

Original Article

Anti-angiogenic effect of rapamycin in mouse oxygen-induced retinopathy is mediated through suppression of HIF-1 α /VEGF pathway

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Received June 20, 2017; Accepted August 16, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: Hypoxia-inducible factor-1 α (HIF-1 α)-vascular endothelial growth factor (VEGF) pathway, a downstream of mammalian target of rapamycin (mTOR), plays a major role in the formation of pathological retinal angiogenesis. Rapamycin (RAPA), a highly specific inhibitor of mTOR, is widely used in cancer studies for its antiangiogenic activity. However, the inhibitory effects of RAPA on the HIF-1 α -VEGF pathway in retinal tissues were rarely researched. The study aimed to investigate the efficacy and potential mechanisms of RAPA in inhibiting retinal neovascularization. Human umbilical vein endothelial cells (HUVECs) were treated with hypoxia and in the presence of different concentrations of RAPA. RAPA was injected intraperitoneally in oxygen-induced retinopathy (OIR) C57BL/6 mice from postnatal day 12 (P12) to P17. The proliferation of HUVECs, the protein and mRNA expressions of HIF-1 α and VEGF were evaluated in HUVECs or OIR mice using MTT assay, ELISA, immunohistochemistry, Western-blot and real-time PCR. Histological methods were used to count blood vessel profiles in the inner retina. RAPA inhibited HUVECs proliferation and retinal neovascularization by reducing protein and mRNA expressions of HIF-1 α and VEGF. RAPA suppresses hypoxia-induced HUVECs cell proliferation and pathological ocular angiogenesis through a mechanism linked to the targeting of HIF-1 α /VEGF signaling.

Keywords: Rapamycin, human umbilical vein endothelial cells, hypoxia-inducible factor-1 α , vascular endothelial growth factor, retinal neovascularization

Introduction

Pathological ocular angiogenesis, or ocular neovascularization (NV), is a characteristic component of several ocular diseases such as diabetic retinopathy (DR), age related macular degeneration (AMD) and retinopathy of prematurity (ROP). It is the leading cause of irreversible blindness [1]. Targeting angiogenesis represents a potent approach in ocular disease treatment, especially for retina characterized by active neovascularization. Given its central role in angiogenesis, vascular endothelial growth factor (VEGF) has become a prime target for angiotherapy. Although anti-VEGF therapy has been clinically used and shown short-term beneficial effects, it cannot completely prevent the formation of new vessels due to aberrant functions of over-expressed protein and systemic side effects, thus limiting their utility in the treatment of ocular disease [2].

Compared to targeting VEGF alone, targeting hypoxia inducible factor-1 (HIF-1) which stimulates the required angiogenic growth factors endogenously seems to be a safer and more effective therapeutic strategy [3]. As an important regulator in response to angiogenesis, HIF-1 belongs to transcription factor family and is composed of HIF-1 α and HIF-1 β subunits. HIF-1 α has been identified as a key element to mediate the function of HIF-1. The activation of HIF-1 α involves regulation of multiple key angiogenic genes, such as VEGF, platelet-derived growth factor (PDGF) and transforming growth factor α (TGF α) [4, 5]. HIF-1 α -VEGF pathway represents an appealing drug target. One possible candidate inhibiting the angiogenic signaling cascade upstream of HIF-1 α -expression is rapamycin (RAPA).

RAPA is a FDA-approved anti-tumor and anti-angiogenic agent with relatively low side effects

[6]. RAPA reduces HIF-1 α -VEGF production in ovarian clear cell [7]. A growing body of experimental evidence suggests RAPA treatment significantly reduced the extent of neovascularization in both choroidal and retinal neovascularization [8]. Clinical trials of RAPA treating patients with AMD or diabetic macular edema have been reported [9]. Yagasa Ki et al indicated that mammalian target of rapamycin (mTOR) inhibitors targeting proliferation of endothelial cells may be promising candidates as anti-angiogenic agents for treating vasoproliferative retinal diseases [10]. However, the studies investigating the inhibitory effects of RAPA in Hypoxia-inducible factor (HIF) induced VEGF expression in retina have not been conducted. The molecular mechanisms through which rapamycin evokes anti-angiogenic properties in the eye remain poorly understood. Some documents proposed that RAPA could potentially inhibit proliferation, migration and capillary-like tubule formation in human umbilical vein endothelial cells (HUVEC) [11, 12]. We hypothesized that the inhibitory effects of RAPA on retinal neovascularization may be achieved through suppressing HIF-1 α -VEGF pathway. Using the HUVECs model and vivo mouse model of oxygen-induced retinopathy (OIR), the present study examined the expressions of HIF-1 α and VEGF in retina with or without RAPA under normoxic and hypoxic conditions to extensively investigate the antiangiogenic effects and possible mechanisms of RAPA.

Materials and methods

Cell culture and treatment

HUVECs were purchased from Shanghai Institutes for Biological Sciences (SIBS, China; Code: ECV304) and cultured according to the manufacturer's instructions. The experiments were used on HUVECs of passages 3 to 5. Cells were grown to confluence, were then randomly divided into normoxic or hypoxic group. The condition of normoxic group was 5% CO₂, 95% air and 37°C. Hypoxic group was comprised of five subgroups according to the different concentrations (0.1, 1, 10, 100, 1000 ng/ml) of treated RAPA [11]. HUVECs were cultured for 12 h in hypoxic environments (3% O₂, 5% CO₂, 92% N₂, 37°C). Data of cells cultured under normoxic condition were regarded as baseline and were defined as hypoxia 0 h. All experiments were repeated three times.

OIR model

The use of animals was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee of Medical University Ophthalmic Center. OIR was induced in C57BL/6J mice (Laboratory animal center of China Medical University, China) as previously described [13]. The experiments were randomly divided into three groups with sixteen mice in each group. Group 1: normal group, postnatal day 7 (P₇) mice and their mothers were maintained in room air from birth to P₁₇. Group 2: OIR group, age-matched mice were exposed to hypoxia (75 \pm 3% oxygen) for 5 days. On P₁₂, the pups were taken out and placed in room air for an additional 5 days. Group 3: RAPA-treated OIR group, OIR mice were daily treated with RAPA (4 mg/kg/day, intraperitoneally) (Sigma, St. Louis, MO) from P₁₂ to P₁₇ [8]. At the end of the 17-day experimental time, eight randomly chosen mice from each group were sacrificed. Histology and immunohistochemistry were performed in the right eye from each mouse. The retinas from the remaining eight mice in each group were collected. The retinas from the left eyes were used for Western-blot array and those from the right eyes were frozen for the upcoming mRNA analysis by real-time PCR.

MTT assay

MTT assay was performed to detect the effect of RAPA on the growth of HUVECs under normoxic and hypoxic conditions for 12 h. Cells were seeded in a 96-well plate at a density of 1 \times 10⁴ cells/well and were continuously cultured for 24 h. As reported [12], the cells were incubated in hypoxic environment with medium plus 0.1, 1, 10, 100 or 1000 ng/ml of RAPA for another 12 h. Thereafter, 20 μ L of MTT (5 g/L, Sigma) was added in each well and incubated for further 4 h. The culture medium was then removed, 150 μ L of DMSO was added in each well. The absorbance was determined using a spectrophotometer reader at 570 nm. The inhibitory rates of cell growth were calculated using the published formula [14].

Detection of HIF-1 α and VEGF secretion by HUVEC under normoxic and hypoxic conditions

According to the MTT experiment, the cells cultured under normoxic or hypoxic condition for

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12 h were treated with RAPA at a concentration of 100 ng/ml. HIF-1 α and VEGF levels in the culture supernatants were determined by enzyme-linked immunosorbent assays (ELISAs). The supernatants were collected after 12 h and the HIF-1 α and VEGF levels were quantified using a HIF-1 α and VEGF ELISA Assay Kit respectively (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Western-blot assay for VEGF and HIF-1 α

As described, cell preparation was treated with RAPA at different concentrations. HUVECs and the retinas in the experimental groups were collected and lysed in RIPA buffer. Bradford method was used to measure the protein concentration with reagents being purchased from Bio-Rad (Hercules, CA). Cell extracts (50 μ g of protein) were analyzed by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with rabbit-anti human/mouse polyclonal antibody (VEGF, 1:300; HIF-1 α , 1:200) and rabbit anti-human/mouse β -actin (loading control) polyclonal antibody (1:300) over night. After being washed in TBST, membranes were incubated with second antibody (goat anti-rabbit alkaline phosphatase-labeled IgG antibody, Santa Cruz, CA) with a dilution ratio of 1:1000. All experiments were performed in triplicate. The specific protein bands were visualized using enhanced chemiluminescence (ECL, Amersham-Pharmacia Biotech, Beijing, China) in line with the manufacturer's instructions. Autoradiograms were quantified by densitometry. The relative levels of protein were derived by comparing the protein level of β -actin.

Quantitative real-time RT-PCR

Total RNA was extracted from HUVECs/mice retinas with Trizol reagent (Life Technologies, Carlsbad, USA) in line with the manufacturer's instructions. The reverse transcription reaction was performed using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The PCR reaction was performed using the SYBR Green Kit (Toyobo, Shang Hai China) in a thermal cycler (Applied Biosystems StepOne, USA). The internal control was β -actin. The primers were:

VEGF (human): forward: 5'-TTC GGA GGA GCC GTG GTC CG-3'; reverse: 5'-GTC CAT GAG CCC GGC TTC CG-3'. HIF-1 α (human): forward: 5'-GGC AGC AACGAC ACA GAA ACT GA-3'; reverse: 5'-TTG GCG TTT CAG CGG TGG GT-3'. β -actin

(human): forward: 5'-GCC CTG GCA CCC AGC ACA AT-3'; reverse: 5'-GAC GAT GGA GGG GCC GGA CT-3'. The PCR reactions consisted of 5 min at 95°C, 35 cycles at 95°C for 15 seconds, 60°C for 20 seconds and 72°C for 20 seconds.

VEGF (mouse): forward: 5'-ACTCGGATGCCGACACGGGA-3'; reverse: 5'-CCTGGCCTTGCTTGC-TCCCC-3'. HIF-1 α (mouse): forward: 5'-ACCGCAACTGCCACCACTGA-3'; reverse: 5'-TGGTGAG-GCTGTCCGACTGTGA-3'. β -actin (mouse): forward: 5'-CCTCTATGCCAACACAGTGC-3'; reverse: 5'-CA-TCGTACTCTGCTTGCTG-3'.

The PCR reactions were: 3 min at 95°C, 40 cycles at 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 15 seconds.

Histological analysis of blood vessel profiles in the inner retina of mice

The eyes were fixed in 4% paraformaldehyde in PBS for at least 24 h and embedded in serial paraffin sections (6 μ m) of whole eyes which were cut sagittally through the cornea parallel to the optic nerve. Randomly chosen eight sections of one eye from each animal were deparaffinized, then stained using hematoxylin and eosin. Blood vessel profiles (BVPs) were counted in a double-blind fashion using an established technique [13, 15, 16] by two independent observers. The vascular cell nuclei were linked with the new vessels anterior to the vitreal side of the internal limiting membrane (ILM). BVPs were counted in the inner retina and included vessels adherent to the ILM. The inner retina was comprised of ILM, ganglion cell layer (GCL) and inner plexiform layer (IPL). A BVP was defined as an endothelial cell or a blood vessel with a lumen.

Immunohistochemistry for HIF-1 α and VEGF

We used the strep avidin-biotin complex method for immunohistochemical staining in randomly selected sections to evaluate the protein expressions of VEGF and HIF-1 α . The applied antibodies were polyclonal HIF-1 α antibody (1:300 in PBS) and polyclonal VEGF antibody (1:400 in PBS) (Santa Cruz Biotechnology, Inc.). Sections incubated in PBS rather than primary antiserum were used as negative controls. Cells positive for VEGF and HIF-1 α showed light yellow or dark brown coloration in the cytoplasm. The integrated areas of VEGF and HIF-1 α staining were determined using automatic

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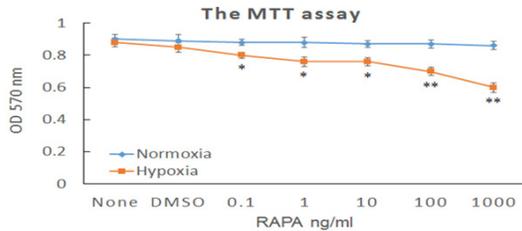


Figure 1. The MMT assay showing the effects of RAPA on HUVEC under normoxia or hypoxia for 12 h. HUVECs were treated with 0, 0.1, 1, 10, 100 or 1000 ng/ml of PRPA. Data are expressed as means \pm SD; * $P < 0.05$, ** $P < 0.01$ compared with the normoxia.

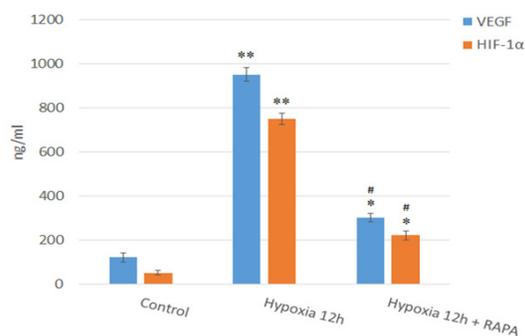


Figure 2. Protein levels of VEGF and HIF-1 α in HUVECs supernatants under normoxia and hypoxia for 12 h (ELISA) with or without RAPA (100 ng/ml). Data are expressed as means \pm SD; * $P < 0.05$, ** $P < 0.01$ compared with normoxia group; # $P < 0.05$ compared with the hypoxia for 12 h group.

microscope and image analysis system (LUZEX-F, Japan). The integral optical density (IOD) values were detected as indicators of VEGF and HIF-1 α expressions.

Statistical analysis

Quantitative data were expressed as mean \pm standard deviation (SD). Data of MTT assay were analyzed using analysis of variance (ANOVA). One-way ANOVA and least significant difference (LSD) were used in multiple comparison. Then, data of the histological analysis were analyzed by a paired t-test. All analyses were performed using SPSS 20.0 software (IBM Corp, Armonk, NY). $P < 0.05$ was considered to be statistically significant.

Results

Effects of RAPA on the proliferation of HUVECs

As shown **Figure 1**, the cell growth of HUVECs was not affected by the treatment of RAPA

under normoxic condition. In contrast, when the HUVECs were exposed to hypoxic condition, the HUVECs growth was significantly inhibited in a concentration-dependent manner. However, the proliferation rates were nearly unchanged in the presence of different concentrations of RAPA (100 or 1000 ng/ml).

Detection of protein levels of VEGF and HIF-1 α in HUVECs

The HIF-1 α and VEGF protein levels under hypoxic condition tended to increase compared with those under normoxic condition ($P < 0.01$). Treatment with 100 ng/ml of RAPA decreased both HIF-1 α and VEGF protein levels compared with those exposed to hypoxia ($P < 0.05$). The levels of the two proteins were greater than those in the controls ($P < 0.05$) (**Figure 2**).

Effects of RAPA on the HIF-1 α and VEGF protein expressions in HUVECs

The protein expressions of HIF-1 α and VEGF were weakly expressed in cells incubated in normoxic condition, but strongly expressed in hypoxic condition ($P < 0.01$). With the increasing dose of RAPA, the protein expressions of HIF-1 α and VEGF were inhibited in a dose-dependent fashion. Compared with the normoxic condition, however, the protein levels of HIF-1 α and VEGF were greater under hypoxic condition ($P < 0.05$) (**Figure 3A, 3B**).

Effects of RAPA on expressions of HIF-1 α and VEGF mRNA in HUVECs

The patterns of HIF-1 α and VEGF mRNA expressions were similar to their corresponding protein expressions. HIF-1 α and VEGF mRNA were weakly expressed in cells exposed to normoxic condition, but their mRNA expressions were markedly increased in hypoxic condition. With the increasing concentration of RAPA for 12 h under hypoxia, RAPA reduced the mRNA levels of HIF-1 α and VEGF in a dose-dependent fashion manner (**Figure 4A, 4B**).

Histopathology and quantification of blood vessel profiles in mice

As shown in **Figure 5A-D**, blood vessels were confined to the inner retina in normal group. There were no nuclei of new vascular endothelial cells breaking through the inner retina compared with those in the OIR group which had large clusters of blood vessels adherent to the

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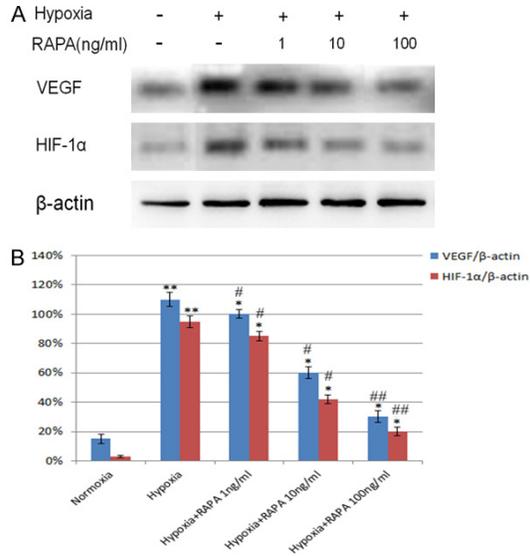


Figure 3. Protein levels of VEGF and HIF-1 α in HUVECs under normoxia and hypoxia for 12 h by Western-blot analysis. A. Western-blot showed the protein expressions of HIF-1 α and VEGF in normoxia and hypoxia for 12 h and treated with different concentrations of RAPA after 12 h exposure to hypoxia. B. The statistical analysis of HIF-1 α and VEGF expressions. The HIF-1 α and VEGF densities were normalized against the β -actin. Data are expressed as means \pm SD; * P < 0.05, ** P < 0.01 compared with normoxia group; # P < 0.05, ## P < 0.01 compared with the hypoxia for 12 h group.

ILM (0.13 ± 0.06 vs 32.54 ± 1.76). In the RAPA-treated OIR group, although the blood vessels attached to the ILM were greater than those in the normal group, the number was reduced by 72% compared with those in untreated OIR mice (9.11 ± 0.13) (P < 0.05).

HIF-1 α and VEGF Expressions by Immunohistochemistry in mice

In normal group, there was little HIF-1 α or VEGF protein expression in the GCL (the IOD were 1.48 ± 1.59 and 3.17 ± 0.82 , respectively) (Figure 6A, 6B). In contrast, increased HIF-1 α and VEGF protein expressions were detected in the OIR group of the inner nuclear layer (INL) and GCL, and more neovascularization was noted breaking through the inner retina than those in normal group (the IOD were 8.84 ± 0.57 and 12.88 ± 1.33 , respectively, P < 0.05) (Figure 6C, 6D). In RAPA-treated OIR mice, RAPA significantly inhibited the expression of HIF-1 α and VEGF compared with those in the ROP group and there were very few HIF-1 α and

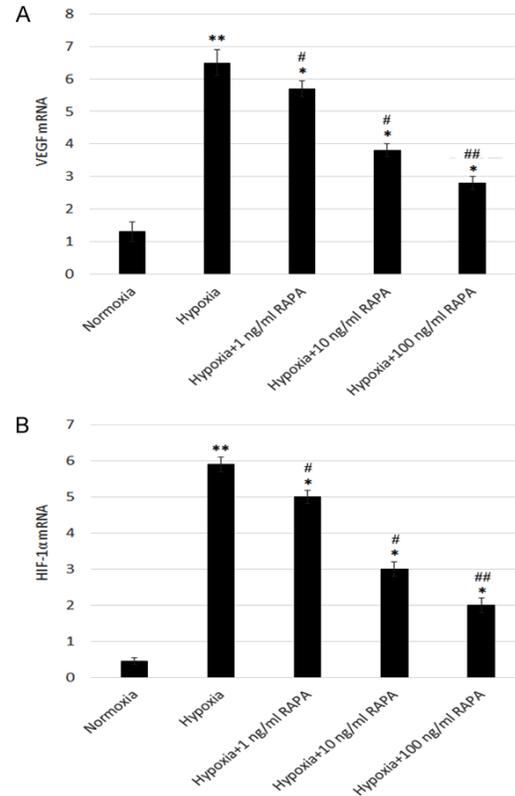


Figure 4. Real-time PCR showing the mRNA levels of VEGF (A) and HIF-1 α (B) in HUVECs under normoxia and hypoxia for 12 h or treated with different concentrations of RAPA after 12 h exposure to hypoxia. Data are expressed as means \pm SD; * P < 0.05, ** P < 0.01 compared with normoxia group; # P < 0.05, ## P < 0.01 compared with the hypoxia for 12 h group.

VEGF protein expressions in the ganglion cell and in the retinal blood vessels (the IODs of 4.09 ± 1.12 and 6.27 ± 1.24 , respectively, P < 0.05) (Figure 6E, 6F).

Protein and mRNA expressions of HIF-1 α and VEGF in OIR mouse retinas

The protein expressions of HIF-1 α and VEGF in the retina of OIR mice were further detected by Western-blot analysis. Compared to normal group, the protein levels of HIF-1 α and VEGF were elevated by 2.15 fold and 2.67 fold respectively in OIR mice. As shown in Figure 7A, 7B, the protein levels of HIF-1 α and VEGF were reduced by the treatment with RAPA, but still greater than those in the normal group. mRNA levels of HIF-1 α and VEGF showed a similar manner of increased levels in the OIR group compared to those in normal group (Figure 8A, 8B).

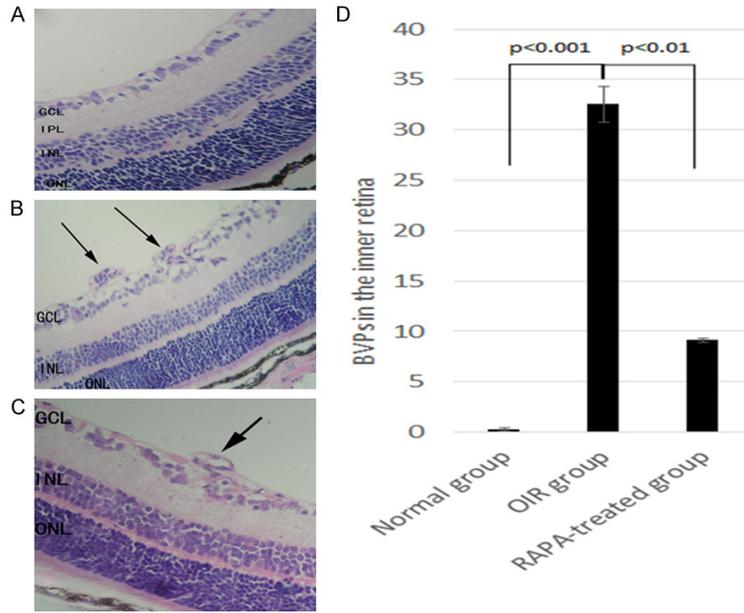


Figure 5. Histopathological analysis and blood vessel profiles of all the groups. In normal group, there were no nuclei of new vascular endothelial cells breaking through the inner retinal layer (A). Large clusters of blood vessels (BVPs) were adherent to the internal limiting membrane in untreated OIR group (B) (arrow). In OIR mice treated with RAPA, the number of nuclei of endothelial cells was significantly reduced, but more than in normal group (C). A small amount of BVPs were still presented in the RAPA-treated OIR group (arrow). Magnification, $\times 200$. (D) The average numbers of BVPs in the inner retina (means \pm SD, $n=8$ retinas per group) of the RAPA-treated OIR group mice were compared with the untreated OIR group and normal group mice using one-way ANOVA.

there were limited VEGF and HIF-1 α protein expressions in the ganglion cell and in the retinal blood vessels (E&F arrows). Magnification $\times 200$.

Discussion

Ocular angiogenesis is an important and complex process resulting from complicated interplay between diverse gene products. Antiangiogenic treatment is one of the most effective therapeutic strategies for the management of retinal neovascularization diseases. Recent evidence indicated that many agents act to suppress retinal neovascularization. Prominent examples of anti-VEGF agents are Avastin (bevacizumab) and Lucentis (ranibizumab). Although the antibodies are effective, they are not efficient because direct inhibition of VEGF cannot completely suppress the formation of new vessels [17] and multiple agents are needed to inhibit the target protein. Developing “broad-spectrum” antiangiogenesis drugs with “multiple targets” might be of interest especially for patients who do not sufficiently benefit from inhibiting single molecule. RAPA was first isolated in 1975 from a soil sample and was later found to possess potent antimicrobial and immunosuppressive qualities as well as significant anti-tumor and anti-angiogenic activities [18, 19]. Growing evidence showed that RAPA targets VEGF, also directly or indirectly induces the production of anti-angiogenic molecules such as pigment epithelium derived factor, tissue inhibitor of metalloproteinase-3 and endostatin [20]. Meanwhile, the signaling pathway of RAPA may involve in binding to intracellular receptor FKBP12, blocking the

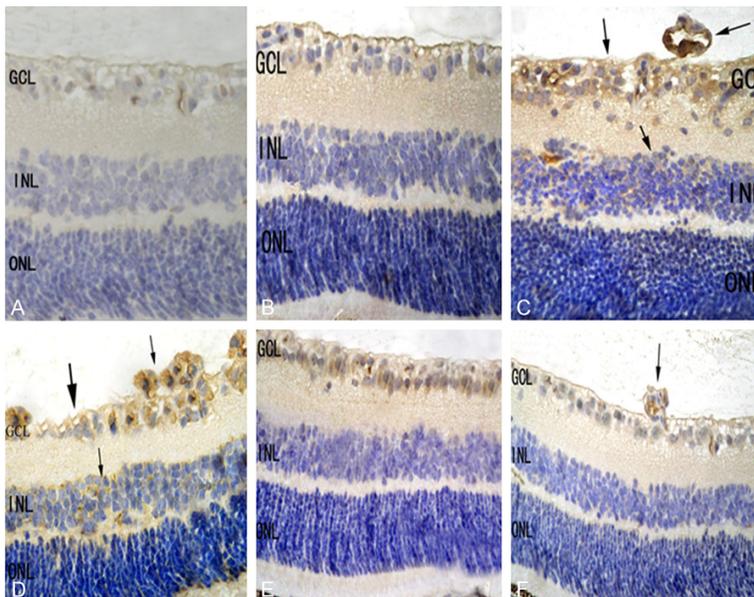


Figure 6. Immunolabeling for VEGF and HIF-1 α protein expressions of all the groups. In normal group, a little VEGF and HIF-1 α protein expression were detected in the GCL (A&B). In untreated OIR group, greater VEGF and HIF-1 α protein expressions were detected in the inner nuclear layer, the ganglion cell layer, as well as neovascularization breaking through the inner retinal layer compared with normal group (C&D arrows). In RAPA-treated OIR mice,

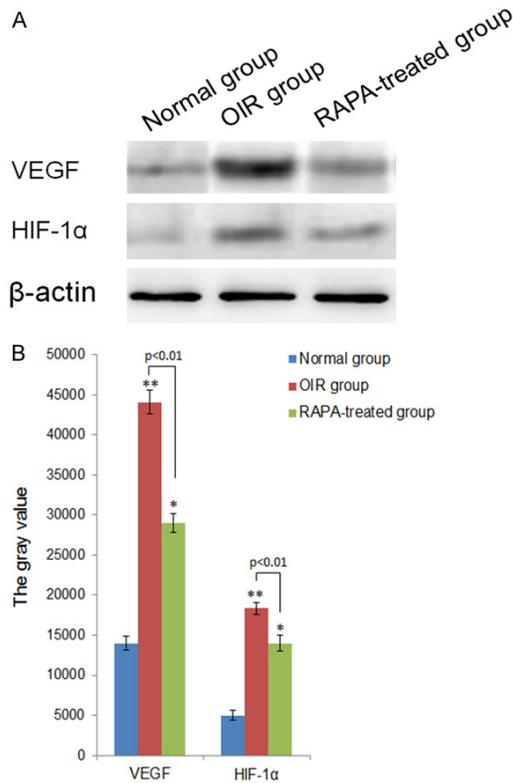


Figure 7. The protein expressions of VEGF and HIF-1α in Retina by western Blot. The protein levels of VEGF and HIF-1α were elevated significantly in the retinas of untreated OIR group compared with those in the normal group (**P < 0.01). After the treatment of RAPA, the expressions of both proteins were decreased compared with those in untreated OIR group (P < 0.01), but the protein levels of VEGF and HIF-1α were still stronger than those in the normal group (n=8 retinas per group) (*P < 0.05).

activity of mammalian target of rapamycin (mTOR), a downstream target of HIF-1α and VEGF which play a key role in modulating cell growth and proliferation and angiogenesis [21]. Another prominent advantage of RAPA is its superior safety profile. Evidence also accumulates from clinical phase I trials for intravitreally treated rapamycin in patients with exudative AMD or diabetic macular edema that no safety issues have been reported [22, 23]. Other groups have confirmed the apparent safety of intravitreally administered RAPA in different animal species [10, 24]. Therefore, RAPA is an exciting potentially therapeutic agent for retinal neovascularization. RAPA has been demonstrated to suppress VEGF and its receptor Flt-1 [8, 25, 26]. However, other mechanisms need to be addressed.

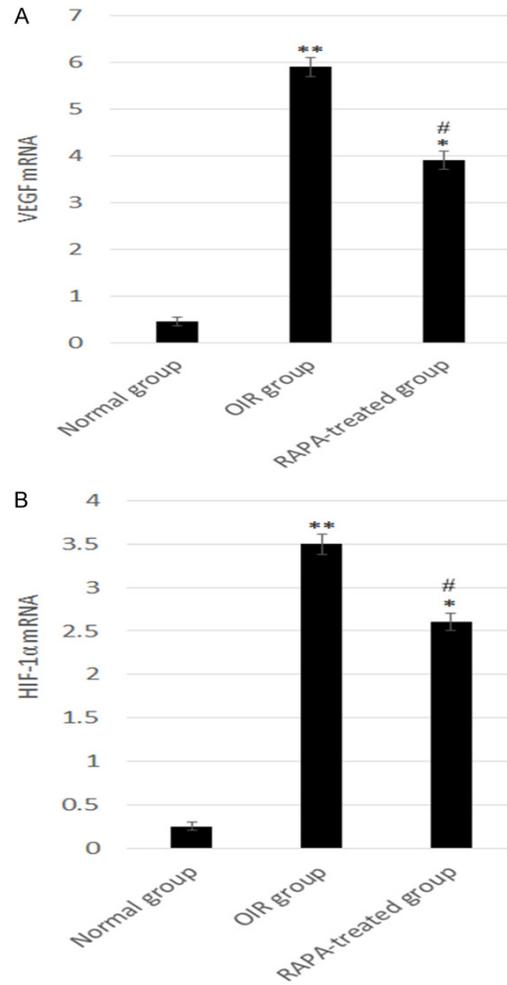


Figure 8. mRNA expressions of VEGF and HIF-1α were determined in Retina by real-time PCR with β-actin as internal control. Data are presented as mean ± SD (n=8), *P < 0.05, **P < 0.01 compared with normal group; #P < 0.05 versus OIR group.

Our data in the present study demonstrated that RAPA may potentially be used as an exciting agent for the diseases with retinal neovascularization. As HIF-1α is a vital upstream regulator for VEGF, targeting HIF-1α/VEGF pathway is vital for the treatment of ocular diseases. We examined the expressions of HIF-1α and VEGF in vitro to determine whether RAPA affected the hypoxic accumulation of HIF-1α and VEGF in HUVECs. We found significant elevation in the expressions of HIF-1α and VEGF when exposed to hypoxia for 12 h compared with those in normoxia using ELISA, Western-blot and real-time PCR techniques, suggesting hypoxia is indeed a prominent factor for the accumulation and the activation of HIF-1α and its downstream

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gene VEGF. We also found that, with increasing dose of RAPA, the expressions of HIF-1 α and VEGF were inhibited in a concentration-dependent manner. Our *in vitro* study showed that RAPA inhibited HUVECs proliferation. The inhibitory effects were dose dependent. A possible mechanism explaining the inhibition is the restoration of the expressions of HIF-1 α and VEGF. Similarly, our subsequent *in vivo* experiments showed that the protein and mRNA levels were increased simultaneously in the retina of OIR mice under hypoxic condition which was associated with the retinal neovascularization. The increase in HIF-1 α and VEGF protein and mRNA levels was reduced by RAPA, which was also observed *in vitro*, confirming that HIF-1 α and VEGF expressions are associated with retinal neovascularization *in vitro* and *in vivo*. The findings are consistent with a previous study suggesting HIF-1 α is stabilized during hypoxia, binds to HIF-1 β , and enters the nucleus to regulate the expression of the target genes VEGF [27].

Our findings suggested that the blockage of the HIF-1 α -VEGF signal passway did not completely suppress retinal neovascularization, indicating the process of retinal neovascularization mediated by hypoxia is complicated and RAPA may have yielded its effects through multiple pathways. Another possible mechanism is that the local concentration of RAPA may be not high enough to completely inhibit retinal neovascularization. To achieve a greater concentration, the concentration or the dose of RAPA needs to be increased or other modes of administration (e.g. subretinal injection or intravitreal injections) should be considered.

In summary, we comprehensively examined the antiangiogenic effects of RAPA *in vitro* and *in vivo* and found that RAPA may effectively suppress HUVECs proliferation and retinal neovascularization through a mechanism related to reduced expressions of HIF-1 α and VEGF. Although RAPA appeared not to completely suppress angiogenesis, it provides a novel insight for future therapeutic strategies against retinal neovascularization.

Acknowledgements

This research was supported by Doctoral Scientific Research Foundation of CHINA (No. 20091115); National Natural Science

Foundation of Liaoning Province (No. 2010-2299).

Disclosure of conflict of interest

None.

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