• Basic Research •

Blockade of insulin receptor substrate-1 inhibits biological behavior of choroidal endothelial cells

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Abstract

• AIM: To investigate the effects of blockade of insulin receptor substrate-1 (IRS-1) on the bio-function of tube formation of human choroidal endothelial cells (HCECs).

• METHODS: Quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were performed to determine the expression level of IRS-1 and phospho-IRS-1 in HCECs. Tube formation of HCECs was analyzed using three dimensional *in vitro* Matrigel assay with or without IRS-1 blockage *via* IRS-1 inhibitor (GS-101) and vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor. In addition, cell counting kit (CCK)-8 and Transwell migration assay were exerted to analyze the effects of blockade of IRS-1 on the bio-function of proliferation and migration of HCECs, respectively. The apoptosis of HCECs was examined using flow cytometry (FCM).

• RESULTS: RT-PCR and Western blot revealed that IRS-1 phospho-IRS-1 were expressed in HCECs and the expression level was enhanced by stimulation of VEGF-A. The number of tube formation was decreased significantly in GS-101 treated groups compared to phosphate buffered saline (PBS) treated control groups. Furthermore, both cell proliferation and migration of HCECs were decreased in the presence of GS-101. FCM analysis showed that the apoptosis of HCECs was enhanced when the cells were treated with GS-101. Western blot also showed that the expression level of cleaved-caspase 3 in GS-101 treated group was higher than that in control group.

• CONCLUSION: Blockade of IRS-1 can inhibit tube formation of HCECs through reducing cell proliferation and migration and promoting cell apoptosis.

• KEYWORDS: insulin receptor substrate-1; choroidal endothelial cells; neovascularization; proliferation

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INTRODUCTION

Choroidal neovascularization (CNV), known as a crucial late complication for pathological myopia and wet age-related macular degeneration (AMD), is characterized by new capillary vessels growing in choroid and breaking through Bruch's membrane and further growing into the subretinal space, frequently causing severe visual impairment[1-4]. It is well known that targeting on vascular endothelial growth factor (VEGF)-A is currently the most efficient treatment to exudative AMD in clinical settings, despite numerous regulatory factors involved in angiogenic, inflammatory and immune modulatory cascade have been confirmed in CNV occurrence and development[5-8]. However, anti-VEGF agents can also induce local and systemic side effects[9]. Therefore, in order to create novel therapeutic targets, it is necessary to obtain better knowledge of the mechanisms of CNV development[10].

Insulin receptor substrate (IRS)-1, currently known as the first indentified member of IRS protein (or cytoplasmic adaptor proteins) family, is extensively expressed in various mammalian cells[11]. It functions as crucial ligand in triggering insulin-induced response in human cells by binding to its cognate receptor[11-12]. Like other members in IRS protein family, IRS-1 has no intrinsic enzymatic property but it can be activated after phosphorylation[12]. It plays key role in lifespan determination and cellular stress resistance[13-14], adipogenesis[15], glucose homeostasis[16-17] and cancer metabolism[18] under physiological and pathological conditions. Accumulating evidence indicated that IRS-1 has an important

role in occurrence and development of neovascularization in some ocular neovascularization diseases[19-21]. It was reported that the occurrence of experimental CNV was restrained in IRS-1 knockout mice when compared to that in wild type mice[21]. Furthermore, there was evidence also showing that application of the antisense oligonucleotide targeting on human IRS-1 mRNA, GS-101 (5'-TATCCGGAGGGCTCG CCATGCTGCT-3'), acting as IRS-1 inhibitor, can suppress corneal neovascularization significantly both in experimental corneal neovascularization animal models and patients with keratitis[22-24]. These findings have proven and highlighted the anti-angiogenic efficacy of IRS-1 inhibitor in treating ocular neovascularization diseases.

Though IRS-1 has an important role in some ocular neovascularization diseases, the mechanism underlying IRS-1 promoting neovascularization or IRS-1 inhibitor suppressing neovascularization in this process has not yet been fully elucidated. Some reports revealed that the pro-angiogenic effects of IRS-1 may be related to interaction with VEGF-A[21,25] and integrin signaling[26-27], but it still need further exploration to verify and delineate the mechanism of this process. In present work, we detected the influences of blockade of IRS-1 on capabilities of tube formation, proliferation and migration of HCECs, and further examined the gene and protein expression of cytokines associated with tube formation of HCECs, then analyzed the exact mechanisms of blockade of IRS-1 affecting the bio-function of HCECs in this process. Our findings provide a novel insight into the mechanism of IRS-1 being involved in ocular neovascularization.

MATERIALS AND METHODS

Reagents And Antibodies The cell line of HCECs were obtained from Yaji Biological Technologies (Shanghai, China). IRS-1 inhibitor of GS101 was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Annexin V-FITC Assay Kit (cat. No.556547) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). The cell counting kit (CCK)-8 was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). Trypsin-EDTA was obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The total RNA extraction kit (RNeasy Mini kit) and reverse transcription kit (Ominiscript RT kit) were obtained from Qiagen Sciences, Inc. (Frederick, MA, USA). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories; GE Healthcare Life Sciences. Transwell plates with 8.0 μm pore polycarbonate membrane insert were obtained from Corning Life Science (New York, USA). Mouse anti-human phospho-IRS-1 antibody (cat. No.3105-100) as well as rabbit anti human IRS-1 antibody (cat. No.3424-100) were purchased from BioVision (Milpitas, CA, USA). Rabbit anti- human phospho-vascular endothelial growth factor receptor 2 (VEGFR2) antibody (cat. No.44-1052), rabbit anti-human VEGFR2 antibody (cat. No.MA5-15157), rabbit anti-human cleaved-caspase 3 (cat. No.PA5-23921) and rabbit anti-human caspase 3 antibody (cat. No.700182) were obtained from Thermo Fisher Scientific (Waltham MA, USA). Mouse anti-human VEGFR2 antibody (cat. No.GTX53462) was obtained from GeneTex (Irvine, CA, USA). Mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (cat. No.AF0006) was obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Cell Culture Human choroidal endothelial cells (HCECs) were cultured in 5 mL DMEM medium (Gibco, Shelton, CT, USA) containing 10% FBS (Gibco) and incubated in 37℃ incubator with humidified atmosphere of 5 percent CO_2 and 95 percent air[28]. Fresh culture medium was then added into the HCECs the next day. After incubation for 3-4d, confluent cells were passaged at a 1:4 dilution, and culture medium was renewed every other day. Functional assays in this study were performed using the cells in their logarithmic growth phase. In some experiments, cells were treated with VEGF-A and IRS-1 inhibitor (GS-101) at indicated concentrations or phosphate buffered saline (PBS) as control for 12 or 24h. The proliferation assay and migration assay were conducted in serum-free DMEM.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described in detail previously[29]. Total RNA from the HCECs was isolated using an RNeasy Mini kit (Qiagen, Inc. Frederick, MA, USA) and cDNA was synthesized with the PrimeScript™ RT Master mMix (TaKaRa Biotechnology Co., Ltd., Dalian, China). The mRNAs encoding *IRS-1*, *VEGF-A*, *VEGFR2* as well as *GAPDH* were amplified using appropriate convenient primers. The sequences of the PCR primer pairs are listed in Table 1. Amplification of PCR was performed using a GeneAmp® PCR System 9700 (Perkin-Elmer, Foster City, CA, USA). The PCR parameters involved in initial denaturation at 94℃ for 2min, followed by 37 or 25 (for *GAPDH*) cycles of denaturation at 95℃ for 30s, annealing at 58℃ for 35s and extension at 72℃ for 35s, and a final extension at 72℃ for 10min. Each sample was assayed in triplicate for both target and internal control (*GAPDH*) genes. These PCR products were fractionated on a 1.0% agarose gel and visualized using ethidium bromide. The intensities of the

Table 1 Sequences of the primers used for reverse transcription-polymerase chain reaction analysis

Gene	Sequence (5′-3′)	Annealing temperature (℃)	Cycles (*n*)
IRS-1	(F) GCAACCAGAGTGCCAAAGTG	58	37
	(R) CCTCTGGCTGCTTCTGGAAA		
VEGF-A	(F) TGGTCCCAGGCTGCACCCAT	58	37
	(R) CGCATCGCATCAGGGGCACA		
VEGFR2	(F) GGTACATGCCAACGACACAG	58	37
	(R) CTCAAAGTCTCTCACGAACACG		
GAPDH	(F) ACCACAGTCCATGCCATCAC	58	25
	(R) TCCACCACCCTGTTGCTGTA		

F: Forward primer; R: Reverse primer.

bands were determined and their ratios to *GAPDH* determined using Image J software, version 2.1.4.7 (National Institutes of Health, Bethesda, MD, USA).

Tube Formation Assay The *in vitro* capillary-like tube formation assay for assessment of the effect of blockade of IRS-1 on the HCECs was examined using matrigel matrix as described in a previous report with some modifications[30]. Briefly, a 96-well plate was incubated on ice and coated with 50 μL per well of fully thawed Matrigel™. The samples were centrifuged at 300×g for 10min at 4℃ to remove the air bubbles. The samples were subsequently incubated at 37°C for 30min in order to allow matrigel solidification. HCECs were cultured in different medium with or without GS-101 and/or VEGFR2 inhibitor. The cells were seeded on the solidified matrigel immediately at a density of 1.5×10^4 cells per well. The plates were placed in a humidified atmosphere of 5% CO_2 and 95% air at 37℃ for 12h to allow formation of capillary-like structures. Angiogenesis is the formation of capillary tubes and was assessed following 12h of cultivation. The tube-like capillary structures were examined under an Olympus TMS inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan). The micrographs were captured using an Olympus digital camera.

Western Blot Analysis As described in detail previously[31], HCECs were harvested using 0.25% Trypsin-EDTA. The supernatant was discarded and lysed in 150 mL lysis buffer, to which a protease inhibitor cocktail was added (Boehringer Mannheim, Indianapolis, IN, USA). The samples were then boiled for 5min and separated using 12.5% SDS-polyacrylamide gel electrophoresis under denaturing conditions. It was then electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were finally incubated at room temperature (RT) for 1h with the following antibodies: Anti-phospho-IRS-1 (1:200), anti-IRS-1 (1:200), anti-phospho-VEGFR2 (1:1000), anti-VEGFR2 (1:1000), anti-cleaved-caspase 3 (1:1000), anti-caspase 3 (1:1000) and anti-GAPDH (1:5000) antibodies. The immunoblot assays were then washed with PBST and incubated at RT for 1h with a horseradish peroxidase-labeled secondary antibody (1:10 000; cat. No.5196-2504 or 5178-2504; R&D Systems; Hercules, CA, USA). Enhanced chemiluminescence was used to visualize the blots (ECL Plus; Amersham; GE Healthcare Life Sciences) according to the manufacturer's protocol. The intensities of the protein bands were determined and their ratios to GAPDH determined using Image J software, version 2.1.4.7 (National Institutes of Health, Bethesda, MD, USA).

Proliferation Assay To evaluate the effect of GS-101 on the proliferation of HCECs, we carried out the cell CCK-8 assay as described in detail previously[32]. HCECs were seeded in a 96-well plate (2×10^3 cells per well). The cells were then treated with or without IRS-1 inhibitor of GS-101. Following incubation for 24h, the medium was replaced with fresh DMEM containing CCK-8 (10 μL per well). The cells were subsequently incubated for an additional 2h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The inhibition rate (IR) of the proliferation of cells in the groups was compared with the control group.

Migration Assay To evaluate the effect of blockade of IRS-1 on the migration of HCECs cells, a modified Boyden chamber assay was performed as described previously[33]. Briefly, 1×10^4 cells in 100 μL DMEM medium were seeded in the upper chambers. After 24h incubation, the medium was replaced and fresh DMEM medium was added. Totally 500 μL of DMEM medium were added in lower chambers and treated with GS-101 for another 24h. The migrated HCECs were fixed prior to staining with 0.5% crystal violet solution. Non-invading cells were swapped with a cotton swab. The infiltrated cells were counted under a phase contrast microscopy. Each assessment of each experimental group was repeated several times.

Flow Cytometrical Analysis Apoptosis was assayed by using the dual staining with Annexin V: FITC (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (PI)[34]. Briefly, cells were harvested at 24h post-addition GS-101. Annexin V: FITC and PI were added to the cellular suspension, according

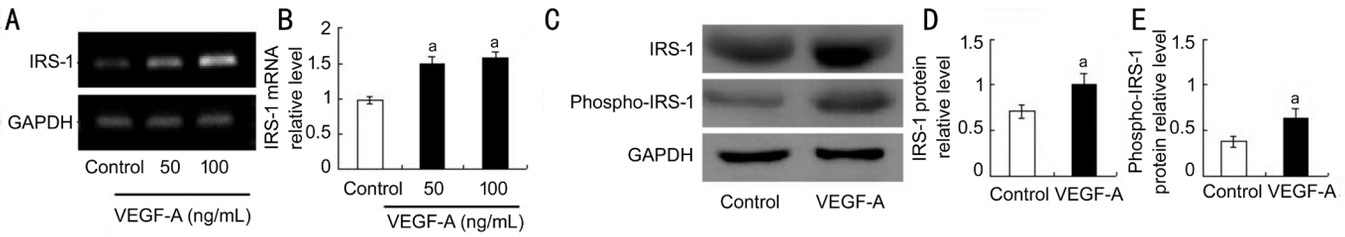

Figure 1 Expression (gene and protein) of IRS-1 in HCECs A: Semi-quantitative RT-PCR analysis was applied to evaluate the mRNA expression of IRS-1; B: Quantitative data of the ratio from three independent experiments; C: Representative Western blot results from three independent experiments; D, E: Ratios of IRS-1 and phospho-IRS-1 to GAPDH protein bands in the control and VEGF-A groups were determined. All values are presented as the mean±standard error of the mean (n=3). ^aP<0.05.

to the manufacturer's instructions, and were analyzed using a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA). Early apoptotic cells were counted for relative apoptotic changes. All experiments were performed at least three times. Fluorescence intensities were determined with the help of FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical Analysis All data were expressed as mean± standard error of the mean (SEM) and analysed statistically by Student's *t*-test (two-tailed) between two groups or by one-way analysis of variance (ANOVA) with Tukey's multiple comparison within multiple groups with statistic software SPSS 18.0 (USA). A value of P<0.05 was considered as statistically significant. Each experiment was independently repeated at least 3 times.

RESULTS

Gene and Protein Levels of IRS-1 in HCECs RT-PCR analysis and Western Blot assay revealed that the gene level of IRS-1 and protein level of IRS-1 as well as phospho-IRS-1 in HCECs was enhanced in VEGF-A-treated groups compared to PBS-treated control group (Figure 1). These results suggested that IRS-1 may have a role in bioactivities of HCECs and is a possible target molecule to inhibit the activities. The observation of the expression of IRS-1 along with phospho-IRS-1 in above HCECs suggested the possible involvement of IRS-1 in HCEC bio-function (Figure 1).

IRS-1 Signaling Blockade *via* GS-101 Reduced Tube Formation of HCECs To determine whether IRS-1 had a role in activity of tube formation of HCECs, the HCEC cells were seeded in plates of 96 wells which coated with Matrigel and were incubated for 12h, the HCECs then formed capillary-like tubes. The results revealed that the HCECs treated with GS-101 showed declined numbers of tube formation in comparison with the numbers in control group (Figure 2). The results of tube formation, which was quantified and statistically analyzed, indicated that GS-101 suppressed tube formation. In addition, HCECs treated with GS-101 and VEGFR2 inhibitor exhibited more decreased tube formation than the cells treated with 40 μmol/L GS-101 alone, suggesting that VEGFR2 signaling is indispensable for angiogenesis, and the treatment of GS-101 combined with VEGFR2 inhibitor enhanced the anti-angiogenesis effect of blockade of IRS-1 signaling.

Blockade of IRS-1 Suppressed Proliferation of HCECs To evaluate the role of IRS-1 in the bio-activities of HCECs, the effect of blockade of IRS-1 on HCEC proliferation was assessed *in vitro*. The HCECs were incubated in the presence of GS-101 for 24h, and cell viability was subsequently examined using CCK-8 kit. The proliferation rates of HCECs which treated with GS-101 were lower than those in the PBS treated control group (Figure 3). The analysis data of quantified optical density (OD) and IR values verified that GS-101 was capable of suppressing cell proliferation. It proposed that the reduction in the proliferation degree of HCECs resulted from blockade of IRS-1 signaling *via* GS-101 attributed to the IRS-1 potential of promoting tube formation of HCEC *in vitro*.

Blockade of IRS-1 Impaired Migration of HCECs We also carried out a migration assay to verify the effects of blockade of IRS-1 on HCECs. When co-cultured with GS-101, the number of infiltrated HCECs which migrated through the upper chambers of plates decreased compared to control group. The numbers of positive-stained migrated cells in IRS-1 groups were 19±2 (10 μmol/L GS-101 group), 16±1 (20 μmol/L GS-101 group) and 15±2 (40 μmol/L GS-101 group) and 25±2 (per mm^2) in control group (Figure 4). This suggests that Blockade of IRS-1 inhibits migration of HCECs.

Declined Expression Levels of VEGF-A and its Receptor VEGFR2 in GS-101-treated HCECs In this work, the expression (gene and/or protein) levels of angiogenic factors of VEGF-A along with its receptor of VEGFR2 in HCECs were detected. The expression (gene and/or protein) levels of VEGF-A, VEGFR2 and phospho-VEGFR2 were lower in the GS-101 treated cells, in comparison with those in the PBS treated control groups (Figure 5). These *in vitro* results indicated that blockade of IRS-1 may suppress HCEC migration, proliferation and tube formation *via* decreasing angiogenesis by down-regulating the gene and/or protein expression of VEGF-A and its receptor of VEGFR2 and/or phospho-VEGFR2.

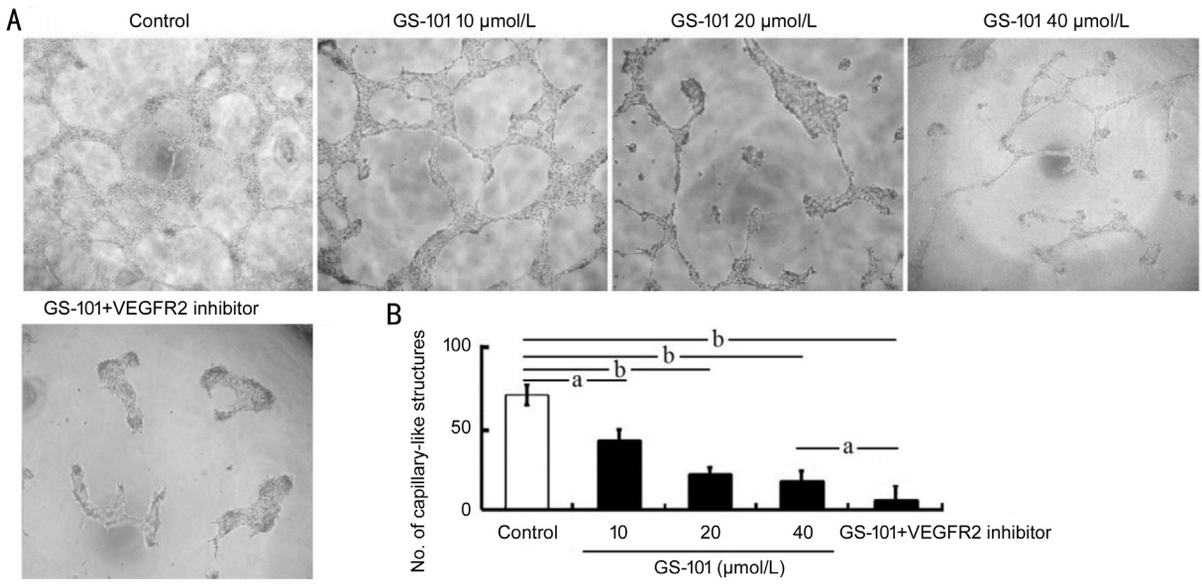

Figure 2 Effect of blockade of IRS-1 on the tube formation of HCECs A: Tube formation assays showed that blockade of IRS-1 significantly suppressed the tube formation of HCECs (magnification, ×200). B: The numbers of capillary-like structures of tube formation were quantified from three independent *in vitro* experiments. All data are showed as the mean±standard error of the mean (n=3). [a]P<0.05; [b]P<0.01.

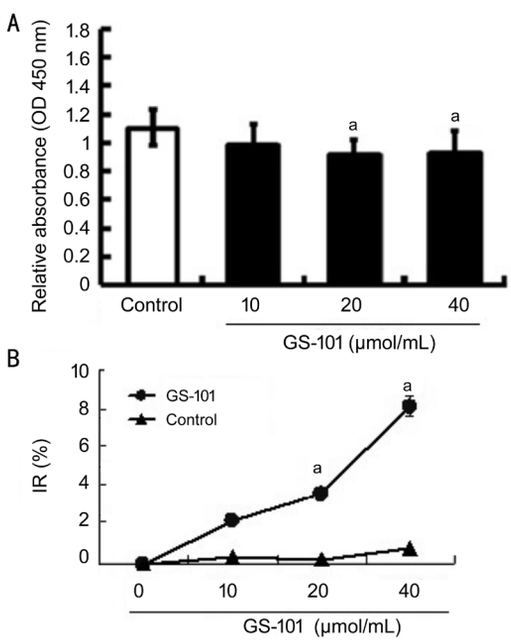

Figure 3 Effect of blockade of IRS-1 on the proliferation of HCECs Cell counting kit-8 assays of examining HCEC proliferation showed that cell proliferation in the GS-101 groups was reduced pronouncedly, in comparison with that in the control group. The data are presented as mean±standard error mean. [a]P<0.05; [b]P<0.01.

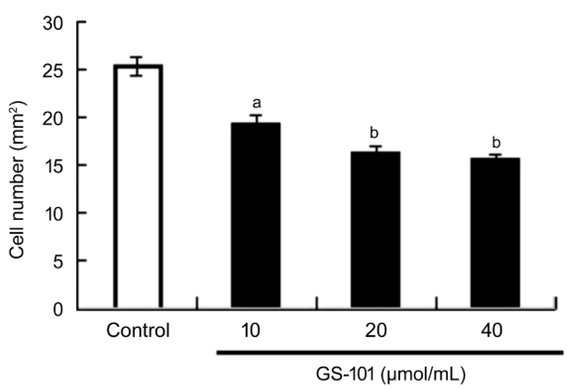

Figure 4 Effect of blockade of IRS-1 on the migration of HCECs Quantitative data of the migrated cells from three independent experiments. All values are presented as the mean±standard error of the mean (n=3). [a]P<0.05 and [b]P<0.01 compared with the control.

Blockade of IRS-1 Increased Apoptosis of HCECs As indicated by flow cytometry (FCM; Figure 6), the GS-101 treated group exhibited higher expression level of FITC-Annexin V in HCECs when compared to the control group, suggesting that blockade of IRS-1 increased apoptosis of HCECs. Additional, the expression level of apoptosis associated protein of cleaved-caspase-3 was also elevated in GS-101 treated group in comparison to control group (Figure 6C). It showed statistically significance between these two groups (Figure 6E). To the best of our knowledge, the level of cleaved-caspase-3 protein expression directly reflects cell apoptosis degree. Thus, our results suggest that GS-101 have negative effect on cell viability by promoting cell apoptosis.

DISCUSSION

The results in present study verified that IRS-1 has an important role in tube formation, proliferation, migration and apoptosis of HCECs, whereas this efficacy of IRS-1 on HCECs was inhibited by IRS-1 inhibitor of GS-101. The results also demonstrated that blockade of IRS-1 affect the bio-function of HCECs through down-regulating VEGF-A/VEGFR2 signaling. Our data implied that IRS-1 would probably be a novel target for treating ocular neovascularization, such as CNV, in clinical settings.

As a signal receptor locating in cytoplasm of various cells, IRS-1 can induce intracellular signaling cascade and subsequently triggering cell bio-activities through its involvement in various

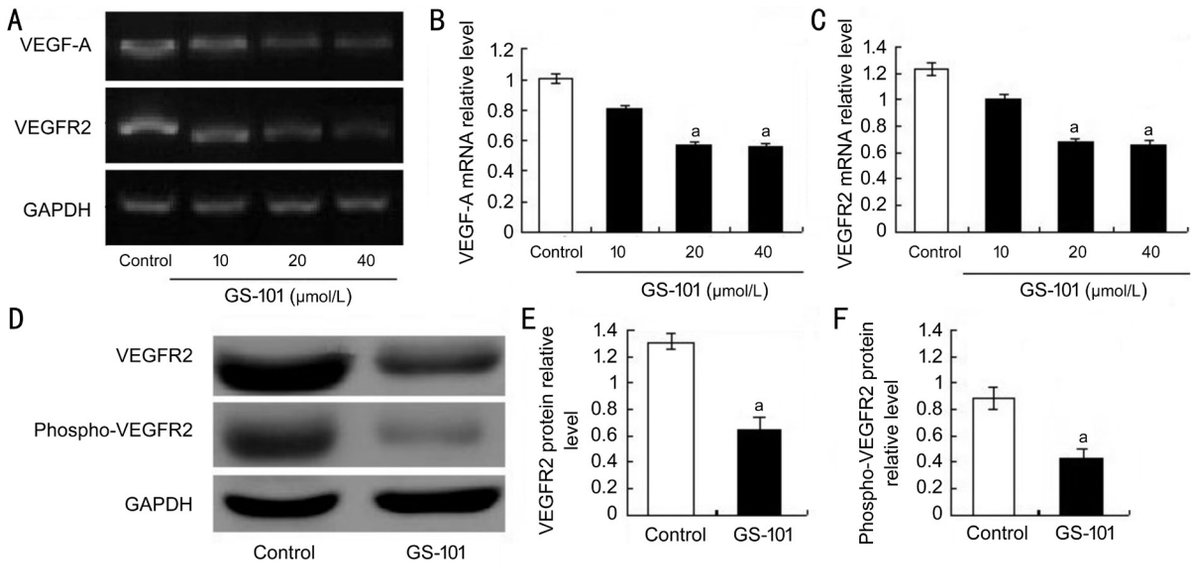

Figure 5 Effect of blockade of IRS-1 on mRNA and protein expression of VEGF-A and VEGFR2 in HCECS A: Semi-quantitative RT-PCR analysis was used to evaluate the mRNA expression of VEGF-A and VEGFR2; B, C: Quantitative data of the ratio from three independent experiments; D: Representative Western blot results from three independent experiments; E, F: Ratios of VEGFR2 and phospho-VEGFR2 to GAPDH protein bands in the control and GS-101 groups were determined. All values are presented as the mean ± standard error of the mean (n=3). ^aP<0.05 compared with the control.

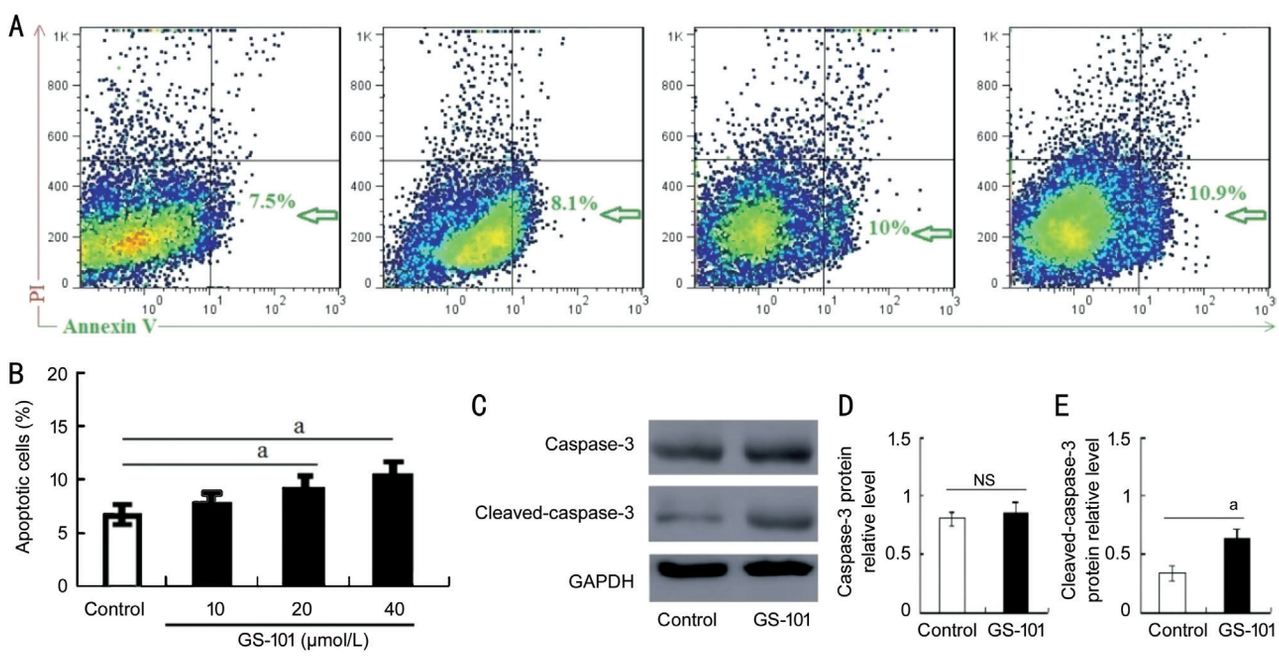

Figure 6 The proportion of Annexin V -positive HCECs and cleaved-caspase 3 expression after treatment with GS-101 A: Representative results from three to four tests of Annexin V positive cells from either control, GS-101 treated HCECs; B: Quantitative data of the ratio from three independent experiments. C: Representative Western blot results from three independent experiments; D, E: Ratios of caspase 3 and cleaved-caspase 3 to GAPDH protein bands in the control and GS-101 groups were determined. All values are presented as the mean±standard error of the mean (n=3). ^aP<0.05 compared with the control.

cellular signal transduction[35]. Bugner *et al*[36] reported that IRS-1 was closely involved in growth of *Xenopus laevis* eye. Some studies also supported that IRS-1 played key role in process of ocular angiogenesis[37]. Berdugo *et al*[22] found that the reduced expression of IRS-1 in subconjunctival tissue by injecting with IRS-1 antisense (IRS-1 antagonist) leads to restrained corneal angiogenesis. Cloutier *et al*[19] revealed that IRS-1 inhibitor have anti-angiogenic potential in suppressing retinal neovascularization (RNV) and CNV when topical administrating with GS-101 in non-human primate and rodent models, suggesting the promoting effect of IRS-1 on RNV or CNV. Additionally, Hos *et al*[38] demonstrated that the blocking effect of GS-101 on IRS-1 signaling in corneas can also inhibit corneal lymph-angiogenesis in animal experiments. These

results together with our findings provided novel insight on IRS-1 pro-angiogenesis or pro-lymph-angiogenesis efficacy in ocular diseases and suggested the latent application for IRS-1 inhibitor to treat ocular neovascularization, such as corneal neovascularization and CNV clinically[37,39].

Several evidences documented that IRS-1 signaling was capable of influence cell migration[40-41] and proliferation when the cells were treated with IRS-1 recombinant protein or IRS-1 inhibitor[42-44]. Besides, other reports revealed that IRS-1 was involved in process of cell apoptosis in breast cancer cells[45] and retinal endothelial cells[46]. In present study, we also carried out relevant *in vitro* experiments to explore the precise mechanism of IRS-1 regulating the tube formation behavior of HCECs. The results verified that blockade of IRS-1 can suppress proliferation and migration, and increase apoptosis of HCECs. These results are consistent with above findings from other reports, suggesting that the anti-angiogenesis effect of blockade of IRS-1 on HCECs tube formation may be through inhibiting cell migration and cell proliferation and promoting cell apoptosis by IRS-1 inhibitor of GS-101.

Since vascular endothelial cells are fundamental for angiogenic process, any components that affect the bio-function of choroidal capillary endothelial cells may alter the development degree of CNV[47-50]. To the best of our knowledge, VEGF-A is the most important factor that promoting vascularization both under physiological and pathological condition. It exerts pro-angiogenesis effects partially *via* facilitating vascular endothelial cell proliferation and migration. For the reason that verifying whether IRS-1 exerts pro-angiogenesis effect through VEGF-A/VEGFR2 signaling pathway, we examined VEGF-A and VEGFR2 expression in HCECs after treating the cells with serial concentrations of IRS-1 inhibitor of GS-101. Our results demonstrated that the gene and protein of VEGF-A is expressed in HCECs and decreased by IRS-1 inhibitor treatment in a dose-dependent way. In addition, the protein level of VEGFR2/phospho-VEGFR2 in HCECs, a receptor for VEGF-A, was also declined when the IRS-1 signaling was blocked by the inhibitor of GS-101. It suggested that blockade of IRS-1 exerting anti-angiogenesis effect may be, at least partly, through VEGF-A/VEGFR2 signaling pathway in the way of down-regulating the levels of VEGF-A and VEGF-A-cognate receptor of VEGFR2/phospho-VEGFR2.

It is well known that migration and proliferation of vascular endothelial cells were initial and vital steps for angiogenesis[51-53]. The angiogenic cascade involves in many complicated and integrated sequential steps, in which vascular endothelial cells proliferation and migration are initial steps in the process of angiogenesis, followed by vascular endothelial cells behaviors of establishing and developing a capillary-like structure[54-55]. Accordingly, the results in this work showed that blockade of IRS-1 exerted negative role in the bio-functions of proliferation and migration of HCECs, and enhanced apoptosis of HCECs, suggesting blockade of IRS-1 signaling exerted anti-angiogenic effects on HCECs *via* in-activating the initial steps of angiogenic cascade.

To summarize, the results of present study confirmed a novel biological role for IRS-1 in HCECs. Blockade of IRS-1 suppressed capillary tube formation through inhibiting cell migration and proliferation of the initial step of angiogenic cascade. The effects may be, at least partly, through down-regulating the expression level of VEGF-A and VEGFR2/phospho-VEGFR2 (VEGF-A receptor), along with promoting cell apoptosis of HCECs. These findings indicate the potential of anti-angiogenesis by GS-101 in HCECs, which may assist to clinical treatment on ocular neovascularization in future.

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