Original Paper

RANK Deficiency Ameliorates Podocyte Injury by Suppressing Calcium/Calcineurin/NFATc1 Signaling

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Key Words

Podocyte • Injury • Receptor activator of NF- κ B • Nuclear factor of activated T cells

Abstract

Background/Aims: Podocyte injury and loss contribute to proteinuria, glomerulosclerosis and eventually kidney failure. Receptor activator of NF- κ B (RANK) belongs to the TNF receptor superfamily, which plays a key role in the pathogenesis of podocyte injury. However, the mechanism underlying the effect of RANK in podocyte injury remains unclear. Here, we sought to explore the possible molecular mechanisms involved in podocyte injury caused by RANK.

Methods: Immortalized mouse podocytes were treated with siRNA targeting RANK for 48 h or ionomycin for 24 h before harvest. Western blot, quantitative RT-PCR and immunofluorescence staining were used to evaluate the expression and function of RANK, nuclear factor of activated T cells c1 (NFATc1), transient receptor potential cation channel, subfamily C, member 6 (TRPC6) and calcineurin in podocytes. The Calcineurin Cellular Activity Assay kit was used to detect the phosphatase activity of calcineurin in cultured podocytes. A Ca^{2+} influx assay was performed to analyze alterations in Ca^{2+} entry under different conditions. Co-immunoprecipitation assays were used to observe the relationship between RANK and TRPC6. **Results:** RANK mRNA and protein expression were markedly increased in injured podocytes (ionomycin stimulation). Further study found that translocation of NFATc1 to the nucleus was significantly reduced after knocking down RANK by siRNA. Meanwhile, we also demonstrated that loss of RANK suppressed the phosphatase activity of calcineurin and attenuated the ionomycin-induced

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increase in Ca^{2+} influx. In addition, we showed that RANK knockdown in cultured podocytes decreased TRPC6 protein expression. Co-immunoprecipitation experiments suggested that RANK binds to TRPC6 and that ionomycin enhanced the binding of RANK to TRPC6.

Conclusion: Our findings demonstrated that RANK deficiency ameliorates podocyte injury by suppressing calcium/calcineurin/NFATc1 signaling, which may present a promising target for therapeutic intervention.

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Introduction

Podocytes are terminally differentiated cells that are located in the glomerular basement membrane (GBM) and play a key role in the selective permeability of the glomerular filtration barrier [1]. Podocyte injury and loss have been proven to be a central pathological mechanism of many kidney diseases including focal segmental glomerulosclerosis (FSGS) [2, 3], membranous nephropathy (MN) [4], minimal change disease (MCD) [5], diabetic nephropathy (DN) [6-8], and others. Although accumulating evidence has identified the signaling pathways that are involved in podocyte injury [9, 10], the mechanisms that damage podocytes are still far from being completely understood.

Nuclear factor of activated T cells (NFAT) transcription factors are the most extensively studied calcineurin substrates and are the major regulators of transcription in response to Ca^{2+} /calcineurin signals [11, 12]. NFAT proteins are generally phosphorylated and found in the cytoplasm of resting cells. Ca^{2+} influx induces calcineurin to dephosphorylate NFAT, which then translocates into the nucleus [13]. Recent studies have indicated that activation of NFATc1 may cause podocyte injury and proteinuria [11, 14]. Meanwhile, multiple studies have implicated activation of calcium/calcineurin/NFATc1 signaling in podocyte injury and glomerulosclerosis [11, 15, 16]. However, the mechanisms mediating Ca^{2+} influx are not fully understood.

Recent studies have demonstrated that receptor activator of NF- κ B (RANK) activates Ca^{2+} influx to stimulate induction of NFATc1 in the early phase of osteoclastogenesis in osteoclasts [17]. RANK, which is a type I membrane protein and belongs to the TNF receptor superfamily, is a central activator of NF- κ B. RANK shares high homology with CD40 and is the only known signaling receptor for receptor activator of NF- κ B ligand [18]. Recently, the RANK signaling pathway in osteoclasts has emerged as a central mechanism of osteoclastogenesis and activation of bone resorption [18, 19]. Moreover, diverse evidence shows that this pathway also plays a crucial role in the migration of cancer cells [20]. Liu et al. [21] reported that RANK is significantly induced in animal models of podocyte injury. Based on these findings, we explored the possibility that RANK mediates podocyte injury by activating NFATc1. We found that RANK deficiency ameliorates podocyte injury by suppressing calcium/calcineurin/NFATc1 signaling.

Materials and Methods

Cell culture and treatment

The conditionally immortalized mouse podocyte cell line was a kind gift from Dr Jochen Reiser of Rush University Medical Center (Chicago, IL, USA) and cultured as previously described [22]. Cells were grown in 33°C in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco BRL, Gaithersburg, MD, USA) and 50 U.mL^{-1} IFN- γ (growth permissive conditions; CYT-358, ProSpec, Tany Technogene Ltd, Ness Ziona, Israel). To induce differentiation, podocytes were reseeded and cultured at 37°C in the culture dish with 12 mg/mL type-I collagen (BD Bioscience, Bedford, MA, USA) and absence of IFN- γ (non-permissive condition), supplemented with 5% FBS for 10 to 14 days. After differentiation, podocytes were confirmed by the identification of synaptopodin, a podocyte specific differentiation marker, cells were synchronized into quiescence by growing cells in serum-free RPMI-1640 medium for 12 h, and

the treated with Ionomycin (a divalent calcium ionophore that is widely used as a tool to investigate the role of intracellular calcium in cellular processes, 2 μ mol/L) for 24h or siRNA (50 nM) for 48h. Each reaction was repeated in triplicates.

Transfection of siRNA

The siRNA sequences that targeted RANK and control-siRNA were designed and synthesized by RiboBio Co, Ltd (Guangzhou, China). Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) was used to transfect siRNA (50 nM) into podocytes following the manufacturer's protocol.

Real-time quantitative-PCR

Total RNA in podocytes was extracted with Trizol Reagent (Invitrogen) according to the supplier's protocol. Reverse transcribed into cDNAs by using the PrimerScript real-time reagent kit (Takara Biotechnology, Dalian, China), and then the cDNA was used for Real-time quantitative-PCR analysis using Power SYBR Green PCR Master Mix (Takara Biotechnology, Dalian, China) following the manufacturer's protocol. The primers used are listed as follows: RANK, forward 5'-CCAGGAGAGGCATTATGAGCA-3', reverse 5'-ACTGTCGGAGGTAGGAGTGC-3'; GAPDH, forward 5'-AGGTGGTGTGAACGGATTG-3', reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Immunofluorescent staining

Cultured podocytes planted on glass coverslips in six-well plates were fixed with pre-cold methanol for 20 min at -20°C, permeabilized with 0.1% Triton X-100 for 10 min and then blocked with 5% bovine serum albumin for 30 min at room temperature. For immunofluorescent staining, following primary antibodies were incubated overnight at 4°C: mouse anti-RANK (abcam, 1:500), rabbit anti-NFATc1 (abcam, 1:1000), goat anti-synaptopodin (Santa Cruz, 1:100). Then the secondary antibodies: Alexa Fluor 488 donkey anti-goat, Alexa Fluor 546 donkey anti-rabbit, Alexa Fluor 546 donkey anti-mouse (Life technologies, 1:250) were applied for 1 h at room temperature. Finally, nuclei were stained by DAPI (Life technologies, 1:1000) for 15 min at room temperature. The laser confocal microscopy (LCSM, Zeiss KS 400, Postfach, Germany) was used to photomicrograph.

Western blotting

Whole and nuclear proteins extracted from podocytes were performed as previously described [11]. According to the manufacturer's protocol, cellular proteins (40 μ g) were concentrated by using a protein assay reagent kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and separated on 7.5% SDS-PAGE gels electrophoresis, then elector-transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Then the membranes were blocked by 5% non-fat milk in TBST at room temperature for 1 h and incubated following primary antibodies overnight at 4°C: mouse anti-RANK (abcam, 1:500), rabbit anti-NFATc1 (abcam, 1:1000), rabbit anti-podocin (sigma, 1:4000), rabbit anti-TRPC6 (abcam, 1:1000), rabbit anti-calcineurin (abcam, 1:2000), rabbit anti-GAPDH (Bioworld, 1:3000), rabbit anti-Histone (Cell Signaling Technology, 1:2000). The membranes were subsequently incubated with anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology, 1:5000) for 1 h at room temperature the next day. ECL reagent (Advansta, Menlo Park, CA, USA) was used to detect the membranes. GAPDH or Histone as the internal control was used to standardize protein expression.

Co-immunoprecipitation

Podocytes were harvested and extracted in IP lysis buffer (87787, Pierce; Rockford, IL) supplemented with protease inhibitors. Equal amounts of proteins were incubated with mouse anti-RANK antibody at 4°C overnight, and then the Dynabeads Protein G (Life technologies, USA) were added and incubated for 2h. Finally, proteins were separated from Dynabeads Protein G and detected by Western blot analysis with rabbit anti-TRPC6 antibody.

Calcineurin phosphatase activity assay

The phosphatase activity of calcineurin in cultured podocytes was analyzed using the Calcineurin Cellular Activity Assay kit (Enzo Life Sciences, Farmingdale, NY, USA) according to manufacturer's

instructions. Briefly, podocytes were harvested via trypsinization (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and extracted in lysis buffer containing protease inhibitors. The lysate was subjected to desalting resin. The protein concentration was evaluated using a protein assay reagent kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and equal amount of proteins were incubated with the calcineurin substrate at 30°C for 30 min. Then, 100ul BIOMOL GREEN Reagent was added to each well and color was developed for 20 min at 30°C before measuring the absorbance value at 620 nm.

Measurement of Ca²⁺ influx

Podocytes transfected with RANK-siRNA or scramble-siRNA were treated with Ionomycin (2uM) for 24 h. Then the cells were incubated with 3uM Ca²⁺ indicator Fluo-8 AM (Cell Signaling Technology, Danvers, MA, USA) in basic RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) at 37°C for 30 min. After incubation, the medium was abandoned and the cells were washed three times with Ca²⁺ free K-H solution contained 136mM NaCl, 5.4mM KCl, 1.0mM MgCl₂, 0.33mM NaH₂PO₄, 10mM HEPES, 10Mm D-glucose. Fluo-8 fluorescence was measured on laser confocal scanning microscopy (Leica, Zeiss KS400, Postfach, Germany). The baseline of the fluo-8 fluorescence was measured at first two minutes and recorded as F₀. And then 2uM thapsigargin (TG, T9033, Sigma-Aldrich) was added to block the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula. To detect the changes in Ca²⁺ influx modulated by the membrane-associated Ca²⁺ channels in different groups, 2nM Ca²⁺ was added to the cells. The calcium influx was represented by F / F₀, where F is the peak of fluo-8 fluorescence intensity. The fluo-8 fluorescence was excited using a 488nm argon laser line and emitted using a wavelength of 525nm.

Statistical Analysis

All the values were presented as mean ± SEM. The statistical package SPSS for Windows Ver. 20.0 (SPSS, Inc., Chicago, IL, USA) was performed to statistical analysis. The dates from two groups were compared by Student's *t*-test, and dates from multiple groups were analyzed using one-way ANOVA followed by Bonferroni adjustment/Tukey test or the Dunnett T3 test. *P*-values <0.05 were considered significant.

Results

The expression of RANK was upregulated in ionomycin-treated podocytes in vitro

Ionomycin is a calcium ionophore which can increase intracellular calcium ions [23] and is widely used as a tool to induce podocyte injury [24]. To observe whether RANK was changed in response to podocyte injury *in vitro*, we studied its expression in ionomycin-treated podocytes. We found that RANK mRNA level and protein expression were increased compared to controls (Fig. 1a, 1b). Similarly, immunofluorescence staining also showed that RANK was significantly induced by ionomycin in podocytes (Fig. 1c).

Loss of RANK increased podocin expression and decreased nuclear localization of NFATc1 in injured podocytes

To study the role of RANK in podocytes, we established a cell model of RANK-silenced podocytes using RANK siRNA. Western blotting and quantitative real-time RT-PCR confirmed efficient siRNA-mediated RANK knockdown in podocytes (Fig. 2a, 2b). As shown in Fig. 2c, the expression of podocin, a specific marker of podocytes, was obviously increased after RANK knockdown in podocytes compared to controls, which indicated that RANK was a factor contributing to podocyte injury. Because accumulating data suggests that NFAT signaling is an important regulatory factor in podocyte function and glomerulosclerosis [11, 15], we next explored whether RANK mediates podocyte injury by regulating NFAT signaling. Western blotting and immunofluorescence staining demonstrated that the expression of nuclear NFATc1 was evidently decreased after 24 h-treatment with ionomycin in RANK-knockdown podocytes (Fig. 2d, 2e).

Loss of RANK decreased calcineurin expression and phosphatase activity in podocytes

We further explored whether RANK knockdown affected the expression of calcineurin, the classical upstream regulator of NFAT signaling in podocytes [16]. As shown in Fig. 3a, the expression of calcineurin protein was markedly decreased after loss of RANK in podocytes. Notably, the activity of NFAT protein is tightly modulated by Ca^{2+} and the Ca^{2+} /calmodulin-dependent serine phosphatase calcineurin [13]. We therefore explored whether loss of RANK also prevented calcineurin phosphatase activity or just its expression. As expected, the inhibition of RANK in podocytes induced less calcineurin phosphatase activity compared to untreated control podocytes or podocytes treated with scrambled RNA (Fig. 3b).

RANK knockdown reduced Ca^{2+} influx in cultured podocytes

To investigate possible alterations in Ca^{2+} entry caused by RANK knockdown, a Ca^{2+} influx assay was performed. Thapsigargin was used to inhibit the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase [25]. Thapsigargin generated a smaller increase in cytosolic free Ca^{2+} levels in RANK-knockdown podocytes indicating that less Ca^{2+} was released from intracellular Ca^{2+} stores. After addition of extracellular Ca^{2+} , an increase in Ca^{2+} influx was observed. However, the knockdown of RANK expression by siRNA suppressed the increase in Ca^{2+} entry (Fig. 4a). The quantification of the Ca^{2+} influx assay (Fig. 4b) is shown as F/F₀. These results demonstrated that RANK knockdown attenuated the ionomycin-induced increase in Ca^{2+} influx in cultured podocytes.

Fig. 1. RANK expression was upregulated in Ionomycin-treated podocytes in vitro. The RANK mRNA (a) and protein (b) expression were upregulated in Ionomycin-treated podocytes. (c) RANK protein (red) was increased after 24 h-treatment of Ionomycin in cultured podocytes, as shown by immunofluorescence with the podocyte marker synaptopodin (green) and DAPI-stained nuclei (blue). Magnification $\times 400$. All above data were from at least three independent experiments. * $P < 0.05$ versus control.

Fig. 2. Loss of RANK decreased nuclear localization of NFATc1 and increased Podocin expression in injured podocytes. (a) RANK was silenced using RANK-siRNA. Quantitative RT-PCR analysis verified that the expression of RANK mRNA was knocked down by RANK-siRNA. (b) Western blot confirmed that the expression of RANK protein was knocked down by RANK-siRNA. (c) The nuclear NFATc1 protein expression was down-regulated in RANK knockdown podocyte. (d) Immunofluorescence staining for NFATc1 (red), podocyte marker synaptopodin (green) and DAPI -stained nuclei (blue) in podocyte. Knockdown of RANK by using RANK-siRNA, nuclear localization of NFATc1 was decreased. Original magnification x400. (e) Compared to scramble, Podocin protein expression, a podocyte marker, was increased in RANK-siRNA knockdown podocytes. All above data were from at least three independent experiments. *P<0.05 versus control.

Fig. 3. Knockdown of RANK prevented the expression of calcineurin and calcineurin phosphatase activity in podocytes. (a) The protein expression of Calcineurin was decreased in RANK knockdown podocytes treated with Ionomycin. (b) RANK knockdown by using RANK-siRNA inhibited the Calcineurin phosphatase activity in Ionomycin-injured podocytes. All above data were from at least three independent experiments. *P<0.05 versus control.

Fig. 4. RANK knockdown reduced Ca^{2+} influx in cultured podocytes. Representative recordings of intracellular Ca^{2+} concentration were shown for podocytes under different conditions. Ca^{2+} labeling with Fluo-8 AM was measured by laser confocal scanning microscopy and the results were expressed as fluorescence intensity. (b) Quantification of the Ca^{2+} influx assay. The peak of fluo-8 fluorescence intensity was normalized to the baseline (F/F₀, n=5-10 cells). Data were expressed as mean $\pm$ S.E.M., *P<0.05 versus control.

RANK formed a protein complex with TRPC6 in podocytes

The above data showed that calcineurin phosphatase activity was reduced and calcium influx was suppressed in RANK-knockdown podocytes. To further examine the underlying mechanism of RANK regulation of NFATc1, we used western blotting to evaluate the expression of transient receptor potential cation channel, subfamily C, member 6 (TRPC6), mutations of which enhance channel currents leading to increased intracellular calcium concentrations and activation of calcineurin [26]. As shown in Fig.5a, TRPC6 protein expression was obviously decreased in RANK-knockdown podocytes compared to controls. To further explore the relationship between RANK and TRPC6, co-immunoprecipitation assays were performed. Immunoprecipitation with anti-RANK followed by immunoblotting with anti-TRPC6 showed that TRPC6 protein was markedly increased in the ionomycin group compared to the control group (Fig.5b). These results indicated that the formation of a RANK/TRPC6 complex was significantly enhanced in podocytes treated with ionomycin.

Fig. 5. RANK formed a protein complex with TRPC6 in podocytes. (a) RANK-siRNA mediated RANK silencing down-regulate TRPC6 protein expression in Ionomycin treated podocytes. (b) Endogenous Co-immunoprecipitation assay in podocytes confirmed that enhanced interaction between RANK and TRPC6 upon Ionomycin treatment. IP with anti-IgG antibody serves as a negative control. All above data were from at least three independent experiments. * $P < 0.05$ versus control.

Discussion

Podocytes, the highly specialized cells of the kidney glomerulus, play a crucial role in the function of the glomerular filtration barrier [27]. Insults to podocytes result in podocyte foot process effacement and podocyte loss, and if not resolved, to more sustained injury including chronic kidney diseases [28]. Recently, multiple signaling pathways have been implicated in podocyte injury, but the mechanisms underlying the injury process remain poorly understood. Therefore, exploring the mechanisms underlying podocyte injury in chronic kidney diseases is imperative.

In this study, we found that RANK mRNA and protein expression were increased in ionomycin-treated podocytes *in vitro*. Moreover, podocin expression, a podocyte marker, was increased and nuclear NFATc1 was decreased in response to ionomycin in RANK-knockdown podocytes. We also demonstrated that protein expression of calcineurin, the upstream target of NFAT signaling in podocytes [16], was decreased after loss of RANK. Additionally, inhibition of RANK prevented calcineurin activation and intracellular calcium influx in podocytes *in vitro*. Further analyses showed that RANK formed a protein complex with TRPC6 and that ionomycin enhanced the binding of RANK to TRPC6. Collectively, these data indicated that the RANK/TRPC6/calcineurin/NFATc1 signaling pathway may play a key role in podocyte injury (Fig. 6).

RANK, as a type I membrane protein which belongs to the TNF receptor superfamily, is a central activator of NF- κ B. Recent research has demonstrated that RANK is involved in bone remodeling and repair [18], migration of cancer cells [20], lymph node development [29] and immune cell function [30], among other activities. Our previous study reported inducible RANK expression in podocytes from proteinuric patients with FSGS, IgA nephropathy and MN, in a PAN rat model and in SCID mice treated with lipopolysaccharide [21]. In addition, Chen et al. reported that RANK was overexpressed in HG-induced podocytes *in vitro* [31]. Here, in our study, we found that RANK expression was also inducible in ionomycin-injured podocytes *in vitro*. In addition, we found that podocin, a podocyte marker, was increased and nuclear NFATc1 was decreased after loss of RANK in ionomycin-injured podocytes. These data indicate that RANK is a factor contributing to podocyte injury and that its underlying mechanism may be promoting NFATc1 translocation into the nucleus.

NFAT is a family of transcription factors consisting of five members: NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5 [32]. Increasing data now support the notion that activation of NFAT in podocytes causes proteinuria and glomerulosclerosis [11, 15, 16, 33]. Clinically, cyclosporine A, an inhibitor of calcineurin, has a direct antiproteinuric effect on proteinuric patients with MCD [34], FSGS [35] or MN [36] by inhibiting the calcineurin-NFAT axis

in podocytes [37, 38]. NFAT proteins are highly phosphorylated and reside in the cytoplasm of resting cells. Upon stimulation, intracellular Ca^{2+} is increased and calcineurin dephosphorylates NFAT proteins, which are then translocated into the nucleus. In the nucleus, NFAT proteins control the transcription of target genes by combining with other nuclear partners in NFAT transcription complexes which exert an injurious effect on podocytes [11, 15]. Calcineurin is the classic upstream activator of NFAT signaling. Here, in our study, we first demonstrated that calcineurin protein expression was decreased in RANK-knockdown podocytes. Similarly, the phosphatase activity of calcineurin was inhibited after loss of RANK in ionomycin-injured podocytes. Moreover, we demonstrated that RANK knockdown reduced Ca^{2+} influx in cultured podocytes. These data confirmed that RANK mediates NFATc1 translocation into the nucleus via the calcium/calcineurin axis.

Previous studies demonstrated that TRPC6 mutations enhanced channel currents leading to increased intracellular calcium concentrations and activation of calcineurin [26]. To further explore the mechanism of RANK deficiency on suppression of the calcium/calcineurin/NFATc1 axis, we studied the interaction between RANK and TRPC6. We found that RANK bound to TRPC6 and ionomycin enhanced the binding of RANK to TRPC6. Meanwhile, TRPC6 protein expression was decreased in RANK-knockdown podocytes. These data indicate that RANK may have a direct role in regulating TRPC6 activity. Thus, these results further confirmed that RANK mediates NFATc1 translocation into the nucleus via the TRPC6/ calcium/calcineurin axis.

In summary, we have demonstrated that RANK was increased in ionomycin-treated podocytes. Knocking down RANK expression increased podocin expression and decreased nuclear NFATc1 accumulation. Moreover, loss of RANK reduced Ca^{2+} influx and inhibited the phosphatase activity of calcineurin. Finally, our findings suggested that RANK bound to TRPC6 and that ionomycin enhanced the binding of RANK to TRPC6. Collectively, our data indicate that inhibition of RANK ameliorates podocyte injury by suppressing calcium/calcineurin/NFATc1 signaling.

Conclusion

Our findings demonstrated that RANK deficiency ameliorates podocyte injury by suppressing calcium/calcineurin/NFATc1 signaling. The RANK/TRPC6/calcium/calcineurin/NFATc1 pathway may potentially play an important role in modulating podocyte injury and glomerular function. It may also provide a basis for valuable therapeutic intervention in various RANK-associated podocytopathies.

Abbreviations

RANK (Receptor activator of NF-Kb); NFATc1 (Nuclear factor of activated T-cell, cytoplasmic 1); siRNA (Small interfering RNA); Ca^{2+} (Calcium).

Fig. 6. The proposed mechanism of RANK deficiency ameliorates podocyte injury. Black arrows indicate positive regulation, whereas red lines indicate negative regulation

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All data that support the conclusions of this manuscript have been presented in the main paper. Please refer to results and figure 1 to 5 in our manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

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