Original Paper

Rosiglitazone Inhibits Angiotensin II-Induced Proliferation of Glomerular Mesangial Cells via the $G\alpha_q$ /PLC β 4/TRPC Signaling Pathway

Linting Wei^a Jiarong Mao^b Jiamei Lu^a Jie Gao^a Dan Zhu^a Lifang Tian^a
Zhao Chen^a Lining Jia^a Li Wang^a Rongguo Fu^a

^aDepartment of Nephrology, Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, ^bDepartment of Pathology, Affiliated Hospital of Shaanxi Institute of Traditional Chinese Medicine, Xi'an, China

Key Words

RSG • PPAR- γ • RGS4 • TRPC • HBZY-1 • Proliferation

Abstract

Background/Aims: Mesangial cell proliferation and extracellular matrix accumulation (ECM) deposition play an important role in the pathogenesis of glomerulosclerosis. TRPC and PPAR- γ can regulate cell proliferation. Angiotensin II (AngII) can induce mesangial cell proliferation and affect TRPC expression. However, the mechanism has not been fully elucidated. This study was designed to investigate the role of TRPC and the effect of rosiglitazone (RSG) in the proliferation of rat glomerular mesangial cells (HBZY-1) that were stimulated by AngII and the underlying mechanisms. **Methods:** Immunofluorescence staining and qRT-PCR were performed to examine the expression levels of TRPCs in HBZY-1. Gene expression levels of TRPC, PPAR- γ , RGS4 (regulators of G protein signaling), the GPCR/ $G\alpha_q$ /PLC β 4/TRPC signaling pathway and major downstream molecules (PCNA, SKP2, P21 and P27) were detected by qRT-PCR and western blotting. Additionally, changes in intracellular Ca^{2+} levels were determined through Fluo-4 Ca^{2+} imaging, and the cell cycle was analyzed by flow cytometry. **Results:** Our results found that TRPC1 and 6 were at higher expression levels in HBZY-1 cells. Following AngII stimulation, there were increased levels of TRPC1 and 6, Ca^{2+} entry, PCNA and SKP2, decreased expression levels of P21 and P27 and a reduced G_0/G_1 percentage. Silencing TRPC1 and 6 by siRNAs led to decrease in Ca^{2+} influx, G_0/G_1 cell cycle arrest and cell proliferation. Notably, PPAR- γ activation by RSG upregulated RGS4 expression, which can interact with the $G\alpha_q$ family to inhibit the $G\alpha_q$ -mediated signaling cascade. The results were similar to silencing TRPC1 and 6 by siRNAs. **Conclusion:** All these results indicate that RSG could inhibit HBZY-1 cell proliferation via the $G\alpha_q$ /PLC β 4/TRPC signaling pathway.

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L. Wei and J. Mao contributed equally to this work.

Rongguo Fu, MD., Ph.D.
and Li Wang, MD., Ph.D.

Department of Nephrology, Second Affiliated Hospital of Xi'an Jiaotong University
Shaanxi province, 710004 (China)
Tel. +86-029-87679917; E-Mail fu_rongguo@sina.com; lemonwine@126.com

Introduction

Mesangial cell proliferation and extracellular matrix accumulation (ECM) deposition play an important role in the pathogenesis of glomerulosclerosis. Growing evidence indicates that abnormal growth of glomerular mesangial cells is an early event in various glomerular diseases and is the main histopathological feature in chronic kidney diseases (CKD) [1, 2]. Various signaling molecules are involved in mesangial cell proliferation and ECM accumulation [3-5]. Nevertheless, to date, no studies have investigated the effects of RSG in glomerular mesangial cell (HBZY-1) proliferation induced by AngII via RGS4 (regulators of G protein signaling) and the $G\alpha_q$ /PLC β 4/TRPC signaling cascade.

Transient receptor potential cationic channels (TRPC) are nonselective calcium channels [6], which can be activated by AngII through its AT₁R receptor-activated $G\alpha_q$ protein. This results in activation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4, 5-diphosphate (PIP₂) to generate 1, 4, 5-triphosphate muscle alcohol (IP₃) and diacylglycerol (DAG). Ca^{2+} is a second messenger molecule that plays an important role in various cellular functions, such as cell differentiation, proliferation, migration, apoptosis and gene expression [7]. Numerous studies have shown that TRPC channels play pivotal roles in cell proliferation [8-12]. Among them, only two studies focused on TRPC3 and 6 in human glomerular mesangial cell proliferation [9, 10].

PPAR- γ , a member of the peroxisome proliferator-activated receptor family, is a transcription factor that can be activated by its ligand and was first discovered and reported by Isseman and Green in 1990 [13]. PPAR- γ is one of the most extensively studied isoforms among the three subtypes of PPAR (PPAR- α , PPAR- β/δ and PPAR- γ), which plays an important role in cell proliferation, apoptosis, cell metabolic balance, tumorigenesis and other physiological processes [14]. Following stimulation by its ligand, PPAR- γ requires heterodimerization with retinoid X receptor (RXR), and then, the heterodimer binds to PPAR response elements (PPRE) in the promoter region of responsive genes to regulate target gene transcription [15]. In kidney, PPAR- γ is mainly expressed in glomerular mesangial cells, endothelial cells, proximal and distal tubules and medullary collecting ducts [16, 17]. Antiproliferative activity of PPAR- γ agonists has been observed in a variety of cells [18, 19]. Previous studies have also found that PPAR- γ agonists can inhibit VEGF, TGF- β 1, PDGF, aldosterone and high glucose-induced mesangial cell proliferation [20-24].

RGSs are GTPase-activating proteins that can promote the deactivation of G proteins and inhibit $G\alpha_q$ signal transduction. RGS4 is an important member of the RGS family and can effectively suppress $G\alpha_q$ - and $G\alpha_i$ -mediated signaling pathways [25-27]. A study showed that RGS5 was a target gene for PPAR- β/δ [28]. In addition, Barish et al. [29] reported that PPAR- δ ligand can increase the expression levels of RGS4 and RGS5 in the aorta. Additionally, Takata et al. [30] demonstrated that an agonist of PPAR- δ can attenuate and alleviate AngII-induced atherosclerosis by upregulating the expression levels of RGS4 and RGS5. Therefore, we postulated that a PPAR- γ agonist may increase the expression of RGS4.

In this study, we investigated the effects of RSG on proliferation of HBZY-1 cells induced by AngII and, subsequently, the effect of RSG on expression of RGS4, which may play a role in $G\alpha_q$ /PLC β /TRPC signal transduction. In addition, we examined if these agents also have effects on intracellular Ca^{2+} influx and cell cycle changes. Elucidating these processes will reveal more mechanistic insights into PPAR- γ agonists and TRPC inhibitors as promising therapeutic agents that could ameliorate mesangial proliferative glomerulonephritis and slow the progression of CKD in patients.

Materials and Methods

Reagents

AngII and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). RSG and GW9662 were purchased from Selleck (Houston, TX, USA). PPAR- γ (166431-1-AP), SKP2 (15010-1-AP) and β -actin

(20536-1-AP) antibodies were purchased from Proteintech (Chicago, IL, USA). RGS4 (sc-39834), PLC β 4 (sc-166131) and PCNA (sc-56) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). TRPC1 (ab-74819), TRPC3 (ab-104534), TRPC4 (ab-84813), G α q (ab-199533), P21 (ab-109199) and P27 (ab-62364) antibodies were purchased from Abcam (Cambridge, MA, USA). TRPC5 (GTX-54861) antibody was purchased from GeneTex (Irvine, SC, USA). TRPC6 (#16716) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson (Los Angeles, CA, USA). All the primers were synthesized by AuGCT (Beijing, China). TRIzol reagent, Lipofectamine 2000 and Fluo-4AM were purchased from Invitrogen (Carlsbad, USA). Reverse transcription kit and SYBR Premix Ex TaqTMII Green Mastermix kit were purchased from TaKaRa (Tokyo, Japan). DMSO was from MP Biomedicals (Santa Ana, CA, USA).

Cell culture

Rat glomerular mesangial cells (HBZY-1) were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). HBZY-1 cells were cultured in DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) and penicillin (100 U/ml) and streptomycin (100 μ g/ml). In addition, cells were maintained in a cell incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

MTT assay

MTT assays were performed to examine the effects of AngII, RSG and GW9662 on proliferation of HBZY-1 cells. HBZY-1 cells were routinely digested, resuspended and then seeded in 96-well plates at a density of 8×10^3 cells/well. After incubation for 12 h, cells were treated with 0, 0.05, 0.1, 0.5 and 1.0 μ M AngII according to their experimental group and incubated at 37°C for 0, 12, 24, 36, 48 and 72 h. In addition, cells were also incubated with different concentrations of RSG and GW9662 as follows: 1, 5, 10 and 15 μ M. After the treatments, 20 μ L MTT solution at a concentration of 5 mg/mL was added and then incubated for 4 h. Then, we discarded the medium and added 150 μ L DMSO/well. The optical density (OD) value of each well was measured spectrophotometrically at 490 nm. Mean readings from three independent experiments were plotted for each time point. Six replicates were performed for each data.

Immunofluorescence staining

HBZY-1 cells were seeded in glass coverslips, which were placed inside 12-well plates. When the cell monolayer reached 70% confluency, cells were fixed in 4% paraformaldehyde for 20 min. After washing with PBS, 0.2% Triton was added for 10 min. Then, cells were blocked with 5% bovine serum albumin and separately incubated with TRPC1 (1:200), 3 (1:250), 4 (1:200), 5 (1:200) and 6 (1:50) antibodies overnight at 4°C. After removing primary antibodies and washing with PBS, FITC-conjugated secondary antibodies were incubated for 1 h in the dark at room temperature. Nuclei were counterstained with DAPI, and after washing with PBS, coverslips were sealed with anti-quenching fluorescent mounting medium. Stained sections were viewed under an inverted Nikon fluorescence microscope (Nikon Ti-S, Japan). Each test was performed at least three times.

qRT-PCR

Total cell RNA was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized using a PrimescriptTM RT reagent kit (TaKaRa, Japan), and 500 ng of total RNA was used. qRT-PCR amplification with primers was conducted in ABI Prism 7300 detection system (Applied Biosystems, CA, USA) using SYBR Premix Ex TaqTMII Green Mastermix (TaKaRa, Japan). GAPDH was used as an internal control. Relative mRNA expression levels were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method. The corresponding primers used in our study are listed in Table 1. All experiments were performed in triplicate.

Western blot

Total cell proteins in each group were extracted using RIPA buffer (Heart, Xi'an, China) following the manufacturer's instructions, and protein concentrations were detected using BCA kits (Heart, Xi'an, China). Protein samples were run on 6-12% SDS-PAGE gels according to the molecular weight. After electrophoresis, proteins were electrotransferred to a PVDF membrane (Millipore, USA) for 1-2 h using a Mini Trans-Blot

Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Then, membranes were blocked in 5% skim milk in PBST for 1 h at room temperature and were incubated with primary antibodies against PPAR- γ (1:600), RGS4 (1:800), G α q (1:1000), PLC β 4 (1:400), TRPC1 (1:1000), TRPC6 (1:1000), PCNA (1:1000), SKP2 (1:600), P21 (1:1000), P27 (1:1000) and β -actin (1:2000) overnight at 4°C. After washing three times with PBST, membranes were incubated with HRP-conjugated secondary antibody (1:100, 000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit (Millipore, USA). Band intensity was quantified by densitometry using ImageJ software. Each test was performed at least three times.

Transient transfection of siRNA

The siRNA oligonucleotides were synthesized by Shanghai GenePharma (Shanghai, China), and their sequences are listed in Table 1. Specific siRNAs targeting rat TRPC1 and 6 were separately cotransfected into HBZY-1 cells using Lipofectamine 2000 according to the manufacturer's instructions. In the control group, cells were transfected with scrambled siRNA. The efficiency was detected using FAM in the control cells.

Intracellular Ca²⁺ imaging

Fluorescence imaging of intracellular Ca²⁺ was performed as follows: HBZY-1 cells were seeded in petri dishes, incubated with buffer containing 5 mM of Fluo-4/AM at 37°C for 40 min and washed and incubated with HEPES-buffered saline containing 2.0 mM of CaCl₂. In addition, intracellular Ca²⁺ fluorescence intensity levels were measured and quantified using a laser scanning confocal microscope (Nikon, Japan). Full images were collected at 5 s intervals. Fluo-4 fluorescence was excited at 488 nm. All data are representative of at least three independent experiments.

Flow cytometry

Cells in different treatment groups were harvested, fixed in 70% cold ethanol and stored overnight at -20°C. After washing with PBS, PI staining solution (50 μ g/mL PI and 100 μ g/mL RNase A) was added to the cells and incubated for 30 min in the dark at 37°C. Then, cells were analyzed using flow cytometry (FACS Calibur from BD Biosciences, USA). FlowJo software was used to analyze the results. Three independent experiments were conducted.

Statistics

SPSS 20.0 statistical software (IBM Corporation, Armonk, NY, USA) was utilized to assess the statistical significance of differences using Student's *t*-test or one-way ANOVA. Data are presented as the mean $\pm$ SD. *P*-values were two-tailed, and a *P*-value < 0.05 was considered statistically significant.

Table 1. Sequences of primers used for qRT-PCR

Gene	Sequence (5' - 3')
PPAR- γ	sense 5'- CCTGTTGACCCAGAGCATGG -3'
	antisense 5'- GGTCCACAGAGCTGATTCCG -3'
RGS4	sense 5'- TTCATCTCTGTGCAGGCAAC -3'
	antisense 5'- GGAAGGATTGGTCAGTCAA -3'
G α q	sense 5'- GCACAATTGGTTCGAGAGGT -3'
	antisense 5'- GATAGGAAGGGTCAGCCACA -3'
PLC β 4	sense 5'- ATCGTGGCCAGTATGACAAG -3'
	antisense 5'- ATCTGCTGCATCTCTTCGC -3'
TRPC1	sense 5'- ACT CAG CCT TGT GGA GGT -3'
	antisense 5'- TCG TTT GTC AAG AGG CTC -3'
TRPC3	sense 5'- GGAGTCTCGCAGCTCAATGTC -3'
	antisense 5'- TCTTCAGCAGCAGCTCGGTCA -3'
TRPC4	sense 5'- AACAGGCGCAGGAGCACTT -3'
	antisense 5'- CCACCACCACCTTCTCCGACTT -3'
TRPC5	sense 5'- GGTGCCCAAGGAAAGCCGAAA -3'
	antisense 5'- ATAGAGTGCTGTGTGCGATGC -3'
TRPC6	sense 5'- TGG CAA GTC CAG CAT ACC TGT C -3'
	antisense 5'- GTG TTT CTG CAG AGG TCC AGG AG -3'
PCNA	sense 5'- CTGAAGAAGGTGCTGGAG -3'
	antisense 5'- ATCGCAGCGGTATGTGTC -3'
SKP2	sense 5'- TTGCGCAGAATGAAAACCTTG -3'
	antisense 5'- GTTCAGCTGGGTGAGCGTAT -3'
P21	sense 5'- AAGAGGCCAGTACCTCCTC -3'
	antisense 5'- GGCCTTTGGAGTGATAGAAA -3'
P27	sense 5'- GATACGAGTGGCAGGAGGTG -3'
	antisense 5'- TCTGACGAGTCAGGCATTG -3'
TRPC1siRNA	sense 5'- CUGUGGAUUAUUGGAUGATT -3'
	antisense 5'- UCAUCCCAUUAUCCACAGTT -3'
TRPC6siRNA	sense 5'- GUCCAUUCAUGAAGUUCGUTT -3'
	antisense 5'- ACGAACUUCAUGAAGUUGACTT -3'
GAPDH	sense 5'- GGCACAGTCAAGGCTGAGAATG -3'
	antisense 5'- ATGGTGGTGAAGACGCCAGTA -3'

Results

AngII promotes proliferation of HBZY-1 cells

The effects of AngII on proliferation of HBZY-1 cells are shown in Fig. 1. HBZY-1 cells were incubated with different concentrations of AngII (0, 0.05, 0.1, 0.5 and 1.0 μM) at different time points (0, 12, 24, 36, 48 and 72 h). MTT assay results revealed that incubation of HBZY-1 cells with 0.1 μM AngII for 24 h significantly increased the OD value and promoted HBZY-1 cell proliferation (Fig. 1A). Meanwhile, treatment with 0.1 μM AngII for 24 h also increased mRNA (Fig. 1B-a and d) and protein (Fig. 1B-b, c and e, f) levels of PCNA. Therefore, incubation with 0.1 μM AngII for 24 h was chosen for all subsequent experiments to establish the proliferative model of HBZY-1 cells. In terms of the RSG and GW9662 effects on proliferation of HBZY-1 cells, 10 μM RSG and 10 μM GW9662 were used in our experiments (for all online suppl. material, see www.karger.com/doi/10.1159/000486056, Suppl. Fig. 2A).

Distribution and expression of TRPCs in HBZY-1 cells

We first examined expression levels of TRPC subtypes in HBZY-1 cells. Immunofluorescence analyses showed that TRPC1 (Fig. 2A a1-c1), TRPC3 (Fig. 2A a2-c2) and TRPC6 (Fig. 2A a5-c5) demonstrated higher levels in HBZY-1 cells, whereas TRPC4 (Fig. 2A a3-c3) and TRPC5 (Fig. 2A a4-c4) displayed relatively low expression levels, especially TRPC5, which was barely detectable in HBZY-1 cells. All these TRPC subtypes were expressed in the membrane and cytoplasm. Furthermore, we also examined expression of TRPCs in HBZY-1 cells with or without 0.1 μM AngII treatment for 24 h by qRT-PCR. As shown in Fig. 2B, TRPC1, 3, 5 and 6 showed elevated mRNA levels in the AngII groups compared with the NC groups, but TRPC4 did not. Additionally, TRPC1 and 6 demonstrated higher mRNA levels in HBZY-1 cells. In contrast, TRPC3, TRPC4 and TRPC5 were barely detectable in HBZY-1 cells.

Gene expression levels of PPAR- γ , RGS4, the G α q/PLC β 4/TRPC signaling pathway and major downstream proliferative molecules with AngII

Initially, we investigated whether the G α q/PLC β 4/TRPC signaling pathway was activated by AngII. As shown in Fig. 3, qRT-

Fig. 1. Ang II promotes proliferation of HBZY-1 cells. MTT, qRT-PCR and Western blot analyses were utilized to select the treatment concentration and time points of Ang II in HBZY-1. Each test was performed at least three times. Data are presented as the mean $\pm$ SD. (A) MTT assay was used to examine the treatment concentration and time of Ang II in HBZY-1. (B) qRT-PCR and Western blotting were performed to measure the mRNA and protein levels of PCNA (one of the proliferation indicators) with different Ang II treatments. (B-a and d) Effects of different treatment times and concentrations of Ang II on mRNA levels of PCNA. (B-b, c and e, f) Effects of different treatment times and concentrations of Ang II on protein levels of PCNA. Each test was performed at least 3 times. * $P < 0.05$ and ** $P < 0.01$, when comparing 12, 24, 36, 48, 72 h with 0 h and 0.05, 0.1, 0.5, 1.0 μM versus 0 μM ; the results indicate significant differences. # $P < 0.05$ and ## $P < 0.01$, when comparing 0, 12, 36, 48, 72 h with 24 h. & $P < 0.05$ and && $P < 0.01$ indicate 0, 0.05, 0.5, 1.0 μM versus 0.1 μM .

PCR and Western blotting results revealed that incubation with AngII can significantly upregulate the expression of $G\alpha_q/PLC\beta_4/TRPC$ signaling molecules. Next, we measured PPAR- γ and RGS4 and found that the mRNA and protein levels were down-regulated when compared with the NC groups (Fig. 3A-C). In addition, the mRNA and protein levels of the major downstream proliferative molecules PCNA and SKP2 were elevated in the AngII group. In contrast, treatment with AngII decreased gene expressions of P21 and P27 (Fig. 3D-F).

Silencing TRPC1 and 6 expressions by RNA interference

TRPC1 and 6 levels were elevated in the AngII stimulation groups. For further exploration of the role of TRPC1 and 6 in proliferation of HBZY-1 cells, specific siRNA sequences were synthesized and chosen. As shown (see online suppl. material) in Suppl. Fig. 1A, three siRNA sequences of TRPC1 and 6 were detected. The results showed that the mRNA levels of TRPC1 and 6 decreased by three siRNAs (see online suppl. material, Suppl. Fig. 1A-a and d). However, only the third siRNA for TRPC1 and the second siRNA for TRPC6 significantly down-regulated protein expression levels of TRPC1 and 6 (see online suppl. material, Suppl. Fig. 1A- b, c and e, f). To validate the results of (see online suppl. material) Suppl. Fig. 1A, consistent results were obtained for TRPC1 and 6 siRNAs, as demonstrated (see online suppl. material) in Suppl. Fig. 1B. For TRPC1, the mRNA and protein levels decreased 68.5% and 64.2%, respectively (Fig. 4A-C). For TRPC6, the mRNA and protein levels declined 65.0% and 56.1%, respectively (Fig. 4D-F). Suppl. Fig. 1C (see online suppl. material) indicates that FAM was expressed inside the cytoplasm in a dispersed scatter dot manner, and the transfection efficiency was approximately 77.4%.

Knockdown of TRPC1 and 6 inhibits AngII -induced proliferation of HBZY-1

Next, we observed the role of TRPC1 and 6 in proliferation of HBZY-1 cells. We found that proliferation was reduced in the TRPC1 (Fig. 5A-C) and 6 (Fig. 5D-F) knockdown groups compared with the AngII treatment groups. qRT-PCR and Western blot analyses showed that PCNA and SKP2 expression levels decreased, and the P21 and P27 levels were upregulated

Fig. 2. The distribution and expression of TRPCs in HBZY-1. (A) Immunofluorescence staining was performed to observe the distribution and expression of TRPC in HBZY-1. Original magnification $\times 200$ (scale bar = 100 μ m). (A-a1, b1 and c1) Distribution and expression of TRPC1 in HBZY-1 and all nuclei were counterstained with DAPI (A, b1-b5). (A-a2, b2 and c2) Distribution and expression of TRPC3 in HBZY-1. (A-a3, b3 and c3) Distribution and expression of TRPC4 in HBZY-1. (A-a4, b4 and c4) Distribution and expression of TRPC5 in HBZY-1. (A-a5, b5 and c5) Distribution and expression of TRPC6 in HBZY-1. (B) Transcription levels of TRPCs were detected with or without 0.1 μ M Ang II treatment for 24 h. Each test was performed at least three times. Values represent the mean $\pm$ SD. ** $P < 0.01$ and *** $P < 0.001$, indicate statistically significant results.

Fig. 3. Gene expression levels of PPAR- γ , RGS4, the G α q/PLC β 4/TRPC signaling pathway and major downstream proliferative molecules (PCNA, SKP2, P21 and P27) were detected by qRT-PCR and Western blotting in HBZY-1 cells with or without 0.1 μ M Ang II treatment for 24 h. HBZY-1 was seeded into 6-well plate, and the stimulus (0.1 μ M Ang II for 24 h) was given after 70-80% confluency. Each test was performed at least three times. Values represent the mean $\pm$ SD. The mRNA (A-a) and protein (A-b and c) levels of PPAR- γ , RGS4, and the G α q/PLC β 4/TRPC signaling pathway molecules in HBZY-1 cells with or without treatment for 24 h Ang II (0.1 μ M). The mRNA (B-a) and protein (B-b and c) levels of PCNA, SKP2, P21 and P27 in HBZY-1 cells with or without 0.1 μ M Ang II treatment for 24 h. *P<0.05 and **P<0.01, Ang II versus NC, indicate statistically significant results.

Fig. 4. Silencing TRPC1 and 6 expressions by RNA interference. HBZY-1 cells were seeded into 6-well plates, and the specific siRNA sequences were cotransfected into the cells after 30-50% confluence utilizing Lipofectamine 2000. In addition, transfected cells were harvested at 24 h for detecting mRNA levels of TRPC1 and 6 by qRT-PCR and 48 h for measuring protein expression levels of TRPC1 and 6 by Western blotting. Each test was performed at least three times. Values represent the mean $\pm$ SD. Three siRNA sequences of TRPC1 and 6 were used for examining mRNA (A and D) levels by qRT-PCR and protein (B, C and E, F) expression levels of TRPC1 and 6 by Western blotting. *P<0.05 and **P<0.01, TRPC1 siRNA and TRPC6 siRNA versus Control siRNA, indicate statistically significant results.

Three siRNA sequences of TRPC1 and 6 were used for examining mRNA (A and D) levels by qRT-PCR and protein (B, C and E, F) expression levels of TRPC1 and 6 by Western blotting. *P<0.05 and **P<0.01, TRPC1 siRNA and TRPC6 siRNA versus Control siRNA, indicate statistically significant results.

in the TRPC1 and 6 siRNA groups. These results further support the hypothesis that TRPC1 and 6 play crucial roles in HBZY-1 cell proliferation.

Fig. 5. Knock-down of TRPC1 and 6 inhibit Ang II-induced proliferation of HBZY-1. HBZY-1 cells were seeded into 6-well plates, and the specific siRNA sequences were cotransfected into the cells after 30-50% confluence utilizing Lipofectamine 2000. Then, the treatments (0.1 μ M Ang II for 24 h) were given after 70-80% confluence. Each test was performed at least three times. Values represent the mean $\pm$ SD. The mRNA (A and D) and protein (B, C and E, F) levels of PCNA, SKP2, P21 and P27 in HBZY-1 cells with or without cotransfection of specific siRNAs followed by treatments with 0.1 μ M Ang II for 24 h. * P <0.05 and ** P <0.01, Ang II versus NC, # P <0.05 and ### P <0.01, Ang II + TRPC1 siRNA or Ang II + TRPC6 siRNA versus Ang II, indicate statistically significant results.

Activated PPAR- γ by RSG inhibits AngII -induced proliferation of HBZY-1 via the G α q/PLC β 4/TRPC signaling pathway

Then, qRT-PCR and Western blot analyses were performed to examine the role of PPAR- γ in proliferation of HBZY-1 cells. As shown in Fig. 6D-F, our results revealed that activation of PPAR- γ by RSG inhibited AngII -induced proliferation of HBZY-1 cells; namely, PCNA and SKP2 gene expression levels were decreased, and P21 and P27 were elevated. In addition, the RGS4 levels were upregulated by treatment with RSG, and the G α q/PLC β 4/TRPC signaling pathway molecules were suppressed (Fig. 6A-C). These findings were probably due to the inhibitory effect of RGS4 on G α q signaling pathways. In contrast, GW9662 showed the opposite effects, except for the P27 mRNA levels.

TRPC1 and TRPC6 knock-down and PPAR- γ activation decrease intracellular Ca²⁺ entry in HBZY-1 cells

Previous studies have shown that the TRPC1 and 6 siRNAs and RSG suppressed proliferation of HBZY-1 cells. To further investigate the possible mechanisms, laser scanning confocal microscopy was applied to determine whether intracellular Ca²⁺ influx was regulated by manipulation of TRPC1 siRNA, TRPC6 siRNA and RSG. The experiments were performed using the same cells as the controls. As shown in Fig. 7A-a, B-a and C-a, basal fluorescence intensity levels of NC cells (0.63 $\pm$ 0.03) were captured through 0-100 s, full images were collected every 5 s interval and the representative images were scanned at 50 s. After the basal levels were recorded, 0.1 μ M AngII at 100 s was applied to the cells, and the intensity levels were recorded at 5 min after AngII addition. Recordings indicated significantly increased intracellular Ca²⁺ entries (2.41 $\pm$ 0.26) (Fig. 7A-b, B-b and C-b), and the images shown were captured at 200 s. To confirm the involvement of TRPC1 and 6, the cells were transiently transfected with TRPC1 and 6 siRNAs. In parallel with the effects on

Fig. 6. Activated PPAR- γ by RSG inhibits Ang II-induced proliferation of HBZY-1 via the G α q/PLC β 4/TRPC signaling pathway. 1: NC; 2: Ang II; 3: Ang II + RSG; 4: Ang II + GW9662. HBZY-1 cells were seeded into 6-well plates, and after 70–80% confluence, RSG (10 μ M) and GW9662 (10 μ M) were used 1 h before Ang II (0.1 μ M) treatment for 24 h. Each test was performed at least three times. Values represent the mean $\pm$ SD. The mRNA expression levels of PPAR- γ , RGS4, the G α q/PLC β 4/TRPC signaling pathway (A) and downstream PCNA, SKP2, P21 and P27 (D) were detected by qRT-PCR. The protein levels of PPAR- γ , RGS4, the G α q/PLC β 4/TRPC signaling pathway (B and C) and downstream PCNA, SKP2, P21 and P27 (E and F) were detected by Western blotting. * P <0.05 and ** P <0.01, Ang II versus NC, # P <0.05 and ## P <0.01, Ang II + RSG or Ang II + GW9662 versus Ang II, indicate statistically significant results.

HBZY-1 cell proliferation, knock-down of TRPC1 (1.44 $\pm$ 0.16) and 6 (1.46 $\pm$ 0.14) significantly reduced AngII-induced fluorescence intensity levels (Fig. 7A c-f and B c-f). In the presence of RSG, 0.1 μ M AngII at 100 s was added to the cells, and the levels were recorded at 5 min after AngII stimulation, which showed a decreased intracellular Ca²⁺ influx (1.32 $\pm$ 0.11) (Fig. 7C c-d). In contrast, the GW9662 groups showed the opposite results (3.75 $\pm$ 0.15) (Fig. 7C e-f). The images exhibited were captured at 180 s. After AngII was washed out, the currents were recovered, as shown in Fig. 7A-e, f B-e, f and C-g, h.

TRPC1 and TRPC6 knock-down and PPAR- γ activation cause G₀/G₁ cell cycle arrest in HBZY-1

Lastly, PI cell cycle analysis was applied to further examine the underlying mechanisms. The results revealed that in parallel with the proliferation stimulatory effect, AngII increased the percentages of S and G₂/M phase cells compared with NC cells and increased the expression levels of PCNA and SKP2 (Fig. 8A a-b, B a-b and Ca-b). TRPC1 (68.61%) and TRPC6 (69.01%) knock-down and RSG (71.2%) led to G₀/G₁ cell cycle arrest compared with the NC groups (66.37%) (Fig. 8A c-d, B c-d and C c, e), and the effects on cell cycle PCNA and SKP2 levels were similarly analyzed. In contrast, GW9662 resulted in a decreased percentage of G₀/G₁ phase cells (63.87%) and increased S phase cells (23.18%) (Fig. 8C d-e). Together, our studies consistently demonstrated that AngII promotes G₀/G₁ progression in HBZY-1 cells and may contribute to the development of mesangial cell proliferation diseases.

Discussion

In the present study, we observed that TRPC participated in the proliferation of HBZY-1 cells, which was induced by AngII via the AT₁R/G α q/PLC β 4/TRPC signaling pathway. Moreover, we first demonstrated that activated PPAR- γ by RSG could upregulate

Fig. 7. TRPC1 and TRPC6 knock-down and PPAR- γ activation decrease intracellular Ca^{2+} entry in HBZY-1. HBZY-1 cells were seeded into petri dishes. In TRPC1 and 6 knock-down groups, specific siRNAs for TRPC1 and 6 were cotransfected into the cells using Lipofectamine 2000. After 70-80% confluence, RSG (10 μM) and GW9662 (10 μM) were added 1 h before Ang II (0.1 μM) stimulation. Each test was performed at least three times. Values represent the mean $\pm$ SD. Basal NC (A-a, B-a and C-a), TRPC1 siRNA (A-c), TRPC6 siRNA (B-c), RSG (C-c) and GW9662 (C-e) cells at 50 s captured by a laser scanning confocal microscope. NC cells treated with 0.1 μM Ang II (A-b, B-b and C-b), TRPC1 siRNA (A-d), TRPC6 siRNA (B-d), RSG (C-d) and GW9662 (C-f) at 180-200 s captured by a laser scanning confocal microscope. Relative fluorescence intensities of Fluo-4 AM were demonstrated in (A-e, f, B- e, f and C-g, h). * $P < 0.05$ and ** $P < 0.01$, Ang II-stimulated NC, Ang II+TRPC1 siRNA, Ang II+TRPC6siRNA, Ang II+RSG and Ang II+GW9662 versus NC baseline cells, # $P < 0.05$ and ## $P < 0.01$, Ang II+TRPC1 siRNA, Ang II+TRPC6siRNA, Ang II+RSG and Ang II+GW9662 versus Ang II-stimulated NC cells, indicate statistically significant results.

Fig. 8. TRPC1 and TRPC6 knock-down and PPAR- γ activation cause G₀/G₁ cell cycle arrest in HBZY-1. HBZY-1 cells were seeded into 6 cm petri dishes. In TRPC1 and 6 knock-down groups, specific siRNA for TRPC1 and 6 were cotransfected into the cells using Lipofectamine 2000 and followed by treatment with 0.1 μ M Ang II for 24 h. For RSG and GW9662, after 70–80% confluence, RSG (10 μ M) and GW9662 (10 μ M) were given 1 h before Ang II stimulation for 24 h. Each test was performed at least three times. Values represent the mean $\pm$ SD. (A) Effect of TRPC1 siRNA on the cell cycle in HBZY-1 detected by flow cytometry. (B) Effect of TRPC6 siRNA on the cell cycle in HBZY-1 detected by flow cytometry. (C) Role of PPAR- γ in the cell cycle process in HBZY-1 detected by flow cytometry. * P <0.05 and ** P <0.01, Ang II versus NC, # P <0.05 and ## P <0.01, Ang II+TRPC1 siRNA, Ang II+TRPC6 siRNA, Ang II+RSG and Ang II+GW9662 versus Ang II, indicate statistically significant results.

Fig. 9. Proposed mechanism of RSG inhibiting proliferation of HBZY-1 induced by Ang II.

RGS4 expression levels (which could suppress the aforementioned Gaq/PLC β 4/TRPC signaling cascade) to inhibit HBZY-1 cell proliferation. In contrast, GW9662 showed the reverse capacities (Fig. 9). These phenomena indicated that PPAR- γ was also involved in

the proliferation of HBZY-1 cells. Additionally, these results presumably correlated with decreasing levels of intracellular Ca^{2+} influx and G_0/G_1 cell cycle arrest.

AngII is a major molecule of the rennin angiotensin system and has various cellular functions in the progression of renal diseases, including proliferation of glomerular mesangial cells and tubular hypertrophy, which may ultimately lead to glomerular sclerosis and tubulointerstitial fibrosis [31-33]. AngII is involved in diverse biological effects by binding to its receptors. AngII mainly consists of two subtype receptors, AT_1R and AT_2R [34]. AT_1R , which belongs to GPCR, couples with $\text{G}\alpha_q$ protein to activate traditional signal transduction mechanisms. AT_1R regulates cell proliferation and smooth muscle contraction [35]. Unlike AT_1R , AT_2R mainly modulates cell apoptosis and smooth muscle relaxation [35].

In our study, AngII-induced HBZY-1 cell proliferation was measured by MTT assays. The results revealed that 0.1 μM AngII treatment for 24 h significantly promoted proliferation of HBZY-1 cells. Significant differences were not observed in the 0.5 and 1.0 μM groups compared with 0.1 μM AngII at 24 h. This was probably due to the dual effects of AngII. The higher AngII concentration groups may show increased binding with AT_2R to mediate cell apoptosis. These results were similar to a former study by Qiu et al. [36], in which 0.1 μM AngII treatment for 24 h was also used to induce HBZY-1 cell proliferation.

The TRPC family is often divided into three groups owing to their structural similarities: TRPC1/4/5, TRPC2 and TRPC3/6/7. TRPC1 can also be categorized as the fourth group, independent of TRPC4/5 [37]. In this study, we screened and determined the expression levels of TRPC subtypes in HBZY-1 cells. IF results found that TRPC1, 3 and 6 all presented higher expression levels in HBZY-1, while only TRPC1 and 6 showed higher mRNA and protein levels. TRPC3 expression levels were relatively low compared with TRPC1 and 6. TRPC4 and 5 were barely detectable. Furthermore, specific siRNAs targeting TRPC1 and 6 affected cell proliferation molecules, followed by decreased intracellular Ca^{2+} entries and G_0/G_1 cell cycle arrest. Consequently, since TRPC1 and 6 knock-down was sufficient to inhibit cell proliferation, reduce Ca^{2+} influxes and arrest the cell cycle, we did not further examine the effects of other subtypes in HBZY-1 cells.

There are some limitations in our studies. First, TRPC3, 6 and 7 were shown to be heteromultimers due to their sensitivity to DAG [38]. Moreover, TRPC3 and 6 may compensate for the loss of each other in knock-out strategies, which was observed in previous studies performed on TRPC6^{-/-} mice; this may compensate for and obscure the functional deficiency [39, 40]. In addition, we did not conduct experiments to observe compensatory phenomenon concerning TRPC3 in HBZY-1 cells treated with specific TRPC6 siRNA. The transfection efficiency of siRNAs in HBZY-1 cells was 77.4% and did not completely inhibit the expression of TRPC6. The mRNA and protein levels of TRPC6 only decreased 65.0% and 56.1%, respectively. Hence, we could not exclude possible roles of TRPC3, 4 and 5 in cell proliferation and other cellular functions.

Second, our study indicated that RSG suppressed AngII-stimulated proliferation of HBZY-1 cells via the $\text{AT}_1\text{R}/\text{G}\alpha_q/\text{PLC}\beta_4/\text{TRPC}$ signaling cascade. Nevertheless, as a second messenger, Ca^{2+} is required to regulate cell cycle progression. Continuous Ca^{2+} entries through the TRPC1 and 6 channels may activate the calcineurin/NFAT (nuclear factor of activated T cells) signaling pathway and then promote proliferation of T lymphocytes [41]. Additionally, translocation of NFATc1 via the TRPC6 channel was also found to be essential in neonatal pig glomerular mesangial cells, although activation of TRPC6 was shown to inhibit cell proliferation and promote cell apoptosis in neonatal pig glomerular mesangial cells [42]. We believe that different animal species and cell conditions may have different profiles of TRPC isoforms, which may result in substantially different consequences. Taken together, it is plausible that the calcineurin/NFAT mechanism may also be involved in Ca^{2+} -mediated proliferation of HBZY-1 cells.

Lastly, cell proliferation is precisely regulated by various molecules, including cyclin, cyclin dependent kinase (CDK) and CDK inhibitors (CKI) [43]. SKP2 is a S phase kinase-interacting protein and is involved in proteolysis of target P21 and P27 [44-46]. P21 and P27 are vital members of CKIs and can block the cell cycle in G_0/G_1 phase by negatively regulating

CDK2/cyclinE and CDK2/cyclinA, respectively [46, 47]. To gain further insight into the potential mechanisms involved in the proliferation of HBZY-1 cells, cell cycle conditions and PCNA, SKP2, P21 and P27 molecules were detected. The results indicated that the pharmacological effect of RSG in HBZY-1 cells was higher in G₀/G₁ phase versus S phase and this was consistent with the TRPC1 and 6 siRNA treatment groups. Gene expression levels of PCNA and SKP2 were elevated in AngII and were down-regulated by treatment with RSG and specific siRNAs of TRPC1 and 6, while P21 and P27 demonstrated the reverse results. Thus, we believe that these issues require further clarification.

In summary, to the best of our knowledge, our results for the first time indicate that activation of PPAR-γ by RSG suppresses proliferation of HBZY-1 cells induced by AngII via the AT₁R/Gαq/PLCβ4/TRPC signaling pathway. These findings may be due to decreased intracellular Ca²⁺ entry and cause G₀/G₁ cell cycle arrest. PPAR-γ agonists and TRPC inhibitors might be promising therapeutic targets that could ameliorate mesangial proliferative glomerulonephritis and slow the progression of CKD in patients.

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Disclosure Statement

The authors declare there are no conflicts of interest.

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