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TRPV4 regulates migration and tube formation of human retinal capillary endothelial cells

Lei Wen[1,2], Yue-Chun Wen[2], Gen-Jie Ke[2], Si-Qin Sun[2], Kai Dong[2], Lin Wang[2] and Rong-Feng Liao[1*]

Abstract

Background: Ca^{2+} entry plays an important role in modulating endothelial cell migration and tube formation. Transient receptor potential cation channel subfamily V member 4 (TRPV4) is a Ca^{2+}-permeable channel that is widely expressed in endothelial cells. It has been reported that TRPV4 is expressed in HRCECs and regulates Ca^{2+} entry. However, the function of TRPV4 in human retinal capillary endothelial cells (HRCECs) remains unknown.

Methods: In this study we used western blot and immunostaining assay to verify TRPV4 expression in HRCECs. And then we pretreated HRCECs with HC067047 and transfected with specific shRNA of TRPV4. The functional presence of TrpV4 was determined by using fluorescence, migration and tube formation assay in TrpV4 knockdown cells or control cells.

Results: Using western blot and immunostaining, we confirmed TRPV4 expression in HRCECs. Moreover, inhibition of TRPV4 using the specific inhibitor HC067047 and the knockdown of TRPV4 with shRNA significantly suppressed tube formation and migration by HRCECs.

Conclusions: TRPV4 is essential for HRCEC migration and tube formation, and maybe a potential therapeutic target for retinal vascular diseases.

Keywords: TRPV4, HRCEC, Migration, Tube formation

Background

As a second messenger, Ca^{2+} plays important roles in cellular functions such as migration, tube formation, and proliferation [1]. To date, > 30 transient receptor potential (TRP) channels have been studied. They are divided into seven subfamilies: TRPA (ankyrin), TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPN (NOMPC-like) [1, 2]. They are Ca^{2+}-permeable, nonselective cation channels, which are widely expressed in endothelial cells (ECs) [3, 4]. They can be activated by a wide variety of stimuli (osmotic, mechanical, and chemical). Now, increasing research has shown that TRPV4 is important in regulating Ca^{2+} influx and plays vital roles in endothelial function. For example, flow-induced TRPV4 activation in lung ECs leads to lung permeability edema [5]; TRPV4 activated by H_2S in mouse aortic ECs regulates vasodilation [6]; TRPV4 plays a minor role in controlling endothelial progenitor cell proliferation [7]; and H_2O_2 induces Ca^{2+} entry via TRPV4 in lung microvascular ECs [8].

Recently, several studies have focused on the function of TRPV4 in the retinal microvascular endothelium of rats and mice [9]. However, human-derived ECs have not been studied. During retinal development, the activation and migration of ECs are important in forming tubular structures. However, retinal neovascularization is also a characteristic pathological consequence of many retinal diseases, including retinopathy of prematurity [10], diabetic retinopathy [11], and retinal vein occlusion [12].

In previous studies, we showed that TRPV4 regulates flow-induced endothelial Ca^{2+} entry [13] and vascular function [14]. Besides, the TRPV4-KCa2.3 signaling

* Correspondence: liaorfayfy@126.com
[1]Department of Ophthalmology, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei, Anhui 230022, China
Full list of author information is available at the end of the article

pathway plays an important role in smooth muscle hyperpolarization and relaxation and may be involved in endothelium-derived hyperpolarizing factor dysfunction in diabetic rats [15]. Here, we show that TRPV4 is expressed in human retinal capillary ECs (HRCECs) and is essential for their migration and tube formation.

Methods

Cell culture

Human retinal capillary endothelial cells (HRCECs) (BeNaCultureCollection, Wuhan, China) and human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were used between passage 10 and 15 and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS)(Sijiqing, Hangzhou, Chna) with 100 U/mL penicillin and 0.1 mg/mL streptomycin (Beyotime, shanghai, China) at 37°C in a humidified incubator under 5% CO_2 [16].

Western blotting

HRCECs and HEK293 were harvested and washed three times in phosphate-buffered saline (PBS). The cells were lysed in RIPA buffer (P0013C, Beyotime, shanghai, China) supplemented with 1% phenylmethylsulfonyl fluoride (Beyotime, shanghai, China) to obtain the proteins, which were separated on SDS-PAGE (10%), and transferred to PVDF membranes. The membranes were incubated with primary antibodies against TRPV4 (1:200, Alomone, Israel) [17] and β-actin (1:3000)(santa cruz biotechnology, California, USA) at 4°C overnight. Then the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the blots were developed with an enhanced chemiluminescence reagent (P0018, Beyotime). Images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD) [16].

Immunostaining assay

HRCECs were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100(Invitrogen, Grand Island, NY, USA) for 10 min at room temperature. After blocking with 5% bovine serum albumin in PBS for at least 30 min at room temperature [18], the cells were incubated with specific primary antibodies against TRPV4 overnight at 4°C, and then with Alexa Fluor secondary antibodies(Invitrogen, Grand Island, NY, USA) for 2 h at room temperature. Cells were washed five times in PBS after each step. The images were captured using a confocal microscope.

Ca^{2+} level measurement

HRCECs,which were grown to 80% confluence, were first pretreated with or without 10 μM HC067047 for 30 min. After that, 10 μmol/L Fluo-4 was loaded into them. Then, the cells were stimulated with 1 nM, 3 nM, 10 nM, 30 nM, 100 nM GSK101670A (G0798, Sigma, Deisenhofen, Germany) and the fluorescent signals were recorded every 7 s using a fluorescence imaging system (IX71 inverted microscope, Olympus) [19].

Migration assay

Transwell chambers with a 0.4-μm pore size membrane. Cells were seeded at 3.8×10^4 in 100 μl serum-free DMEM in the top of a transwell chamber (353,097, corning, NY, USA) [20]. DMEM with 10% FBS (100 μl) was added to the lower chamber. After 48 h incubation at 37°C in a humidified atmosphere of 5% CO_2, cells migrated to the lower surface of the chambers. And then, the chambers were removed and washed twice in PBS. The cells were fixed in 4% paraformaldehyde for 30 min and stained with crystal violet. Non-migrating cells were removed from the upper chamber with cotton-tipped swabs. Images were captured with a video camera (Coolpix 54, Nikon, Japan).

Tube-formation assay

50 μl Matrigel (354,234, BD Bioscience) was added to each well of a 96-well plate and incubated for 30 min at 37°C in a humidified atmosphere of 5% CO_2 [19]. Until matrigel was polymerized, Cells (4×10^4 in 100 μl DMEM) were seeded in each well. Capillary-like tubes were formed within 8 h and recorded with the video camera (Coolpix 54, Nikon, Japan).

Cell transfection assay

HRCECs for infection were plated in 6-well plates, after 24 h, 100 μl lentivirus-3(LV3) packed LV3-TRPV4 shRNAs (GenePharma, Suzhou, China) were added to the cells. After 3 days, the cells can be used for further experimental study. HEK293 were transfected with empty vector and TRPV4 plasmid, using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) as previously described [13, 21].

Results

TRPV4 expression in HRCECs

To confirm that TRPV4 is expressed in HRCECs, we performed western blots (Fig. 1a) and immunostaining assays (Fig. 1b) [22] on cell lysates of HEK293 cells and HRCECs. All the data showed that HRCECs express TRPV4.

TRPV4 regulates Ca^{2+} entry into HRCECs

Our previous experiments had demonstrated that TRPV4 is expressed in HRCECs, so we then investigated its function using Ca^{2+} measurements. We found a robust increase in intracellular Ca^{2+} levels after

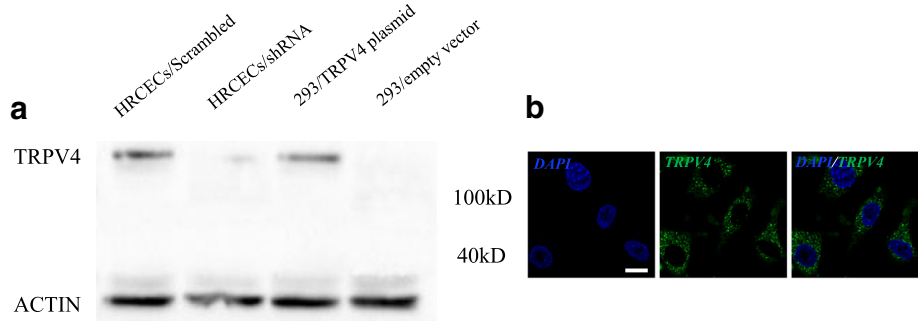

Fig. 1 TRPV4 is expressed in HRCECs. **a** Western blot analysis. **b** Immunofluorescence using confocal microscope. Shown are representative nuclear DAPI staining (Left, blue), TRPV4 staining (center, green)merged images (Right). scale bar: 25 μm

stimulating cells with 100 nM GSK (a specific TRPV4 agonist) (Fig. 2a, c), while pretreatment with HC067047 (a specific TRPV4 antagonist) [23] significantly inhibited this effect (Fig. 2b, c). These data showed that TRPV4 is important in GSK-induced Ca^{2+} influx into HRCECs.

Effect of TRPV4 on HRCEC migration

To evaluate the role of TRPV4 in HRCEC migration, we performed transwell assays with or without a TRPV4 inhibitor. The results showed increased Ca^{2+} influx in GSK-stimulated HRCECs (Fig. 3). However, this effect was blocked when HRCECs were pretreated with the specific TRPV4 inhibitor HC067047 and the specific shRNA. These results demonstrated that TRPV4 plays a key role in the migration of HRCECs.

Effect of TRPV4 on HRCEC tube formation

In addition to migration, we studied the role of TRPV4 in tube formation by HRCECs. As expected, GSK increased tube formation (Fig. 4b), while pretreatment with HC067047 and shRNA blocked the effect (Fig. 4c, d). The results showed that almost no tubular structures formed after co-incubation of HRCECs with HC067047 and GSK, and also we verified the same results with HRCECs, which were transfected with specific shRNA of TRPV4. (Fig. 4f, g). All these results suggested that TRPV4 is involved in tube formation by HRCECs.

Discussion

In this study, we assessed the role of TRPV4 in regulating HRCEC migration and tube formation. Using western blotting and immunostaining, we first confirmed that TRPV4 is expressed in HRCEC. The results of Ca^{2+} imaging demonstrated that TRPV4 regulated Ca^{2+} influx in HRCECs. By using the specific TRPV4 inhibitor HC067047 and using shRNA, we showed that inhibition or knockdown of TRPV4 significantly suppressed migration and tube formation by HRCECs.

Recently, several studies have demonstrated that TRP channels play vital roles in retinal epithelial, corneal, and endothelial cells. For instance, the decreased level of TRPV4 induced by hyperglycemia and diabetes contributes to diabetes-induced endothelial dysfunction and retinopathy in retinal microvascular endothelium [9]. Thermo-sensitive TRPV4 activation protects human corneal ECs [24]. Activation of TRPV4 modulates the extracellular melatonin in human non-pigmented ciliary ECs [25]. Activation of TRPV4 is necessary for the correct establishment of tight junctions in corneal epithelia as well as the regulation of both the barrier function of

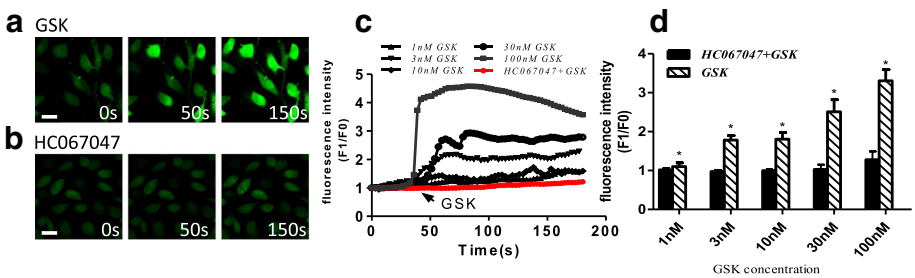

Fig. 2 TRPV is important for GSK-induced Ca^{2+} influx. **a**, **b** Representative Ca^{2+} images of HUCECs stimulated by GSK with (**a**) and without (**b**) HC067047 treatment. **c** Representative images of of time course of intracellular Ca^{2+} levels (F1/F0). **d** Quantification of time course of intracellular Ca^{2+} levels (F1/F0) as in A and B ($n \geq 6$). (scale bar, 25 μm). Mean ± SEM, ($n \geq 6$; *$p < 0.05$, compared with HC067047 + GSK)

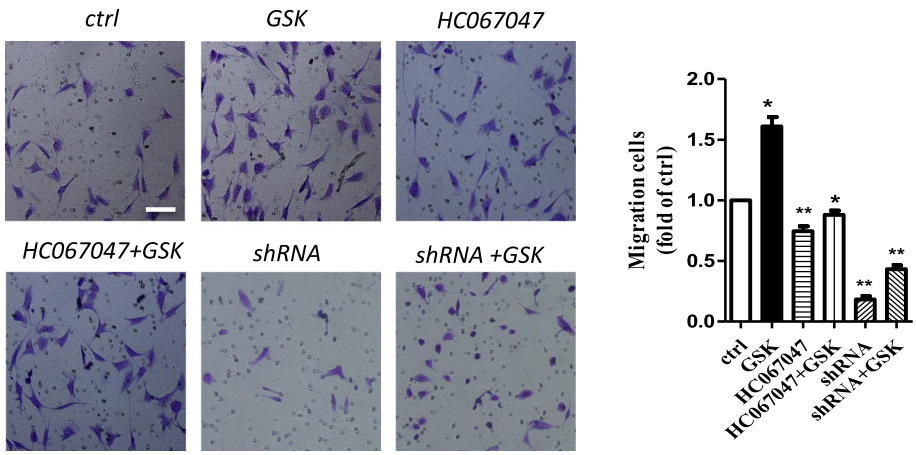

Fig. 3 TRPV4 plays a role in HRCEC migration. Left panels: representative images of migration assays of HRCECs (scale bar, 25 μm). Right panel: data analysis. Mean ± SEM, ($n = 6$; *$p < 0.05$, **$p < 0.01$ compared with control)

tight junctions and their ability to respond to epidermal growth factor [26]. However, no studies had focused on TRPV4 function in HRCECs. This is the first study, to our knowledge, to examine the role of TRPV4 in regulating HRCEC function.

It is well known that TRPV4-regulated Ca^{2+} influx is important for endothelial migration and tube formation, which are vital components of angiogenesis [27]. Also, dysfunctional angiogenesis may lead to several retinal diseases [1, 28]. For example, functional expression of TRPV4 in retinal microvascular ECs are reduced in hyperglycemia and diabetes [9]; activation of TRPV4 in retinal ganglion cells leads to glaucoma and retinal detachment [29]; and TRPV4 is a target for treating ocular hypertension and conventional outflow by regulating Ca^{2+} homeostasis and cytoskeletal remodeling [30]. These studies inspired us to test the hypothesis that TRPV4 is associated with human retinal diseases and can it be a potential therapeutic target.

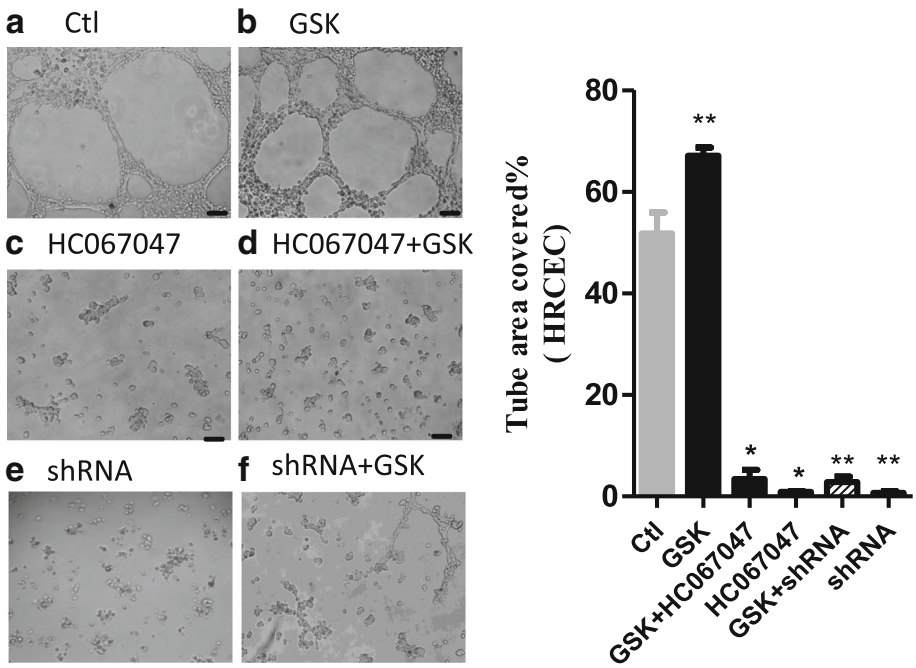

Fig. 4 TRPV4 is necessary for tube formation. HRCECs were seeded on matrigel and visualized 8-12 h late. **a** In control condition. **b** Treated with GSK. **c** treated with HC067047. **d** treated with HC067047 and GSK. **e** treated with shRNA. **f** treated with shRNA and GSK. Data analysis shown in the right. (scale bar, 200 μm). Mean ± SEM, ($n \geq 10$; *$p < 0.05$, **$p < 0.01$ compared with control)

Conclusions

In summary, we have provided direct evidence of functional TRPV4 expression in HRCECs and demonstrated that TRPV4 is involved in migration and tube formation in HRCECs.

Additional file

Additional file 1: Raw materials of western blot, what I used is framed in the red box. Raw materials of transwell and tube formation, every first picture is what I used in the article. (PPTX 10465 kb)

Abbreviations

DMEM: Dulbecco's modified Eagle's medium; ECs: Endothelial cells; FBS: Fetal bovine serum; HEK293: Human embryonic kidney 293 cells; HRCEC: Human retinal capillary endothelial cells; PBS: Phosphate-buffered saline; TRPV4: Transient receptor potential cation channel subfamily V member 4

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional file 1.

Authors' contributions

LW was a major contributor in writing the manuscript. YCW SQS, KD, LW, ZYW and GJK were major contributors in data processing. RFL was a major contributor in modify the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Author details

[1]Department of Ophthalmology, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei, Anhui 230022, China. [2]Department of Ophthalmology, Anhui Provincial Hospital, Hefei, Anhui, China.

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