologic levels of mTORC1 and mTORC2 activity play key roles in maintaining podocyte and glomerular functions. Aberrant activation of mTORC1 or loss of mTORC2 activity in podocytes may be involved in the pathogenesis of glomerular diseases [18]. In this study, we focused on the distinct role of mTOR signaling, and investigated how TRPC6 contributes to Adriamycin (ADR)-induced podocyte damage. We provide evidence that the functional activation of TRPC6 is mediated by the mTORC2/Akt/NF κ B pathway, which may participate in ADR-induced podocyte apoptosis.

Materials and Methods

Antibodies

The following antibodies were used: rabbit anti-TRPC6 (ab12249), mouse anti-beta actin (ab6276), mouse anti-mTOR (ab87540), and rabbit anti-phospho-mTOR^{Ser2448} (ab109268) or phospho-mTOR^{Ser2481} (ab137133, Abcam), mouse anti-phospho-p70S6K^{Thr389} (#9206), rabbit anti-p70S6K (#9202), rabbit anti-phospho-Akt^{Ser473} (#4060), and mouse anti-Akt antibody (#2920, Cell Signaling), mouse anti-NF κ B/p65 (sc-8008), rabbit anti-Histone H3 (sc-10809), rabbit anti-caspase3 antibody (sc-7148), rabbit anti-cleaved caspase3 antibody (sc-22171), and rabbit anti-I κ B α (sc-371, Santa Cruz), HRP-conjugated goat anti-rabbit or mouse IgG (32230, 32260; Thermo Scientific).

Cell culture and treatment

Mouse podocyte clone was a kind gift from Prof. Peter Mundel. Podocyte was cultured at 33°C in RPMI 1640 media (Catalog number: 61870-036; Invitrogen) supplemented with 10% fetal calf serum (Catalog number: 10438-026; Gibco), 100 U/ml of Penicillin/Streptomycin (Catalog number: 15140-122; Gibco), and 10 U/ml of recombinant mouse γ -interferon (Catalog number: 11276905001; Sigma Aldrich). To induce apoptosis, podocyte was treated with 0.5 μ g/ml of Adriamycin (ADR; D1515, Sigma Aldrich) for the indicated time periods. In the experiments using the inhibitor rapamycin (10 nM; Catalog number: 553210; Sigma Aldrich), ku0063794 (1 μ M; S1226, Selleckchem), pyrrolidinedithiocarbamate (PDTC, 4 μ M; P8765, Sigma Aldrich), or triciniribine (20 μ M; Catalog number: 124038; Sigma Aldrich), podocyte was pre-treated with the above inhibitor for 30 min. ADR was then added in the presence of inhibitors (Table 1).

Table 1. Inhibitors that were used in this study

Inhibitor	Pathway	Final concentration
Rapamycin	mTORC1	10 nM
ku0063794	mTORC1 and mTORC2	1 μ M
PDTC	NFkB	4 μ M
Triciniribine	Akt	20 μ M

Knockdown assay

Specific validated siRNA targeting mouse TRPC6 (sc-42673), Raptor (sc-108002), or Rictor (sc-61479) and non-targeted control siRNA (sc-37007) was used for knockdown assay (Santa Cruz). To increase transfection efficiency, proliferative podocytes were used in this study. Podocyte was cultured at 33°C in 6-well plate, and each well 100 pmol of siRNA duplexes was introduced using 10 μ l of RNAiMAX Reagent according to manufacturer's instruction (Catalog number: 13778-075; Invitrogen). After 48 hours of transfection, cells were collected for Immunoblot assay and Calcium Flux studies.

Apoptosis detection assay

Apoptotic cells were labeled by using FITC-conjugated Annexin V and Propidium Iodide (Catalog number: 556547; BD Biosciences) in live podocytes. Briefly, 1×10^5 cells were washed twice with cold phosphate-buffered saline by centrifugation at 1,000 rpm for 5 mins. Cell pellets were re-suspended in 1 μ g/ml of FITC-Annexin V for 30 min on ice, and then 5 μ l of Propidium Iodide (50 μ g/ml) was added immediately prior to detection with flow cytometry (FACScan).

Real time PCR

Total RNA was extracted with Trizol (Catalog number: 15596-018; Invitrogen), and 1.5 μ g of RNA was used for reverse transcription to make complementary DNA (cDNA) by using the First-strand cDNA synthesis kit (Catalog number: 12328-032; Invitrogen). Real time PCR for TRPC6 expression was performed in 25 μ l reaction volume containing 2 μ l of cDNA, 1 μ l of 5 μ M specific PCR primers (forward: 5'-tggtgcggaagatgctagaa-3'; reverse: 5'-aaagcatccccaactcgaga-3'), 12.5 μ l of 2x SYBR Green Master Mix (Catalog number: 4309155; Applied Biosystems). The level of TRPC6 was normalized to the housekeeping gene β -actin (forward: 5'-agccatgtacgtagccatcc-3'; reverse: 5'-gctgtggtggtgaagctga-3'), and the fold change was compared.

Immunoblot assay

The RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), and 10% glycerol] was used to extract total cellular protein. The protease and phosphatase cocktail inhibitors (Catalog number: 05892970001, 4906845001; Roche) were added immediately prior to lysing cells. Nuclear Protein Extraction Kit (ab113474, Abcam) was used for evaluation of NFkB translocation. The 100 μ g of protein was electrophoresed on 7.5 or 12.5% SDS-PAGE. Protein was then semi-transferred to nitrocellulose membranes (GE Healthcare Bioscience). The membranes were blocked for 1 hour in 0.05% Tween-20 Tris-buffered saline solution (TTBS) containing 2% BSA/3% non-fat milk. Membranes were incubated overnight at 4 °C with the indicated primary antibodies. After 5 washes with TTBS, HRP conjugated secondary antibody was applied for 1 hour. After washes with TTBS, blots were developed with an ECL chemiluminescence detection kit (Catalog number: 32109; Pierce). The specific band was scanned and quantified with Image J.

Calcium Flux studies

The fluorescence intensity of free Ca^{2+} indicator Fluo-3AM (F1241, Invitrogen) was used to measure the level of intracellular free Ca^{2+} . Podocytes were seeded in 96-well plate, and incubated with 5 μM of Fluo-3AM for 30 mins at 37°C. After 3 times washes with PBS, the baseline of intracellular free Ca^{2+} level was measured using FLX800 spectrophotofluorometer (BioTek) equipped with a filter of 480 nm excitation and 510 nm emission. Of note, two wells with PBS alone were used as blank control. Cells were then stimulated by addition of 10 μM of hyperforin (H1792, Sigma Aldrich) to initiate activation of TRPC6, and the intracellular free Ca^{2+} level was recorded for 2 minutes at a 15-second interval. The intracellular free Ca^{2+} level from three independent experiments was averaged, and the relative fluorescence intensity to the baseline was compared.

Statistical analysis

Data are present as mean $\pm$ SD. Statistical analysis was performed with Prism 6 (GraphPad Software, Inc.) by using One-Way ANOVA for comparing multiple time-points or Two-Way ANOVA for comparing multiple groups. A p value equal to or less than 0.05 was considered as significant difference.

Results

Induction of TRPC6 is involved in ADR-mediated podocyte apoptosis

As reported previously [19], we applied 0.5 $\mu\text{g}/\text{ml}$ of ADR to cultured mouse podocyte cell line. Apoptotic cells were labeled with FITC-Annexin V and propidium iodide, and the percentage of apoptotic cells was then assessed using flow cytometry. As compared with control, podocyte apoptosis was significantly induced at 24 (14.58 ± 2.86 vs 2.98 ± 1.02 ; $p < 0.001$) and 48 (23.03 ± 2.57 vs 2.98 ± 1.02 ; $p < 0.001$) hours following ADR application (Fig. 1A). TRPC6, a receptor-activated non-selective calcium permeant cation channel, plays an important role in the process of podocyte injury through increasing the entry of Ca^{2+} to cells [6]. Results from real time RT-PCR show that ADR significantly ($p < 0.05$) increased the mRNA level of TRPC6 at 24 and 48 hours (Fig. 1B). Consistently, induction of TRPC6 at protein level was also detected ($p < 0.01$) at 24 and 48 hours in ADR-treated podocytes (Fig. 1C). To verify the role of TRPC6 in ADR-treated podocyte, TRPC6 expression was successfully downregulated using the specific TRPC6 siRNA (Fig. 1D,E). The effects of TRPC6 knockdown on cellular apoptosis were then assessed in both wild type and ADR-treated podocytes. TRPC6 knockdown showed no significant effect on apoptosis in wild type podocytes (Fig. 1F). Nevertheless, the percentage of apoptotic cells was decreased significantly (8.83 ± 1.12 vs 12.38 ± 1.49 ; $p < 0.01$) by TRPC6 knockdown in ADR-treated podocytes although the apoptosis in ADR-treated podocytes with TRPC6 knockdown was still higher (8.83 ± 1.12 vs 2.98 ± 1.02 ; $p < 0.01$) than that in wild-type cells. Of note, control siRNA showed no effects (12.55 ± 1.06 vs 12.38 ± 1.49 ; $p > 0.05$) on ADR-induced apoptosis (Fig. 1F). Caspase-3 is a critical executioner of apoptosis in many types of cells. We assessed the activated caspase3 level using immunoblot assay by detecting the cleaved caspase3 in ADR-treated podocytes. Our data showed that the cleaved caspase3 abundance increased significantly ($p < 0.05$) at 24 hours in ADR-treated podocytes, which was prohibited by TRPC6 knockdown (Fig. 1G).

The mTORC2 activation is related to ADR-induced podocyte apoptosis

Due to distinct components and substrates, mTOR complexes are categorized into two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 activation predominantly at Ser2448 leads to the phosphorylation of its downstream target p70S6K, while the mTORC2 activation predominantly at Ser2481 leads to the phosphorylation of Akt at Ser473 [14, 17]. In ADR-treated podocytes, the phosphorylation of mTOR at Ser2481 increased significantly in a time-dependent manner, whereas the phosphorylation of mTOR at Ser2448 showed no change (Fig. 2A), indicating that the mTORC2 complex is activated following ADR treatment in cultured podocytes. In consistent, we detected a time-dependent increase of phospho-Akt^{Ser473}, but not phospho-p70S6K^{Thr389} in ADR-treated podocytes (Fig.

Fig. 1. TRPC6 upregulation is related to ADR-induced podocyte apoptosis. A – C: Podocytes were treated with Adriamycin (0.5 µg/ml) for the indicated time periods. Cellular apoptosis was assessed using FITC-Annexin V and propidium iodide staining by flow cytometry (A). The abundance of TRPC6 was evaluated using real time RT-PCR (B) and Western blot (C) in ADR-treated podocytes. D – G: The siRNA targeting to mouse TRPC6 (siTRPC6) was introduced using RNAiMAX to knockdown TRPC6 expression. The control siRNA (siCTL) has no any significant sequence similarity to mouse gene sequences. 24 hours after transfection, cells were treated with Adriamycin (ADR, 0.5 µg/ml) or cultured media alone as control (CTL) for another 24 hours. The level of TRPC6 was assessed using real time RT-PCR (D) and Western blot (E), respectively. Effect of TRPC6 knockdown on cellular apoptosis was examined (F). The cleaved caspase3 level was assessed using immunoblot assay (G). A, F: n = 4 independent experiments and triplicates were used for each experiment. B – E and G: triplicates were used, and the representative blots were provided. n.s.: no significant difference.

2B). To assess the distinct role of the two different mTOR complexes in ADR-induced podocyte apoptosis, the cultured podocytes were exposed to rapamycin, an inhibitor of mTORC1, and ku0063794, a dual inhibitor of both mTORC1 and mTORC2. As the data showed, rapamycin application dramatically inhibited the phosphorylation of mTOR at Ser2448 and p70S6K at Thr389, while showed no effects on the phosphorylated mTOR at Ser2481 and Akt at Ser473 (Fig. 2C). The level of phospho-mTOR at Ser2448 and phospho-p70S6K at Thr 389 was remarkably decreased by ku0063794. Of note, ADR-induced upregulation of mTOR at Ser2481 and Akt activation at Ser473 was significantly prevented by ku0063794 (Fig. 2C). We also evaluated the effects of distinct mTOR complexes inhibition on ADR-induced podocyte injury. Firstly, our results showed that there was no significant difference of cellular apoptosis between wild-type podocytes and wild-type podocytes treated with rapamycin or ku0063794 alone (Fig. 2D). Compared to control, ADR significantly induced podocyte apoptosis (12.7 ± 1.51 vs 4.2 ± 0.71 ; $p < 0.001$) at 24 hours following treatment. In comparison to ADR alone, the administration of ku0063794 significantly decreased ADR-induced podocyte apoptosis (7.5 ± 1.41 vs 12.7 ± 1.51 ; $p < 0.001$), while rapamycin treatment displayed no significant effects (11.4 ± 1.02 vs 12.7 ± 1.51) (Fig. 2D).

NFκB activation is associated with TRPC6 induction and cellular apoptosis in ADR-treated podocyte

NFκB is a protein complex that controls transcription of DNA, cytokine production and cell survival [20, 21]. It has been reported that NFκB is involved in up-regulation of TRPC6 in angiotensin II-induced podocyte damage [10]. Non-activated NFκB binds to IκB proteins,

Fig. 2. The mTORC2 activation is involved in ADR-induced podocyte apoptosis. A, B. Podocytes were treated with Adriamycin (ADR, 0.5 μ g/ml) for the indicated time periods. Total cellular protein was extracted and immunoblot assay was performed to evaluate the activation level of mTOR complexes (A) as well as p70S6K and Akt (B), respectively. C, D. Podocytes were pre-treated with rapamycin (Rap; 10 nM) or ku006394 (Ku; 1 μ M) for 1 hour. ADR was then added (final concentration 0.5 μ g/ml) and incubated for 24 hours in the presence of the indicated inhibitors. Immunoblot assay was performed to examine the level of phospho-mTOR-Ser2448, phospho-mTOR-Ser2481, phospho-p70S6K, and phospho-Akt (C). Effect of rapamycin and ku006394 on podocyte apoptosis was assessed using FITC-Annexin V and propidium iodide staining by flow cytometry (D). A - C: three individual experiments were performed, and the representative blots were provided. D: n = 4 independent experiments, and triplicates were used for each group. n.s.: no significant difference.

while activation of the NF κ B is initiated by the signal-induced degradation of I κ B proteins. With the degradation of I κ B, the NF κ B complex is then released and thus enters the nucleus where it can turn on the expression of specific genes that have DNA-binding sites for NF κ B [20]. In this study, nuclear fraction was obtained from ADR-treated podocytes, evidenced by the absence of GAPDH using immunoblot assay (Fig. 3A). The abundance of NF κ B/p65 in the nuclear fractions was increased significantly in a time-dependent manner (Fig. 3A), whereas the cytosolic I κ B- α was decreased remarkably (Fig. 3B). To evaluate the role of nuclear

Fig. 3. NFκB activation is responsible for TRPC6 induction and cellular apoptosis in ADR treated podocytes. A, B. Podocytes were treated with Adriamycin (ADR, 0.5 μg/ml) for the indicated time periods. Nuclear protein and total cellular protein was extracted to examine the level of nuclear NFκB/p65 and cytosolic IκB-α, respectively. C - E. Podocytes were pre-treated with the NFκB inhibitor pyrrolidinedithiocarbamate (PDTC; 4 μM) for 30 minutes. ADR (final concentration 0.5 μg/ml) was then added and incubated for 24 hours in the presence of PDTC. Immunoblot assay was performed to assess the level of nuclear NFκB (C). The abundance of TRPC6 was examined using real time RT-PCR (D) and Western blot assay (E), respectively. Effect of PDTC on cellular apoptosis in wild-type (CTL) and ADR-treated podocyte was assessed using FITC-Annexin V and propidium iodide staining by flow cytometry (F). A - E: three individual experiments were performed, and the representative blots were provided. F: n = 3 independent experiments and triplicates were used for each experiment.

translocation of NFκB in ADR-induced podocyte apoptosis, we applied the NFκB inhibitor PDTC to ADR-treated podocytes. At 24 hours after ADR treatment, the nuclear NFκB/p65 was significantly inhibited by the application of PDTC (Fig. 3C). We further investigated the effects of NFκB inhibition on TRPC6 expression in ADR-treated podocytes. Real time RT-PCR and Western blot revealed that ADR-induced up-regulation of TRPC6 both at mRNA and protein level was dramatically prevented by PDTC (Fig. 3D,E). Of note, the administration of PDTC significantly decreased (7.6 ± 0.81 vs 12.7 ± 0.77 ; $p < 0.01$) cellular apoptosis induced by ADR at 24 hours although the percentage of cellular apoptosis was still higher (7.6 ± 0.81 vs 3.0 ± 1.0 ; $p < 0.001$) in podocytes treated with ADR and PDTC than control (Fig. 3E). In addition, PDTC showed no significant effect (3.2 ± 0.83 vs 3.0 ± 1.0 ; $p > 0.05$) on apoptosis in wild type podocytes (Fig. 3E). These findings imply that NFκB is activated in ADR-treated podocytes, necessary for the up-regulation of TRPC6 expression and induction of cellular apoptosis.

The mTORC2-dependent Akt activation is associated with NFκB activation in ADR-treated podocytes

In prostate cancer cells, Akt-dependent regulation of NFκB is controlled by mTOR [22]. Therefore, we applied the Akt inhibitor triciribine (20 μM) to ADR-treated podocytes. Immunoblot assay showed that ADR-induced up-regulation of phospho-Akt^{Ser473} was significantly prevented by triciribine, and that the nuclear NFκB/p65 level was also decreased in both ADR and triciribine-treated podocytes (Fig. 4A), suggesting that Akt activation may be related to the nuclear translocation of NFκB in ADR-treated podocytes. We also investigated the effects of Akt inhibition on cellular apoptosis induced by ADR in cultured podocytes. The application of TCN alone showed no significant (3.4 ± 0.94 vs 3.0 ± 1.0 ; $p > 0.05$) effects on apoptosis in wild-type podocytes, while significantly decreased ADR-induced podocyte apoptosis (9.1 ± 0.78 vs 13.9 ± 0.95 ; $p < 0.01$) (Fig. 4B). Moreover,

Fig. 4. The mTORC2-dependent Akt activation is associated with NFκB translocation in ADR treated podocytes. A, B. Podocytes were pre-treated with the Akt inhibitor triciribine (TCN; 20 μM) for 30 minutes. ADR was then added (final concentration 0.5 μg/ml) and incubated for 24 hours in the presence of TCN. Immunoblot assay was performed to assess the level of phospho-Akt^{Ser473} and nuclear NFκB/p65 (A). Effect of TCN on cellular apoptosis in wild-type (CTL) and ADR-treated podocytes was assessed using FITC-Annexin V and propidium iodide staining by flow cytometry (B). C - E. To specifically block the mTOR pathway, knockdown of Raptor and Rictor was obtained using siRaptor (siRAP) and siRictor (siRIC), respectively. The control siRNA (siCTL) has no any significant sequence similarity to mouse gene sequences. Expression of mTOR activation was evaluated using anti-phospho-mTOR^{Ser2448} and anti-phospho-mTOR^{Ser2481} (C). Effect of Raptor and Rictor knockdown on the activation of Akt and nuclear translocation of NFκB was examined using Western blot assay (D). 24 hours after siRNA transfection, cells were treated with Adriamycin (ADR, 0.5 μg/ml) or cultured media alone (CTL) for another 24 hours (E). A, C, D: three individual experiments were performed, and the representative blots were provided. B, E: n = 3 independent experiments and triplicates were used for each experiment. n.s.: no significant difference.

we specifically knocked down the expression of the distinct marker of mTOR complexes. Our results revealed that knockdown of Rictor and Raptor specifically down-regulated the level of phospho-mTOR^{Ser2481} and phospho-mTOR^{Ser2448}, respectively (Fig. 4C). Furthermore, effect of Raptor and Rictor knockdown on the activation of Akt and nuclear translocation of NFκB/p65 was examined in ADR-treated podocytes. Notably, we found that Rictor knockdown, but not Raptor knockdown, significantly prevented the increase of phospho-Akt^{Ser473} and nuclear NFκB/p65 induced by ADR treatment (Fig. 4D). Knockdown of Rictor, not Raptor, significantly decreased (8.7 ± 1.2 vs 14.3 ± 0.71 ; $p < 0.01$) ADR-induced podocytes apoptosis (Fig. 4E), while Rictor and Raptor knockdown alone showed no significant effect (Rictor: 3.9 ± 0.65 , Raptor: 3.7 ± 0.63 vs control: 3.4 ± 0.65 ; $p > 0.05$) on cellular apoptosis in wild type podocytes (Fig. 4E).

The increased Ca²⁺ influx is mediated by TRPC6 in ADR-treated podocytes

Up-regulation of TRPC6 was detected in ADR-induced podocyte apoptosis. TRPC6 is a transient receptor potential ion channel. Activation of TRPC6 can induce the entry of Ca²⁺ into the cell, while excessive entry of Ca²⁺ into a cell may induce damage or even cause it to

Fig. 5. The increased influx of the cytosolic free calcium is mediated by TRPC6 in ADR-treated podocytes. A, B: Knockdown of TRPC6 (TRPC6 KD) was obtained by introducing the siRNA specifically targeting to mouse TRPC6 in wild-type podocytes (WT). C, D: 24 hours after introduction of TRPC6 siRNA, ADR was added (final concentration 0.5 μ g/ml) and incubated for another 24 hours. The control siRNA (CTL KD) has no any significant sequence similarity to mouse gene sequences. The baseline of the intracellular free Ca^{2+} level was measured with the indicator Fluo-3AM as described in Methods. Activation of TRPC6 was then initiated by addition of 10 μ M of hyperforin, and the intracellular free Ca^{2+} level was recorded for 2 minutes at a 15-second interval. A, C: *, #, Δ : $p < 0.05$ vs 0 min in the same group. B, D: At 30 seconds following addition of hyperforin, the intracellular free Ca^{2+} level was compared with 0 min. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. n.s.: no significant difference. n = 3 independent experiments, and triplicates were used for each experiment.

undergo apoptosis, or death [6, 8]. Here, we examined the functional alteration of TRPC6 by evaluating the cytosolic Ca^{2+} level with the indicator Fluo-3AM. Firstly, we investigated the effects of TRPC6 knockdown on the cytosolic Ca^{2+} level in cultured podocytes. To examine if the Ca^{2+} influx is TRPC6-dependent, the specific TRPC6 agonist hyperforin was applied [10]. Following addition of hyperforin (10 μ M), we recorded the dynamic change of cytosolic Ca^{2+} level for 2 minutes at a 15-second interval. Our data showed that the basal Ca^{2+} level was significantly ($p < 0.05$) lower in TRPC6 knockdown cells than that in wild-type and control siRNA-transfected podocytes (Fig. 5A,B). After stimulation with hyperforin, the Ca^{2+} influx was increased significantly ($p < 0.05$) in wild-type and control siRNA-transfected podocytes, peaking at 30 seconds and persisting to 90 seconds. Increased Ca^{2+} influx was detected in TRPC6 knockdown cell whereas the cytosolic Ca^{2+} level was significantly lower ($p < 0.05$) in TRPC6 knockdown cell than that in wild-type and control siRNA-transfected podocytes, particularly at 30 seconds and only persisting to 75 seconds (Fig. 5A,B). The change of basal intracellular Ca^{2+} level was further investigated in ADR-treated podocytes with or without TRPC6 knockdown. The cytosolic Ca^{2+} level was higher significantly ($p < 0.05$) in podocytes treated by ADR (0.5 μ g/ml) for 24 hours than that in untreated wild-type podocyte. ADR-induced increase of cytosolic Ca^{2+} level was prevented significantly ($p < 0.05$) by knockdown of TRPC6, but not by control siRNA (Fig. 5C,D). This result suggests that the functional change of TRPC6 may be involved in ADR-induced podocyte damage. In wild-type podocytes, the Ca^{2+} influx was increased significantly ($p < 0.05$) since 30 seconds, and then decreased following stimulation with hyperforin (Fig. 5C). In comparison with un-treated wild-type cells, hyperforin increased the Ca^{2+} influx significantly ($p < 0.05$) since 15 seconds with a peak of value at 30 seconds in ADR-treated podocytes. Of note, the increased Ca^{2+} influx

Fig. 6. Inhibition of the mTORC2/Akt/NFκB pathway decreases influx of calcium in ADR-treated podocytes. A - D: Podocytes were pre-treated with rapamycin (Rap; 10 nM) or ku0063794 (Ku; 1 μM) for 1 hour. Cells were cultured for 24 hours in the media containing the indicated inhibitors alone (A, B) or both inhibitors and 0.5 μg/ml of ADR (C, D). E - H: Knockdown of Raptor (siRaptor) and Rictor (siRictor) was obtained in podocytes by introducing the validated siRNA specifically targeting to mouse Raptor and Rictor (E, F). 24 hours after introduction of siRNA, ADR was added (final concentration 0.5 μg/ml) and incubated for another 24 hours (G, H). The control siRNA (CTL KD) has no any significant sequence similarity to mouse gene sequences. I - L: Podocytes were pre-treated with the NFκB inhibitor pyrrolidinedithiocarbamate (PDTC; 4 μM) for 30 minutes. Cells were cultured for 24 hours in the media containing PDTC alone (I, J) or both PDTC and 0.5 μg/ml of ADR (K, L). The baseline of the intracellular free Ca^{2+} level was measured with the indicator Fluo-3AM as described in Methods. Activation of TRPC6 was then initiated by addition of 10 μM of hyperforin, and the intracellular free Ca^{2+} level was recorded for 2 minutes at a 15-second interval. Line graph: *, #, Δ, ▼: $p < 0.05$ vs 0 min in the same group. Bar graph: At 30 seconds following addition of hyperforin, the intracellular free Ca^{2+} level was compared with 0 min. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. n.s.: no significant difference. $n = 3$ independent experiments, and triplicates were used for each experiment.

initiated by hyperforin in ADR-treated podocytes was decreased significantly ($p < 0.05$) by TRPC6 knockdown at all time points, especially at 30 seconds, while control siRNA showed no obvious effects (Fig. 5C, D). These data imply that induction of TRPC6 may be related to the increase of cytosolic Ca^{2+} influx in ADR-treated podocytes.

Inhibition of the mTORC2/Akt/NFκB pathway decreases the Ca^{2+} influx in ADR-treated podocytes

In this study, we also assessed the role of the mTOR/Akt/NFκB pathway on regulation of the Ca^{2+} influx by using distinguished pathway inhibitors in ADR-treated podocytes. Firstly, we investigated the effects of mTOR inhibition on the cytosolic Ca^{2+} level in cultured podocytes. Ku0063794 (1 μM), the dual inhibitor of both mTORC1 and mTORC2, and rapamycin (10 nM), the specific mTORC1 inhibitor, were used in this study. We found that the basal Ca^{2+} level showed no significant difference ($p > 0.05$) between control and ku0063794- or rapamycin-treated podocytes (Fig. 6A,B). After stimulation with hyperforin, the Ca^{2+} influx

Fig. 7. The schematic of the potential signaling pathways related to ADR-induced podocyte damage. Increased TRPC6 expression and channel activity (Ca^{2+} influx) contributes to ADR-induced podocyte apoptosis, which is prevented by the application of ku0063794, tricinibine, PDTC, siRictor, and siTRPC6, respectively. Hence, the activation of mTORC2/Akt/NF κ B signaling pathway is associated with TRPC6 induction and apoptosis in ADR-treated podocytes. ADR: Adriamycin; Rap: rapamycin, the mTORC1 inhibitor; Ku: ku0063794, the dual mTORC1 and mTORC2 inhibitor; TCN: tricinibine, an Akt inhibitor; PDTC: a NF κ B inhibitor.

was increased significantly ($p < 0.05$) in both control and rapamycin or ku0063794-treated podocytes. Nevertheless, the increased Ca^{2+} influx was significantly lower ($p < 0.05$) in ku0063794-treated cells than that in control and rapamycin-treated podocytes, particularly at the peaking time 30 seconds (Fig. 6A,B). In podocytes treated with ADR (0.5 $\mu\text{g}/\text{ml}$) for 24 hours, the influx of Ca^{2+} was increased significantly ($p < 0.05$) following stimulation with hyperforin (10 μM), which was prevented significantly ($p < 0.05$) by ku0063794 (Fig. 6C,D). However, treatment with rapamycin (10 nM) did not change the hyperforin-induced Ca^{2+} influx in ADR-treated podocytes (Fig. 6C,D).

Similarly, we found that the basal Ca^{2+} level showed no significant difference ($p > 0.05$) between control and Raptor- or Rictor- knockdown podocytes (Fig. 6E,F). After stimulation with hyperforin, the increased Ca^{2+} influx was significantly lower ($p < 0.05$) in Rictor siRNA-transfected cells than that in control and Raptor siRNA-transfected podocytes, particularly at the peaking time 30 seconds (Fig. 6E,F). Moreover, knockdown of Rictor, the marker of mTORC2 complex, inhibited the Ca^{2+} influx significantly ($p < 0.05$) in ADR-treated podocytes in comparison with control siRNA, while knockdown of Raptor, the marker of mTORC1 complex, showed no obvious influence on the Ca^{2+} influx in ADR-treated podocytes (Fig. 6G,H). Therefore, these findings suggest that the mTORC2 complex may be related to the increased influx of Ca^{2+} in ADR-induced podocyte injury.

Additionally, we examined the effects of the NF κ B inhibitor PDTC (4 μM) on the Ca^{2+} influx in ADR-treated podocytes. The basal Ca^{2+} level showed no significant difference ($p > 0.05$) between control and PDTC-treated podocytes (Fig. 6I,J). After stimulation with hyperforin, the increased Ca^{2+} influx was slightly but significantly lower ($p < 0.05$) only at 30 seconds in PDTC-treated cells than that in control podocytes (Fig. 6I,J). Notably, we found that the application of PDTC significantly ($p < 0.05$) decreased the Ca^{2+} influx induced by ADR (Fig. 6K,L).

Discussion

Adriamycin (ADR), an anthracycline antibiotic, is a podocyte toxin used to induce experimental FSGS [23]. Time-dependent increase of podocyte apoptosis was detected in ADR-treated podocytes (Fig. 1A). Notably, ADR treatment significantly enhanced TRPC6 expression (Fig. 1B, C), which was also reported by other groups [19, 24]. Our data from knockdown assay showed that TRPC6 knockdown only marginally reduced ADR-induced podocyte apoptosis (Fig. 1D-F). ADR-induced increase of cleaved caspase3 was also partially inhibited by TRPC6 knockdown (Fig. 1G). This suggests that TRPC6 may contribute and there may be other unknown factors that may mediate ADR-induced apoptosis. Therefore, the role

of TRPC6 in injured podocytes needs be further investigated in other podocyte injury model. TRPC proteins, which belong to the larger TRP superfamily of channels, form Ca^{2+} -permeable channel that are important players in the pathogenesis of renal and cardiovascular diseases [6 - 9]. A gain-of-function mutation in TRPC6 is associated with the onset of hereditary FSGS [6 - 9]. TRPC6 has been functionally and pharmacologically shown to be involved in Ca^{2+} influx in podocytes [25, 26]. In this study, we applied hyperforin and evaluated the functional channel activity of TRPC6 in ADR-treated podocyte with or without TRPC6 knockdown. Hyperforin is a specific agonist of TRPC6, which elevates the intracellular Ca^{2+} level by activating TRPC6 channels without activating the other isoforms [14]. Firstly, our data showed that hyperforin application significantly resulted in Ca^{2+} influx in wild-type podocytes, while hyperforin-induced Ca^{2+} influx was decreased significantly in TRPC6 knockdown cells (Fig. 5A, B). This result displayed a baseline TRPC6 activity in podocytes as reported previously [3, 10, 11, 14]. We then found that ADR significantly increased both the basal intracellular Ca^{2+} level and hyperforin-induced Ca^{2+} influx in podocytes, which was inhibited by TRPC6 knockdown (Fig. 5C, D), suggesting that functional activation of TRPC6 is related to ADR-induced podocyte damage.

We further examined how TRPC6 is induced in ADR-treated podocytes. NF κ B, a protein complex that controls transcription of DNA, cytokine production and cell survival, is involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis [20]. NF κ B is a homo- or hetero-dimeric complex formed by the Rel-like domain-containing proteins RelA/p65, RelB, NF κ B1/p105, NF κ B1/p50, Rel and NF κ B2/p52 and the heterodimeric p65-p50 complex [20]. In a conventional activation pathway, I κ B is phosphorylated by I κ B kinases in response to different activators, subsequently degraded thus liberating the active NF κ B complex which then translocate to the nucleus and bind to target genes [20]. In Ang II-induced podocyte apoptosis, the nuclear translocation of NF κ B/p65 was detected [10]. Similarly, the nuclear translocation of NF κ B was detected in ADR-treated podocytes, which was supported by reduced cytosolic I κ B α (Fig. 3A, B). Podocyte-specific NF κ B inhibition ameliorates proteinuria in ADR-induced mice nephropathy [27]. Consistently, the application of NF κ B inhibitor PDTC significantly prevented increase of TRPC6 in ADR-treated podocytes (Fig. 3C-E). Our results also show that PDTC decreased TRPC6 channel activity and cellular apoptosis in ADR-treated podocytes (Fig. 6K,L; Fig. 3F). This suggests that ADR-induced upregulation of TRPC6 is NF κ B-dependent.

Previous studies suggest that Akt regulates transcriptional activity of NF κ B by inducing phosphorylation and subsequent degradation of I κ B [28, 29]. In this study, we applied Akt inhibitor triciribine [30], and found that Akt inhibition decreased ADR-induced nuclear translocation of NF κ B and cellular apoptosis (Fig. 4A,B). A key effector of Akt-induced signaling is the regulatory protein mTOR. The mTOR downstream from Akt controls NF κ B activity in PTEN-inactive prostate cancer cells via interaction with I κ B kinase. The mTORC1-associated protein Raptor is required for the ability of Akt to induce NF κ B activity [29]. Akt-dependent activation of mTORC1 can affect transcription of p70S6K or 4EBP1. In many cancers, this pathway is overactive, thus reducing apoptosis and allowing proliferation. In addition, the mTORC2 can induce activation of Akt at both Ser473 and Ser450 regulating cellular cytoskeleton and metabolism [29]. In this study, the activation of mTORC2 was identified, showing that phospho-mTOR^{Ser2481} and phospho-Akt^{Ser473} was significantly increased, while there was no obvious change of phospho-mTOR^{Ser2448} and phospho-p70S6K levels in ADR-treated podocytes (Fig. 2A, B). It was reported that mTOR was activated by reduced AMPK phosphorylation in high-glucose-induced podocytes apoptosis [31], while it is unclear which mTOR complex is activated. In addition, the mTORC1 is hyper-activated in podocytes from both diabetic mice and patients with diabetes. Nevertheless, complete deletion of mTORC1 activity in podocyte specific Raptor knockout mice leads to early onset of proteinuria [32, 33]. These findings suggest that although over-activated mTORC1 is causative for podocyte injury, the basal mTORC1 activity is required for maintaining the physiologic functions of podocyte. In normal podocyte, knockdown of mTORC2 complex protein Rictor, not mTORC1

complex protein Raptor, suppresses TRPC6 expression and Ca^{2+} influx [14]. Here, we investigated the distinct role of mTOR complex in ADR-treated podocytes. The application of rapamycin, the sensitive inhibitor of mTORC1, did not influence phospho-Akt^{Ser473} level (Fig. 2C) and cellular apoptosis following ADR treatment (Fig. 2D), suggesting that Akt activation may be mTORC1-independent. Ku0063794, a dual inhibitor of mTORC1 and mTORC2, was applied in the current study. Our data show that ADR-induced activation of mTORC1 and mTORC2 as well as their downstream effectors including p70S6K and Akt was remarkably inhibited by ku0063794 (Fig. 2C). Furthermore, ADR-induced apoptosis was also decreased by ku0063794 (Fig. 2D), implying that the mTORC2 pathway was involved in ADR-induced podocyte apoptosis.

To further explore the role of mTOR signaling pathways, we specifically knockdowned Raptor and Rictor, respectively. Our data show that only Rictor knockdown prevented ADR-induced activation of Akt and nuclear translocation of NF κ B/p65 (Fig. 4C, D). Importantly, we also examined the role of distinguished mTOR complex in regulating TRPC6 channel activity. Rapamycin and Raptor knockdown showed no obvious effects on Ca^{2+} influx in ADR-treated podocytes, whereas ku0063794 and Rictor knockdown significantly decreased ADR-induced activation of TRPC6 (Fig. 6A-H). In podocyte specific Rictor knockout mice, where mTORC2 activity is abolished in podocytes, did not show any obvious phenotypes [33]. Similarly, we did not observe increase of apoptosis in wild-type podocytes with either TRPC6 knockdown or specific mTOR pathway inhibition (Fig. 1G, Fig. 2D, Fig. 4E). However, upon bovine serum albumin overloading stress, proteinuria level was increased significantly in podocyte Rictor knockout mice compared with control [33], suggesting that the function of mTORC2 in podocytes is required for their adaptation under certain stress conditions.

Taken together, we provide evidence that the mTORC2/Akt/NF κ B pathway-mediated activation of TRPC6 may contribute to ADR-induced podocyte apoptosis (Fig. 7). Nevertheless, it has been demonstrated that ADR enhances TRPC6 expression in angiotensin II (AngII)-dependent manner [25]. Furthermore, ADR-induced podocyte damage requires TRPC6-mediated Ca^{2+} influx and the activation of the Ca^{2+} -dependent protein phosphatase calcineurin and its substrate nuclear factor of activated T cells (NFAT) [25]. In podocytes, it was also reported that insulin increases TRPC6 expression by a calcineurin-dependent pathway [34]. This suggests that a co-activation of both mTORC2/Akt/NF κ B and AngII/calcineurin/NFAT pathways might occur post ADR-induced podocyte injury. On the other hand, activation of Akt leads to inactivation of the effector GSK-3 β , resulting in degradation of NFAT by the proteasome and subsequent inhibition of cancer cell migration [35]. Therefore, there may be a crosstalk between the two pathways in ADR-induced podocyte injury. The molecular mechanism of ADR-driven TRPC6 expression needs be further investigated to underline the critical role of TRPC6 in the pathogenesis of podocyte injury and proteinuria.

Disclosure Statement

We declare that we have no conflict of interest.

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