

# Occludin downregulation in high glucose is regulated by SSTR<sub>2</sub> via the VEGF/NRP1/Akt signaling pathway in RF/6A cells

MENGLING LI<sup>1</sup>, SHUAIWEI WANG<sup>1</sup>, SONGJIANG WANG<sup>1</sup>, LEI ZHANG<sup>1</sup>, DONGDONG WU<sup>1</sup>, RUISHENG YANG<sup>2</sup>, AILING JI<sup>1</sup>, YANZHANG LI<sup>1</sup> and JUN WANG<sup>1</sup>

<sup>1</sup>Department of Physiology, Henan University School of Medicine, Kaifeng, Henan 475004; <sup>2</sup>Department of Clinical Laboratory, The First Affiliated Hospital of Henan University, Kaifeng, Henan 475001, P.R. China

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**Abstract.** Occludin is a tight junction protein that forms the permeability barrier, which is typically disturbed in ischemic associated diseases. The aim of the present study was to determine whether somatostatin receptor 2 (SSTR<sub>2</sub>) in RF/6A cells is involved in the modulation of the downregulation of occludin induced by high glucose, and to evaluate the implicated molecules. RF/6A cells were maintained in Dulbecco's modified Eagle medium and treated with 0 or 30 mM D-glucose. SSTR<sub>2</sub> agonist octreotide (OCT), OCT with SSTR<sub>2</sub> antagonist cycle-somatostatin (c-SOM) and neuropilin 1 (NRP1) inhibitor ATWLPPR, respectively, were administered to RF/6A cells under high glucose conditions. Cell apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling. Western blot analysis was used to detect the protein expression level of SSTR<sub>2</sub>, occludin, vascular endothelial growth factor (VEGF), protein kinase B (Akt), phosphorylated Akt (p-Akt), extracellular signal-related kinases (ERK) and p-ERK proteins. The amount of VEGF released was determined by ELISA. Notably, the level of occludin reduced significantly under high glucose conditions. The results indicated that the administration of OCT prevented the reduction of occludin induced by high glucose, and co-administration with c-SOM reversed the effect of OCT. Increased VEGF secretion and expression of VEGF, p-Akt and p-ERK in RF/6A cells induced by high glucose were inhibited by OCT. ATWLPPR also prevented the downregulation of occludin, but did not inhibit p-Akt and p-ERK levels under high glucose conditions. The current study concluded

that the activation of SSTR<sub>2</sub> prevents high glucose-induced occludin downregulation in RF/6A cells, and VEGF, NRP1, p-Akt and p-ERK were implicated in this process. The pharmacological effects of SSTR<sub>2</sub> targeting to endothelium may be used to assess the role of resistance of permeability and anti-inflammation.

## Introduction

Diabetic retinopathy (DR) is one of the primary causes of blindness globally. More than 300 million people are estimated to have diabetes worldwide, and 40% have retinopathy to some extent, which includes ~8.2% with vision-threatening retinopathy (1,2). Primary pathological hallmarks of DR are retinal ischemia and proliferation, induced by microcirculatory disturbance and microangiopathy. The process includes oxidative stress, vascular endothelial growth factor (VEGF)-induced damage and inflammatory changes (3).

Previous studies have indicated that somatostatin (an endogenous peptide) or its analogue exhibit anti-inflammatory effects and neuroprotection against DR or retinal ischemia-reperfusion injury (4-6). Somatostatin receptors (SSTR1-5) are widely distributed in the retina (7,8). Activation of retinal SSTR<sub>2</sub> may reduce VEGF release from damaged neurons and limit the VEGF response (9). It has been demonstrated in an oxygen-induced retinopathy model that activation of SSTR<sub>2</sub> may downregulate VEGF and exert an anti-angiogenesis effect via the SHP-1/STAT3 pathway (10). VEGF combined with its receptors, VEGF receptor (VEGFR<sub>2</sub>) and neuropilin 1 (NRP1), has an important role in the disruption of the blood-retinal barrier (BRB), which is a core pathological change in retinal dysfunction of DR (11-13). Tight junctions of vascular endothelial cells are key components of BRB. Occludin, a transmembrane protein in the tight junctions, exerts its role by forming the permeability barrier. The high glucose conditions of DR may lead to VEGF release and occludin degradation in cultured human retinal endothelial cells (5).

A recent study indicated that SSTR<sub>2</sub> was scarcely expressed in VEGF-containing cells, and SSTR<sub>2</sub> or VEGF barely localized in retinal vessels (10). However, under hypoxic conditions, SSTR<sub>2</sub> and VEGF immunoreactivity was noted in retinal capillaries (10). This is consistent with

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*Correspondence to:* Dr Jun Wang or Professor Yanzhang Li, Department of Physiology, Henan University School of Medicine, Jin Ming Avenue, Northern Segment, Kaifeng, Henan 475004, P.R. China  
E-mail: whb21c@henu.edu.cn  
E-mail: yanzhang206@163.com

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another study that demonstrated VEGF was released from damaged neurons and entered vessels under hypoxic conditions (9). Therefore, it is not clear whether SSTR<sub>2</sub> exists in normal vascular endothelial cells, and whether activation of SSTR<sub>2</sub> is able to regulate the degradation of occludin induced by high levels of glucose in vascular endothelial cells. The present study aimed to elucidate this and determine whether SSTR<sub>2</sub> modulates the occludin degradation induced by high glucose via the VEGF/NRP1/protein kinase B (Akt) signaling pathway.

## Materials and methods

**Materials.** RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). D-glucose, penicillin and streptomycin were obtained from DingGuo Biotech Co., Ltd., (Shanghai, China). Octreotide (OCT) and cyclo-somatostatin (c-SOM; both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were dissolved and diluted in 0.9% NaCl to 1 mM.

**Cell culture and experimental grouping.** Rhesus monkey retinal fovea vascular endothelial cells (RF/6A) were obtained from Wuhan Boster Biological Technology, Ltd., (Wuhan, China). The RF/6A endothelial cells were maintained in Dulbecco's modified Eagle medium (DMEM; Wuhan Boster Biological Technology, Ltd.) culture medium supplemented with 10% FBS, streptomycin (100 µg/ml) and penicillin (100 IU/ml) in a 5% CO<sub>2</sub> incubator at 37°C. Cells were seeded in 6-well plates at a density of 1x10<sup>5</sup> under normal (5.6 mM) and high glucose (30 mM) conditions, respectively. Passages 4-8 of the cells were used in the present study. The experimental groups in the current study were as follows: i) Control (normal conditions); ii) high glucose (HG; 30 mM D-glucose); iii) HG + Octreotide (OCT; 1 µM); iv) HG + OCT + c-SOM (OCT, 1 µM; c-SOM, 1 µM); and v) HG + VEGF co-receptor NRP1 antagonist (ATWLPPR; GL Biochem, Ltd., Shanghai China; 0.1 mM). Cells were observed and photographed with an inverted microscope (CX40RF200; Olympus Corp., Tokyo, Japan).

**Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL).** TUNEL assay was performed using an *in situ* cell apoptosis detection kit (MK1022; Boster Biological Technology, Pleasanton, CA, USA) based on the manufacturer's protocol. RF/6A cells grown in normal and high glucose medium for 5 days were fixed with 4% paraformaldehyde at room temperature for 30 min. Following washing (3 times for 2 min) with phosphate-buffered saline (PBS) and distilled water, Proteinase K (1:200) was added to cells and incubated at 37°C for 10 min. Cells were washed with PBS and incubated with Labeling Buffer mixed with terminal deoxynucleotidyl and digoxigenin-11-deoxyuridine triphosphate (both provided in the kit) in a moist chamber at 37°C for 2 h. Cells were then washed with PBS (3 times for 2 min) and incubated at room temperature for 30 min with anti-digoxigenin peroxidase (provided in the kit) prior to treatment with SABC-AP and chromogenic substrate (BCIP/NBT; provided in the kit).

**Culture media VEGF measurement.** A sandwich mouse VEGF ELISA kit (EK0541; Boster Biological Technology) was used to determine the level of VEGF according to the protocol provided by the manufacturer. Briefly, following collection of the cell culture fluid, with a 5-min centrifugation at 2,000 x g (4°C), the supernatant was diluted with sample diluent (provided in the kit) to a proportion of 1:2. From each sample, 100 µl supernatant was transferred to wells, which were coated with VEGF monoclonal antibody (1:100; provided in the kit). Subsequently, the avidin-biotin-peroxidase complex, ABC and chromogenic substrate were added in sequence according to the manufacturer's protocol. VEGF levels were determined by the absorbance at 450 nm using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Inc.) and normalized to the standard recombinant VEGF.

**Western blot analysis.** The culture solution was discarded and the RF/6A cells were washed with PBS three times and lysed by radioimmunoprecipitation lysis buffer containing EDTA-Na<sub>2</sub>, sodium fluoride and phenylmethylsulfonyl fluoride (all 1 mM). Cell extracts were collected and centrifuged for 5 min at 20,000 x g and 4°C. The supernatant was transferred into a 1.5-ml Eppendorf tube. Protein concentrations were measured using a Bradford Protein Assay kit (Beyotime Institute of Biotechnology, Co., Ltd., Haimen, China). Each protein sample (50 µg) was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Following washing in TBST (2 times for 1 min), the membranes were blocked in 5% non-fat milk in Tris-buffered saline with Tween-20 and incubated overnight at 4°C with the primary antibodies, as follows: SSTR<sub>2</sub> (Boster Biological Technology; BA1406-2; 1:400), Occludin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-8144; 1:400), Akt (Boster Biological Technology; BA0631; 1:400), phosphorylated-Akt (p-AKT; Santa Cruz Biotechnology, Inc.; sc-33,437; 1:500), extracellular signaling-regulated kinase (ERK), and p-ERK (both CST Biological Reagents Company Ltd., Shanghai, China; 9102s and 9101s; both 1:1,000). The secondary antibody was horseradish peroxidase-goat anti-rabbit IgG (Boster Biological Technology; BA1080; 1:2,000).

**Statistical analysis.** Results were presented as the mean ± standard deviation. Statistical analyses were performed by using SigmaStat (version 3.2; Systat software, Inc., San Jose, CA, USA). Different groups were compared using one-way analysis of variance (Turkey test or Kruskal Wallis were used as post-hoc tests) or Student t-test. P<0.05 was considered to determine statistically significant differences.

## Results

**Observation of RF/6A cell appearance in high glucose conditions.** Morphological observation demonstrated that the RF/6A cells exhibited a shuttle-like and polygonal appearance when they were not crowded in the well. As the concentration increased, a number of cells exhibited a cobblestone appearance. No difference in appearance was observed between normal and high glucose conditions (Fig. 1A). TUNEL assay was performed on day 5 to

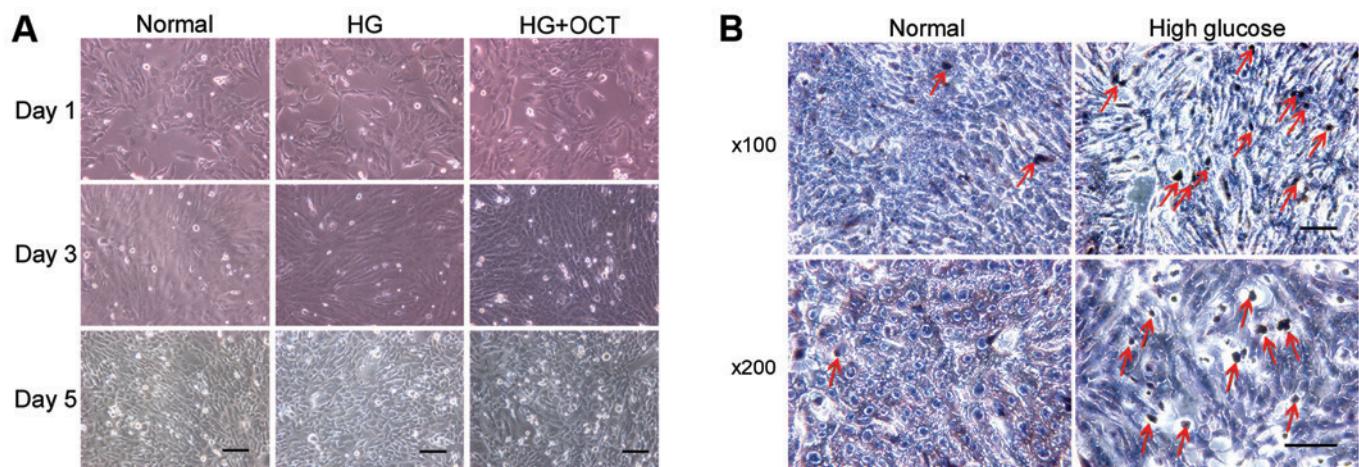


Figure 1. Observation of RF/6A cells in differing glucose concentrations. (A) Observation of RF/6A cells under normal, HG (30 nM) and HG + OCT conditions. Scale bar, 200  $\mu$ m. (B) There was a marked increase in the number of TUNEL-positive cells in the HG group on day 5, as compared with the normal group. Red arrows indicate TUNEL-positive cells. Scale bar, 200  $\mu$ m. HG, high glucose; OCT, octreotide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

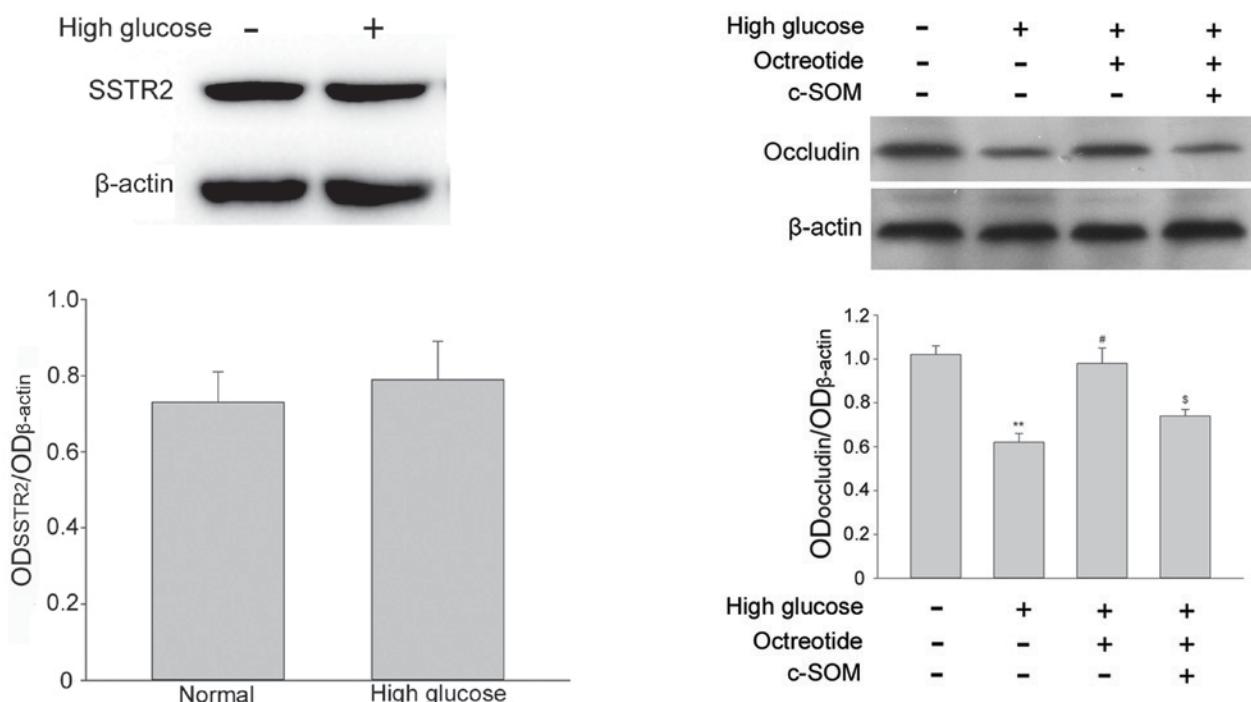


Figure 2. Evaluation of SSTR<sub>2</sub> expression under normal and high glucose conditions using western blot analysis and  $\beta$ -actin was used as the loading control. Each bar represents the mean + standard deviation of OD data from three samples. SSTR<sub>2</sub>, somatostatin receptor 2; OD, optical density.

determine whether RF/6A cells were influenced by the high glucose conditions. The number of apoptosis-positive cells in high glucose conditions was higher than those exposed to normal conditions (Fig. 1B). *Expression of SSTR<sub>2</sub> in RF/6A cells.* To evaluate the expression of SSTR<sub>2</sub> in RF/6A cells, western blot analysis was performed on normal control cells and high glucose-treated cells. SSTR<sub>2</sub> was expressed in the RF/6A cells both in normal and high glucose conditions. No significant difference in expression was observed in RF/6A cells treated with 30 mM D-glucose for 5 days, as compared with the untreated cells (Fig. 2).

Figure 3. Involvement of SSTR<sub>2</sub> in modulation of the degradation of occludin induced by high glucose, as evaluated by western blot and densitometric analysis. A high concentration of glucose induced degradation of occludin, this was restored by administration of the SSTR<sub>2</sub> agonist OCT, and the effect of octreotide was reversed by SSTR<sub>2</sub> antagonist c-SOM. \*\*P<0.01 vs. untreated normal condition; #P<0.05 vs. high glucose group; \$P<0.05 vs. high glucose + OCT group. Each column represents the mean + standard deviation of OD data from three samples.  $\beta$ -actin was used as the loading control. SSTR<sub>2</sub>, somatostatin receptor 2; OCT, octreotide; c-SOM, cyclo-somatostatin.

*SSTR<sub>2</sub> modulates the degradation of occludin induced by high glucose.* Occludin is a transmembrane tight junction protein expressed in endothelial cells and is also a key factor in the formation of the permeability barrier. Under 30 mM D-glucose conditions, occludin expression was significantly reduced when compared with cells exposed to normal conditions (Fig. 3; P<0.05). For RF/6A cells under high glucose

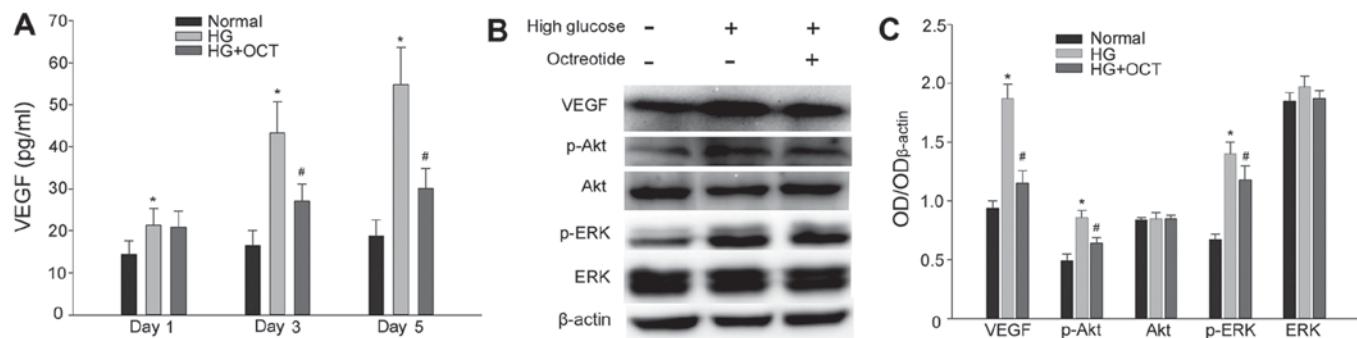


Figure 4. Effect of HG on VEGF expression and secretion in RF/6A cells, and the VEGF autocrine response was inhibited by OCT. (A) HG conditions resulted in an increase of VEGF release in a time-dependent manner. The increase of VEGF release may be inhibited by administration of OCT. (B) Expression of VEGF, Akt, p-Akt, ERK and p-ERK in RF/6A was evaluated by western blot and densitometric analysis and subsequently (C) quantified. VEGF, p-Akt and p-ERK upregulation in HG conditions was inhibited by treatment with OCT. Each column represents the mean + standard deviation of OD data from three samples.  $\beta$ -actin was used as the loading control. \*P<0.05 vs. normal control; #P<0.05 vs. high glucose group. VEGF, vascular endothelial growth factor; Akt, protein kinase B; ERK, extracellular signal-related kinase; p-Akt, phosphorylated Akt; p-ERK, phosphorylated ERK; HG, high glucose; OCT, octreotide; OD, optical density.

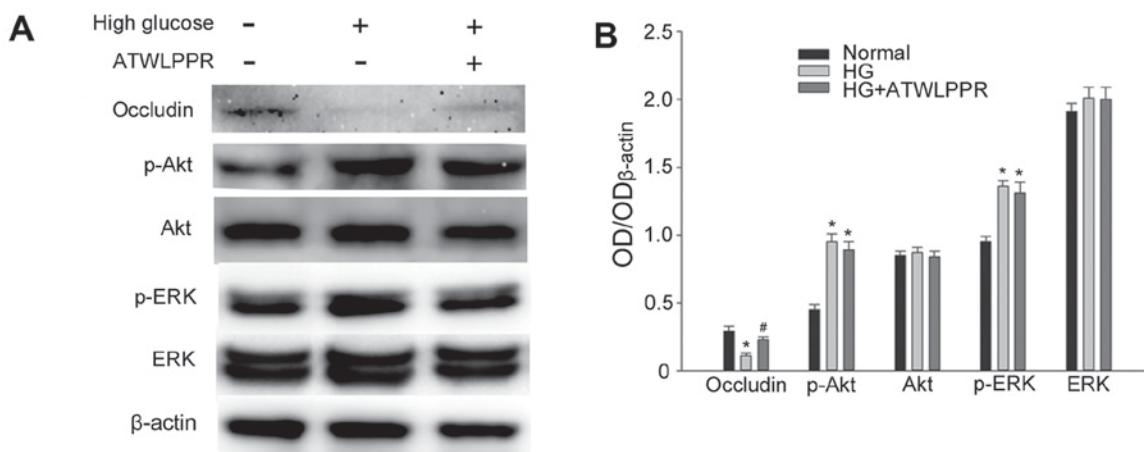


Figure 5. NRPI inhibitor ATWLPPR prevented HGe-induced occludin downregulation. This was assessed using (A) western blot analysis, which was (B) quantified. Each column represents the mean + standard deviation of OD data from three samples.  $\beta$ -actin was used as the loading control. \*P<0.05 vs. normal control; #P<0.05 vs. high glucose group. NRPI, neuropilin 1; OD, optical density; Akt, protein kinase B; ERK, extracellular signal-related kinase; p-Akt, phosphorylated Akt; p-ERK, phosphorylated ERK; HG, high glucose.

conditions, treatment with the SSTR<sub>2</sub> agonist OCT (1  $\mu$ M) restored the level of occluding, and this change was significant when compared with the high glucose group ( $P<0.05$ ). Co-administration of SSTR<sub>2</sub> antagonist c-SOM (1  $\mu$ M) with octreotide significantly inhibited the effect of OCT ( $P<0.05$ ). The results demonstrated that the specific SSTR<sub>2</sub> was involved in modulation of occludin levels in the RF/6A cells under the high glucose conditions.

**OCT inhibits the VEGF expression and secretion involved in the activation of Akt and ERK in the high glucose condition.** A sandwich ELISA assay was performed to evaluate the secretion level of VEGF from RF/6A cells. As presented in Fig. 4A, cells exposed to 30 mM glucose exhibited in a significant increase in VEGF levels from day1 ( $P<0.05$ ). In cells subjected to high glucose, treatment with 1 mM OCT significantly inhibited the release of VEGF into the culture medium from day 3 ( $P<0.05$ ; Fig. 4A). Expression levels of VEGF, Akt, p-Akt, ERK and p-ERK were determined by western blot analysis. As presented in Fig. 4B and C, high glucose conditions significantly up-regulated the expression of VEGF in RF/6A

cells ( $P<0.05$ ). At the same time, the expression of p-Akt and p-ERK also increased significantly compared with the cells under normal conditions ( $P<0.05$ ). The increase in expression of VEGF, p-Akt and p-ERK was significantly inhibited by treatment with OCT ( $P<0.05$ ). VEGF is able to activate its downstream signaling molecules (Akt and ERK) by binding to its membrane receptor. Thus, these results demonstrated that OCT inhibited VEGF expression and secretion and subsequently the autocrine response induced by a high concentration of glucose.

**Involvement of NRPI in the effects of high glucose-induced occludin downregulation.** VEGF is secreted from RF/6A cells and bound to its receptors VEGFR<sub>2</sub>/NRPI, exerting its role of downstream signal transduction. Western blot analysis indicated that treatment with NRPI (VEGFR<sub>2</sub> co-receptor) inhibitor ATWLPPR (0.1 mM) significantly prevented occludin downregulation under high glucose conditions ( $P<0.05$ ; Fig. 5). p-Akt and p-ERK increased significantly in high glucose conditions ( $P<0.05$ ; Fig. 5B); however, this was not influenced by ATWLPPR. These results indicate that

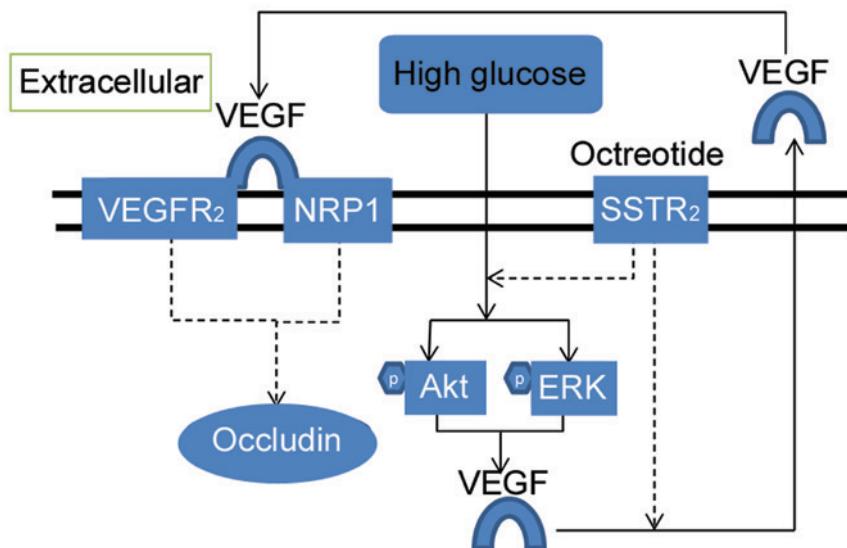


Figure 6. Schematic diagram of the pathway linking SSTR<sub>2</sub> activation to the prevention of occludin decrease induced by high glucose. Solid arrows, activation; dashed arrows, inhibition; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; NRP1, neuropilin 1; SSTR<sub>2</sub>, somatostatin receptor 2; Akt, protein kinase B; ERK, extracellular signal-related kinase.

NRP1 may be involved in the modulation of the VEGF autocrine or paracrine responses, as demonstrated in Fig. 6.

## Discussion

The results of the present study indicate that SSTR<sub>2</sub> exists in RF/6A cells cultured in normal and high glucose medium. Furthermore, no significant difference in SSTR<sub>2</sub> expression was observed using densitometric analysis between the two culture conditions. Activation of SSTR<sub>2</sub> prevented occludin downregulation under high glucose conditions and this process is involved in the increased expression of p-Akt, p-ERK and VEGF. Administration of the NRP1 inhibitor, ATWLPPR, inhibited the decrease of occludin induced by high glucose.

A previous study demonstrated that the SSTR<sub>2</sub> agonist, OCT, has anti-inflammation and anti-oxidation effects and alleviated retinal edema in retinas following ischemia-reperfusion (5). It has been indicated that OCT was associated with the integrity of blood vessel endothelium, as confirmed by previous studies (14-16). Damage of endothelial cells is often observed in the early stage of DR (3,17,18). Somatostatin and its receptors are widely distributed in the retina and serves important and complex physiological roles (7,19). The current study focused on an endothelial cell line, RF/6A, and determined the existence of SSTR<sub>2</sub> initially. A previous study assessing hypoxic retinas indicated that SSTR<sub>2</sub> immunostaining was increasingly noted in capillaries (20). It has also been indicated that SSTR<sub>2</sub> and SSTR<sub>5</sub> are expressed in proliferative endothelial cells but not in quiescent cells (21). Whether SSTR<sub>2</sub> in the endothelium serves a downstream role and modulates the integrity of endothelium remains unknown. The results of the present study indicated that high glucose conditions did not change the expression level of SSTR<sub>2</sub> in RF/6A cells. Tight junctions among the endothelium in capillary vessels are the functional base of BRB (3). Occludin is an important tight junction transmembrane protein, responsible for the permeability of the barrier (22). Tissues or cells exposed to ischemia, hypoxia and high glucose conditions

may exhibit a reduction of tight junction proteins, such as occludin, and this process forms the primary pathological base of BRB injury as it is demonstrated by enhanced permeability (3,22-24). In the current study, the condition culture medium with 30 mM glucose led to the decreased expression of occludin in RF/6A cells and this reduction was regulated by SSTR<sub>2</sub>. A study on the intestinal epithelia indicated that activation of SSTR<sub>2</sub> led to protective effects on the intestinal barrier by modulation of the expression of tight junction proteins (25). Accumulated evidence has indicated that the administration of somatostatin or SSTR<sub>2</sub> agonist had anti-inflammatory and neuroprotective effects in ischemic retinopathy (4,5). However, whether SSTR<sub>2</sub> acts directly on capillaries epithelium, and the mechanism involved, remains unclear.

It is well-known that high glucose conditions are able to increase VEGF expression (22,26). The current study also indicated that not only the release of, but also the expression of VEGF in RF/6A cells was enhanced by high glucose. VEGF is an important factor that contributes to the permeability and the integrity of blood vessels (24). In patients with diabetes, increased VEGF in the eyes has been implicated in elevated vascular permeability and breakdown of BRB (27,28). VEGF-induced permeability is mediated by the downregulation of tight junction proteins (occludin), and the molecular mechanisms may be associated with urokinase plasminogen activator, nitric oxide and protein kinase C (24,29-32). The results of the current study demonstrated that high glucose induced an increase in VEGF expression, which may be inhibited by OCT, a SSTR<sub>2</sub> preferring agonist. It is therefore hypothesized that SSTR<sub>2</sub> regulates occludin, potentially by controlling the expression and secretion of VEGF. The results of the present study were consistent with other research performed in a mouse model of retinopathy (33). Although there remains a lack of such reports in RF/6A cells, previous studies have demonstrated that octreotide prevents the upregulation of VEGF induced by hypoxia (10,33). VEGF, as a specific mitogen, has been demonstrated to have an effective role in autocrine regulation of endothelial cells (34,35). Thus,

the VEGF autocrine response may contribute to the vessel permeability changes in DR (22).

Akt and ERK are important cellular signal molecules that serve their biological functions when activated by phosphorylation. Activation of Akt may inhibit apoptosis and promote cell survival and lumen formation (36). ERK is involved in regulating angiogenesis (37). A high concentration of glucose may induce oxidative stress, tumor necrosis factor- $\alpha$  upregulation and apoptosis in the endothelium (38). The results of the current study indicated that high glucose induced an increase of p-Akt and p-ERK in RF/6A cells. Treatment with OCT was then demonstrated to reduce the activation of Akt and ERK. The present study was not able to illustrate whether the effects of OCT were from the inhibition of VEGF or other pathways, leading to anti-oxidative stress. Numerous studies have demonstrated that Akt and ERK are common downstream signal molecules of VEGF and its receptors (13,24,39,40). Increased p-ERK and p-Akt were typically observed in retinal ischemia diseases and retinal neovascularization (41,42), and they were also required factors for the expression of VEGF (41,43).

NRP1 acts as a co-receptor for VEGF by forming a complex with VEGFR<sub>2</sub>. NRP1 may promote VEGF binding and potentiate VEGFR2 activation and intracellular signaling (44). To illustrate whether NRP1 was involved in the high glucose-induced VEGF autocrine response and occludin reduction, a NRP1 inhibitor, ATWLPPR was administered to RF/6A cells. The results indicated that ATWLPPR reduced the increase of occludin, but had no significant effects on the activation of Akt and ERK. This was consistent with a previous study that demonstrated NRP1 to be a required component for the regulation of vascular permeability, induced by VEGF (40). Evans *et al* (44) suggested that treatment of small interfering RNA on NRP1 in HUVEC cells did not affect the phosphorylation of AKT and ERK mediated by VEGF. This was in accordance with the results of the present study. Activation of Akt and ERK was unaffected by the inhibition of NRP1, this was potentially due to lower thresholds of the receptors activity.

In conclusion, the results of the present study demonstrated that SSTR<sub>2</sub> in RF/6A cells prevented the high glucose-induced decrease of occludin. NRP1, increased expression of VEGF, and activation of Akt and ERK were involved in this process. This may account for the regulating mechanisms of OCT on vascular permeability in diabetes or high glucose-mediated VEGF autocrine or paracrine response.

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