

Adenoviral 15-lipoxygenase-1 gene transfer inhibits hypoxia-induced proliferation of retinal microvascular endothelial cells *in vitro*

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Abstract

• **AIM:** To investigate whether 15-Lipoxygenase-1 (15-LOX-1) plays an important role in the regulation of angiogenesis, inhibiting hypoxia-induced proliferation of retinal microvascular endothelial cells (RMVECs) and the underlying mechanism.

• **METHODS:** Primary RMVECs were isolated from the retinas of C57/BL6J mice and identified by an evaluation for FITC-marked CD31. The hypoxia models were established with the Bio-bag and evaluated with a blood-gas analyzer. Experiments were performed using RMVECs treated with and without transfer Ad-15-LOX-1 or Ad-vector both under hypoxia and normoxia condition at 12, 24, 48, 72 hours. The efficacy of the gene transfer was assessed by immunofluorescence staining. Cells proliferation was evaluated by the CCK-8 method. RNA and protein expressions of 15-LOX-1, VEGF-A, VEGFR-2, eNOs and PPAR-r were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot.

• **RESULTS:** Routine evaluation for FITC-marked CD31 showed that cells were pure. The results of blood-gas analysis showed that when the cultures were exposed to hypoxia for more than 2 hours, the Po₂ was 4.5 to 5.4 Kpa. We verified RMVECs could be infected with Ad-15-LOX-1 or Ad-vector via Fluorescence microscopy. CCK-8 analysis revealed that the proliferative capacities of RMVECs in hypoxic group were

significantly higher at each time point than they were in normoxic group ($P < 0.05$). In a hypoxic condition, the proliferative capacities of RMVECs in 15-LOX-1 group were significantly inhibited ($P < 0.05$). Real-time RT-PCR analysis revealed that the expressions of VEGF-A, VEGF-R2 and eNOs mRNA increased in hypoxia group compared with normoxia group ($P < 0.01$). However, the expressions of 15-LOX-1, PPAR-r mRNA decreased in hypoxia group compared with normoxia group ($P < 0.01$). It also showed that in a hypoxic condition, the expressions of VEGF-A, VEGF-R2 and eNOs mRNA decreased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). However, 15-LOX-1 and PPAR-r mRNA increased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). There was no significant difference of the mRNA expressions between vector group and hypoxia group ($P > 0.05$). Western blot analysis revealed that the expressions of relative proteins were also ranked in that order.

• **CONCLUSION:** Our results suggested that 15-LOX-1 and PPAR-r might act as a negative regulator of retinal angiogenesis. And the effect of 15-LOX-1 overexpression is an anti-angiogenic factor in hypoxia-induced retinal neovascularization (RNV). Overexpression 15-LOX-1 on RMVECs of hypoxia-induced RNV blocked signaling cascades by inhibiting hypoxia-induced increases in VEGF family. PPAR-r effect on VEGFR₂ could be an additional mechanism whereby 15-LOX-1 inhibited the hypoxia-induced RNV.

• **KEYWORDS:** 15-Lipoxygenase-1; hypoxia; retinal microvascular endothelial cells; retinal neovascularization
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INTRODUCTION

Retinal neovascularization (RNV) is a major cause of vision loss at all ages. It causes vision loss in retinopathy of prematurity (ROP) in children, in diabetic

retinopathy (DR) in young adults, and in age-related macular degeneration (AMD) in the elderly^[1,2]. Intraocular concentrations of VEGF which is an angiogenic protein and vasopermeability factor are strongly correlated with DR, AMD and ROP^[3]. VEGF has been demonstrated to play an important role in RNV^[4,5]. The inhibition of VEGF action indicated to provide a viable approach for therapeutic intervention on RNV^[6-8]. However, the exact mechanism underlying the pathogenesis of retinal RNV is not yet well-understood.

Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes which catalyze the stereoselective dioxygenation of polyunsaturated fatty acids to their hydroperoxy derivatives^[9,10]. In mammals, LOXs are categorized with respect to their positional specificity of arachidonic acid oxygenation into 5-, 8-, 12-, and 15-LOXs^[11]. Within the 15-LOXs, two isoforms have been identified^[12]. 15-LOX-1 is a lipid-peroxidizing enzyme capable of introducing molecular oxygen to polyunsaturated fatty acids either in free form or in more complex compounds, such as biological membranes, phospholipids, cholesterol esters, and plasma lipoproteins^[13-15]. 15-LOX-2 has limited tissue expression in prostate, lung, skin, and cornea^[16]. In terms of enzymatic characteristics, 15-LOX-1 preferentially metabolizes linoleic acid primarily to 13- (S)-HODE also metabolizes arachidonic acid to 15-(S)-HETE^[17]. 15-LOX-1 is involved in many pathological conditions, such as in cell differentiation, in inflammation, in atherogenesis and carcinogenesis^[14]. Expression and function of 15-LOX-1 have been studied in endothelial cells, smooth muscle cells, monocytes, and 15-LOX-1 has been shown to play key roles in vascular remodeling and the progression of atherosclerosis^[18-20]. 15-LOX-1 has also been implicated for its role in angiogenesis. However, the evidence is controversial, even contradictory. Several studies in tumor models have shown that 15-LOX-1 could promote or inhibit angiogenesis.

Angiogenesis in 2 xenograft models is inhibited in transgenic mice overexpressing 15-LOX-1 in ECs under the control of preendothelin promoter^[21]. In contrast, the PC-3 human prostate cancer cell line, which overexpresses 15-LOX-1, secretes high levels of VEGF and enhances angiogenesis as compared with the parental PC-3 cell lines^[22]. Considering the angiogenic process is complex and the role of 15-LOX-1 in the development of RNV has not been well investigated, we explore the changes in 15-LOX-1 expression on RMVECs in hypoxia condition and determine whether 15-LOX-1 activity impact on proliferation of RMVECs and the underlying mechanism in this study.

MATERIALS AND METHODS

Materials

Recombinant adenoviral vector construction Two recombinant Adenoviral vectors based on the pDC315-EGFP vector (purchased from Shanghai GeneChem Co.Ltd) were constructed, respectively, expressing the mouse 15-LOX-1 gene and the green fluorescence protein gene under the control of the mouse CMV promoter. Stock solution of Ad-15-LOX-1 and Adenoviral vector were 1.25×10^{11} and 2.50×10^{11} plaque formation unit (PFU)/mL, respectively. Working solution were prepared to make 1 μ L of vehicle contained approximately 1.0×10^6 PFU.

RMVECs culture and identification Primary RMVECs (MIC-CELL-0046, PriCells, China) were isolated from the retinas of C57/BL6J mice. The third generation of RMVECs was used in the experiments.

Methods

Transfection recombinant adenoviral vectors into RMVECs RMVECs were seeded in a 6-well plate. Twenty-four hours later, RMVECs obtained a final cell density of 6×10^5 mL⁻¹ and changed 2mL fresh medium. Each micropipet was calibrated to deliver approximately 1.0×10^6 PFU of Ad-15-LOX-1 or Adenoviral vector. RMVECs were analyzed with 100 \times magnification by fluorescence microscopy (OLYMPUS BH2-RFL-T3) to ensure that the transfected cells express EGFP. EGFP fluorescence was detected at 510-532nm with excitation at 488nm.

In vitro hypoxia model and assessment The experiments were divided into four groups: (1) normal control group (normoxia group), (2) untreated hypoxia group (hypoxia group), (3) hypoxia treated with *Ad-15-LOX-1* group (15-LOX-1 group), and (4) hypoxia treated with Adenoviral vector group (vector group). RMVECs of hypoxia group, 15-LOX-1 group, vector group were placed inside a bio-bag (Mitsubishi Chemical Corporation, Japan) and placed in an anaero pack to induce hypoxia. Cultures were exposed in a hypoxic condition for 12, 24, 48 and 72 hours. As a control, cultures were also incubated under a normoxic condition for the same length of time. Each group was placed into an incubator at 37 $^{\circ}$ C. Culture supernatants were aspirated with a blood-gas sampling needle. The Po₂, Pco₂, and pH values of the different hypoxia groups were measured using a blood-gas analyzer (ABL555; Radiometer, Copenhagen, Denmark).

Examination of cell proliferation by the CCK-8 method RMVECs were seeded in a 96-well plate. Each group had 5 replicates holes. Each of the 96-well plate was located in the blank control group (added 100 μ L culture medium, without cell). Cell proliferation was evaluated

using a CCK-8 assay according to the method of Yang *et al.*^[23]. The duration of hypoxia was 12, 24, 48 and 72 hours. As a control, cultures were also incubated in a normoxic condition for the same lengths of time. Optical density was measured with an ELISA Reader (Rayto RT-6500) at 450nm. All experiments were carried out in triplicate.

Real-time RT-PCR Total RNA was isolated from the RMVECs of each group according to standard techniques^[24]. Reverse transcription was performed according to the manufacturer's guidelines (Fermentas, Life sciences). The sequences of primers were designed using the Primer Express software (PE Biosystems). The primer sequences were 15-LOX-1: 5'- ATCCGTCCAAGATGACCAAG-3' (forward) and 5'-AAGAGTCAACTGGCCTGAA-3'(reverse), VEGFA:5'-CAGGCTGCTGTAACGATGAA-3'(forward) and 5'-TTTGACCCTTTCCTTTCCT-3'(reverse), VEGFR2:5'-TTCTGGACTCTCCCTGCCTA-3' (forward) and 5'-AAGACCATCCCACTGTCTG-3' (reverse), eNOs:5'-AGCATACCCCACCTTCTGTG-3' (forward) and 5'-GAAGATATCTCGGGCAGCAG-3' (reverse), PPAR-r: 5'-AAGAGCTGACCCAATGGTTG-3' (forward) and 5'-ACCCTTGCATCCTTCACAA G-3'(reverse), β -actin:5'-CACGATGGAGGGGCGGACTCATC-3'(forward) and 5'-TAAAGACCTCTATGCCAACACAGT-3' (reverse). The PCR reaction was performed in a volume of 25 μ L using SYBR green mix (Toyobo, Shanghai, China) on the Rotor-Gene 3000 Real-time PCR instrument (Corbett Research, Sydney, Australia). The thermal cycling program consisted of 1 min at 95 $^{\circ}$ C, 40 cycles of 15 sec at 95 $^{\circ}$ C, 15 sec at 58 $^{\circ}$ C, 45 sec at 72 $^{\circ}$ C. Relative quantification of the gene expression was performed using the $2^{-\Delta\Delta CT}$ method^[25]. All experiments were carried out in triplicate.

Western blot analysis The RMVECs cells were homogenized by 200 μ L of lysis buffer (20mmol/L Tris, pH 7.4, 150mmol/L NaCl, 1mmol/L EDTA, 1mmol/L orthovanadate, 1mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin and 10 μ g/mL aprotinin) on ice. The protein concentration of the sample was determined using the Bradford assay. Equal amounts (50 μ g) of proteins from each sample were loaded on SDS-PAGE and then transferred onto nitrocellulose membrane (Hybond-C, Amersham, Arlington Heights, IL) at 200mA for 1 hour. After blocking of nonspecific binding sites with 5% skim milk for 1 hour, the membrane was incubated with the primary antibodies as the following dilutions: mouse monoclonal antibody to 15-LOX-1 at 1:200 (Abcam, Cambridge, MA, USA), rabbit polyclonal antibodies to VEGFA at 1:1000 (Bioss Biotechnology, Beijing, China), rabbit polyclonal antibodies to VEGFR₂ at 1:800 (Bioss Biotechnology, Beijing, China), rabbit polyclonal antibodies to eNOs at 1:500 (Bioss Biotechnology, Beijing,

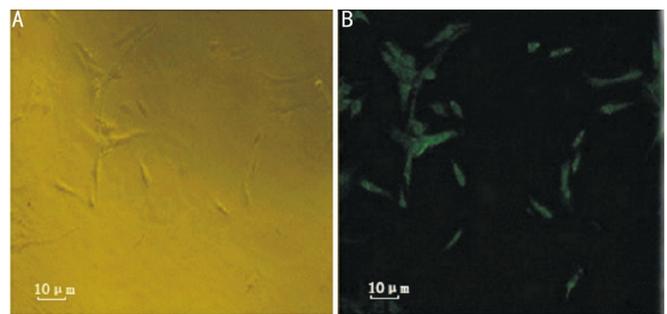


Figure 1 RMVECs culture and identification A: RMVECs forming a colony of spindle shaped cells in primary culture (400 \times); B: Expression of CD31 in RMVECs with FITC staining.

China) and rabbit polyclonal antibodies to PPAR-r at 1:200 (Bioss Biotechnology, Beijing, China) overnight at 4 $^{\circ}$ C. Antibody dilutions were made in a solution of 5% skim milk /0.1% Tris-buffered saline Tween-20. Then, membranes were washed with TBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Boster Biotechnology, Wuhan, China) in blocking buffer for 2 hours at room temperature. After four washes, the proteins were detected with ECL kit (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK). β -actin staining served as the internal standard for all membranes.

Statistical Analysis The SPSS 13.0 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The results are expressed as the means \pm standard error of mean (SEM) and were subjected to One-way analysis of variance (*ANOVA*). Differences between two groups were determined by Student's *t* test. *P* value of <0.05 indicates statistical significance.

RESULTS

RMVECs Culture and Identification Primary RMVECs that were isolated from the retinas of C57/BL6J mice formed a colony of spindle shaped cells after 3 days in culture. Confluence of RMVECs of the primary culture exhibited contact inhibition and presented a typical paving stone appearance. Routine evaluation for FITC-labeled CD31 showed that cells were pure (Figure 1).

Fluorescence Microscopy Identification after Transfection

RMVECs were transfected with Ad-15-LOX-1 or Adenoviral vector. The expressions GFP were visualized by fluorescence microscopy at 12, 24, 48 and 72 hours post-transfection. Ad-15-LOX-1 or Adenoviral vector was transfected into RMVECs (Figure 2).

In vitro Hypoxia Model and Assessment The Po₂, Pco₂, and pH values of the different hypoxia groups were measured with a blood-gas analyzer. Experiments showed that when the cells had been exposed to hypoxic conditions for less than 1 hour, the Po₂ was 11.22 to 12.5 Kpa, the Pco₂ was 4.58 to 5.57 Kpa, and the pH was 7.30 to 7.40.

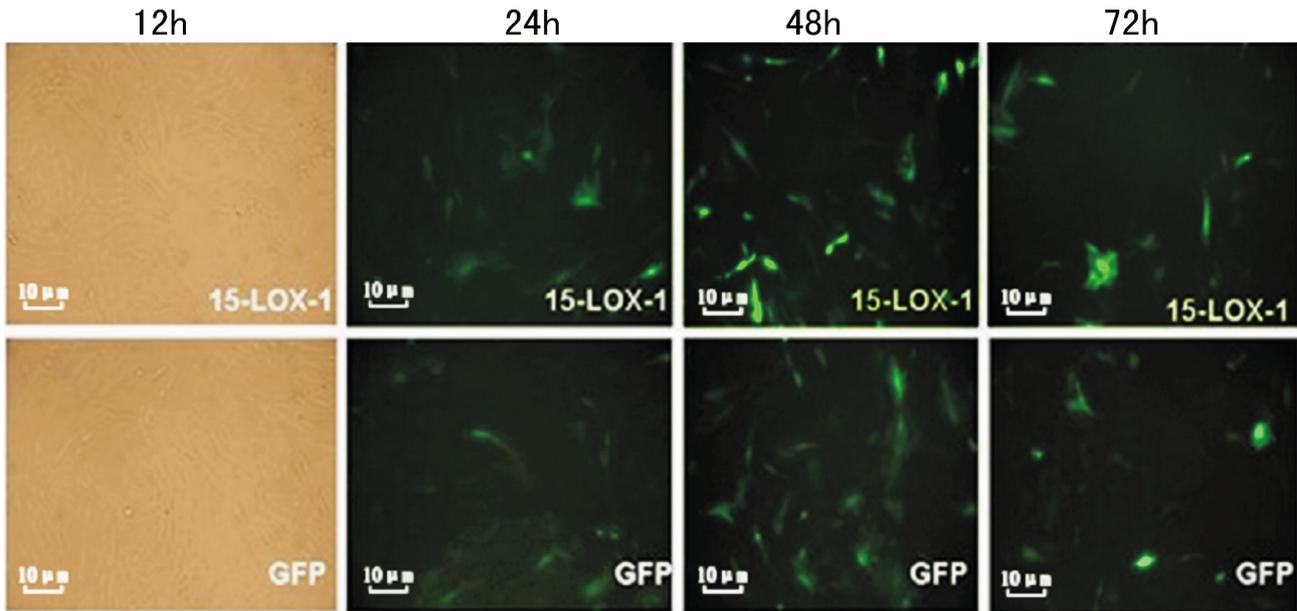


Figure 2 Photographs of *Ad-15-LOX-1* or *Ad-GFP* transfected RMVECs. RMVECs were transfected with *Ad-15-LOX-1* or *Ad-GFP*. The expressions of EGFP and GFP were visualized by fluorescence microscopy (100×) at 24, 48 and 72 hours post transfection.

When the cells had been exposed to hypoxic conditions for 2 hours, the Po₂ was 5.6 Kpa, the Pco₂ was 6.0 Kpa, and the pH was 7.01. When the cultures had been exposed to hypoxic conditions for more than 2 hours, the Po₂ was 4.5 to 5.4 Kpa, the Pco₂ was 6.13 to 6.54 Kpa, and the pH was 6.8 to 7.0 (Figure 3).

Examination of Cell Proliferation by the CCK -8 Method

The proliferative capacities of RMVECs that had been incubated in a hypoxic environment were significantly higher at each time point than they were in cells that had been incubated in a normoxic environment ($P < 0.05$). In a hypoxic environment, the proliferative capacities of RMVECs in 15-LOX-1 group were significantly inhibited. The proliferative capacities of RMVECs in 15-LOX-1 group significantly decreased compared with the hypoxia group ($P < 0.05$); No significant differences of the proliferative capacities were found between vector group and hypoxia group ($P > 0.05$) (Figure 4).

Real-time RT-PCR analysis All points were measured relative to β -actin and normalized to the normoxia group. All data were presented as the means \pm SE. The analysis was performed as follows: $\Delta CT = CT(\text{test gene}) - CT(\beta\text{-actin})$; $\Delta\Delta CT = \Delta CT(\text{hypoxia group RNA}) - \Delta CT(\text{normoxia group RNA})$; Relative Expression = $2^{-\Delta\Delta CT}$. Real-time RT-PCR analysis revealed that the expressions of VEGF-A, VEGF-R2 and eNOs mRNA increased in hypoxia group compared with normoxia group ($P < 0.01$). However, the expressions of 15-LOX-1, PPAR-r mRNA decreased in hypoxia group compared with normoxia group ($P < 0.01$). This analysis also showed that in a hypoxic condition, the

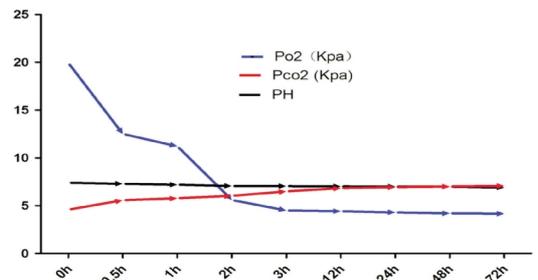


Figure 3 The hypoxia model *in vitro* was assessed by a blood-gas analyzer. The Po₂, Pco₂, and pH values of the hypoxia groups were measured with a blood-gas analyzer at each time point. When the cultures had been exposed to hypoxic conditions for more than 2 hours, the Po₂ was 4.5 to 5.4 Kpa, the Pco₂ was 6.13 to 6.54 Kpa, and the pH was 6.8 to 7.0.

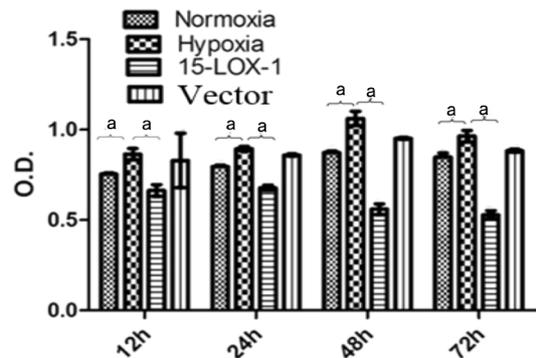


Figure 4 The proliferative capacities of RMVECs were evaluated by CCK-8 method. The proliferative capacities of RMVECs in hypoxic group significantly increased compared with the normoxic group at each time point; the proliferative capacities of RMVECs in 15-LOX-1 group significantly decreased compared with the hypoxia group ($P < 0.05$); no significant differences of proliferative capacities were found between GFP group and hypoxia group ($P > 0.05$). All data represent mean \pm SEM ($n = 3$); ^a $P < 0.05$.

expressions of VEGF-A, VEGF-R2 and eNOs mRNA decreased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$); However, the expressions of 15-LOX-1 and PPAR-r mRNA increased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). No significant differences of the mRNA expressions were found between vector group and hypoxia group ($P > 0.05$) (Figure 5).

Western –blotting Analysis As show in Figure 6, the expressions of VEGF-A, VEGF-R2 and eNOs proteins were higher in the hypoxia group than those in normoxia group at 12, 24, 48 and 72 hours. However, the expressions of 15-LOX-1, PPAR-r proteins decreased in the hypoxia group compared with the normoxia group at each time point. This analysis also showed that in a hypoxic condition, the expressions of VEGF-A, VEGF-R2 and eNOs proteins decreased in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). In a hypoxic condition, the expressions of 15-LOX-1 and PPAR-r proteins increased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). No significant differences of proteins expressions between vector group and hypoxia group ($P > 0.05$). The band were quantified by densitometry and normalized by β -actin (Figure 7).

DISCUSSION

RNV occurs in a number of oxygen induced retinopathy (OIR) including AMD, PDR and ROP [26]. For many years, treatments for RNV in ocular diseases were limited to laser and cryosurgery of the avascular zone, which were effective for reducing severe vision loss, whereas could lead to serious side effects at the same time. It is very important to develop new therapies for the pathological angiogenesis. In recent years, progress has been made in the pharmacological inhibition of the angiogenic stimulators such as VEGF. For instance, anti-VEGF strategies have recently emerged as valuable new therapies for ocular neovascularization diseases [27-29].

We have previously used the Bio-bag to make a hypoxia-induced RNV model of RMVECs and showed that hypoxia triggers the induction of VEGF-A expression in RMVECs, which, through an autocrine mechanism, binds to the up-regulated receptor VEGFR₂ and the messenger eNOs (Figure 8), to promote the cells proliferation and ultimately lead to angiogenesis which results in retinal pathological neovascularization.

In recent years, progress has been made by inhibition of the angiogenic factors like VEGF.

15-LOX-1 was found being implicated in angiogenesis, but previous studies apparently contradicted on the angiogenic

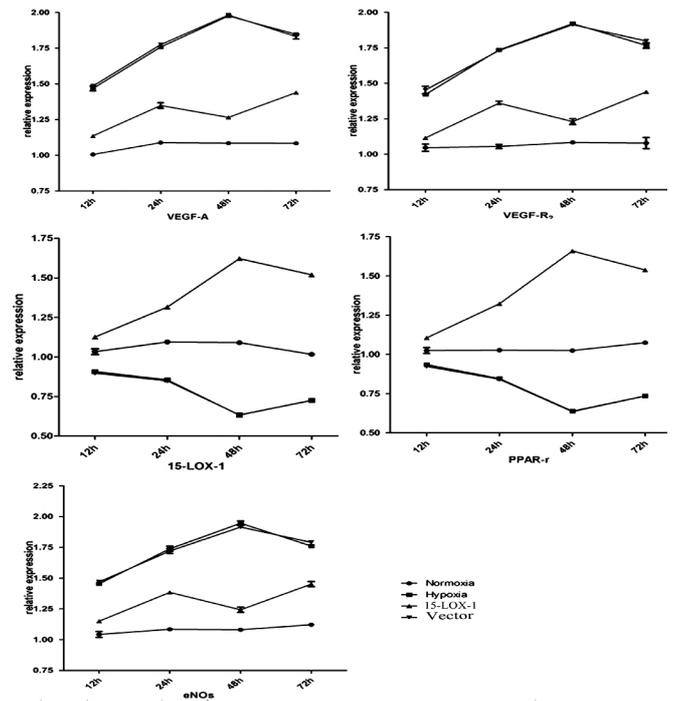


Figure 5 The expressions of 15-LOX-1, PPAR-r, VEGF-A, VEGF-R₂ and eNOs mRNA at each time point were determined by real-time RT-PCR. The expressions of VEGF-A, VEGF-R₂ and eNOs mRNA increased in hypoxia group compared with normoxia group ($P < 0.01$). However, the expressions of 15-LOX-1, PPAR-r mRNA decreased in hypoxia group compared with normoxia group ($P < 0.01$). In a hypoxic condition, the expressions of VEGF-A, VEGF-R₂ and eNOs mRNA decreased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$); However, the expressions of 15-LOX-1 and PPAR-r mRNA increased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). No significant differences of the mRNA expressions were found between vector group and hypoxia group ($P > 0.05$). All data represent mean \pm SEM ($n = 3$). ^b $P < 0.01$.

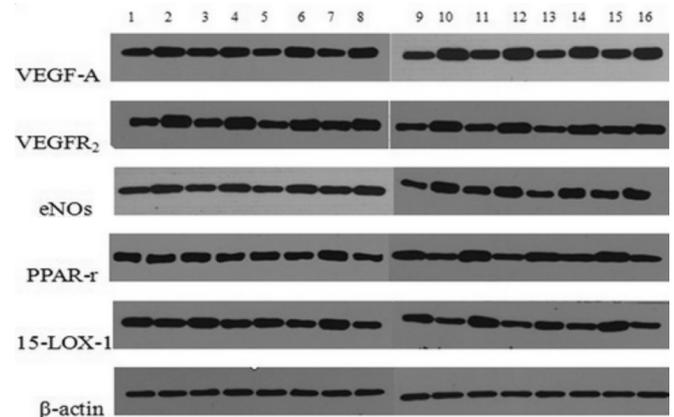


Figure 6 Shown is the Western blot analysis of 15-LOX-1, PPAR-r, VEGF-A, VEGF-R₂, eNOs and β -actin proteins at each time point. From the left: (1-4) 12h; (5-8) 24h; (9-12) 48h; (13-16) 72h; (1, 5, 9, 13) normoxia group; (2, 6, 10, 14) hypoxia group; (3, 7, 11, 15) 15-LOX-1 group; (4, 8, 12, 16) GFP group.

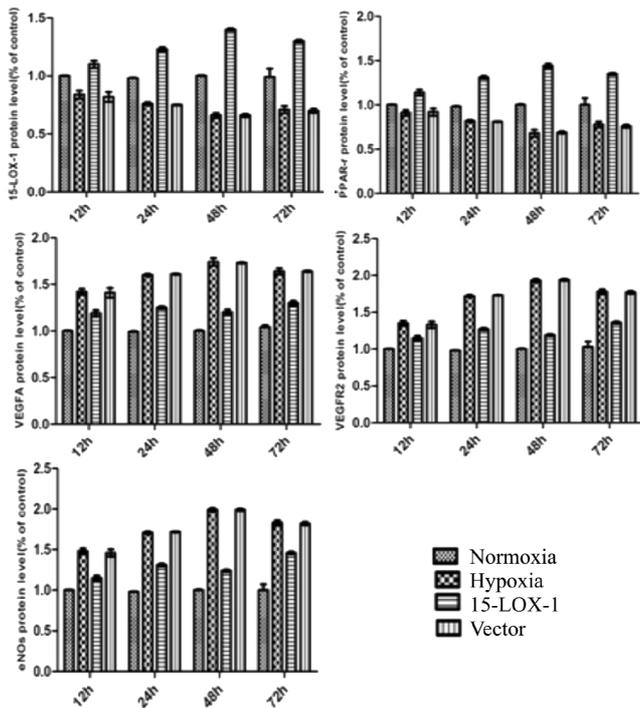


Figure 7 The relative expressions of 15-LOX-1, PPAR-r, VEGF-A, VEGF-R2 and eNOs proteins at each time point by western blot. The bands were quantified by densitometry and normalized by β -actin. The expressions of VEGF-A, VEGF-R2 and eNOs proteins increased in the hypoxia group than those in normoxia group at 12, 24, 48 and 72 hours. However, the expressions of 15-LOX-1, PPAR-r proteins decreased in the hypoxia group compared with the normoxia group at each time point. This analysis also showed that in a hypoxic condition, the expressions of VEGF-A, VEGF-R2 and eNOs proteins decreased in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). However, the expressions of 15-LOX-1 and PPAR-r proteins increased significantly in 15-LOX-1 group compared with hypoxia group ($P < 0.01$). No significant differences of proteins expressions between GFP group and hypoxia group ($P > 0.05$). All data represent mean \pm SEM ($n=3$).

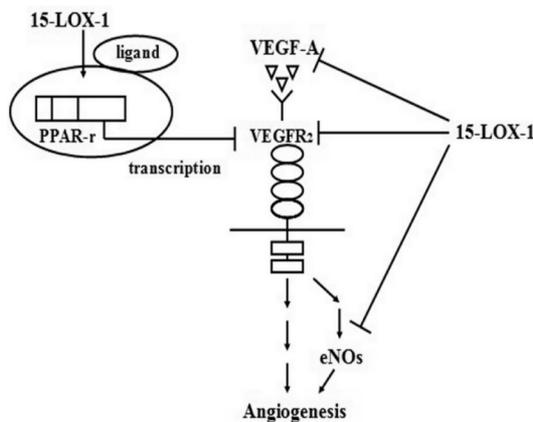


Figure 8 Proposed mechanism for the inhibitory effect on RNV by 15-LOX-1.

properties of 15-LOX-1 [22], varied from promoting angiogenic effect to its suppression of angiogenesis [21,30]. The levels of 15-LOX-1 in normal and pathological conditions

have not been revealed previously and the role of 15-LOX-1 in hypoxia-induced RNV needs further investigation. To the best of our knowledge, this is the first study to describe changes in the expression of 15-LOX-1 during angiogenesis model *in vitro* which results in retinal pathological neovascularization. The major findings of our study include (1) decreased expression of 15-LOX-1 and PPAR-r during angiogenesis model *in vitro*; (2) retinal 15-LOX-1 levels are negatively correlated with the proliferation of RMVECs; (3) inhibition of VEGF family expression by overexpression 15-LOX-1; (4) activation of PPAR-r effect on VEGFR₂ by overexpression 15-LOX-1.

Our studies demonstrated decreased 15-LOX-1 and PPAR-r expressions in the hypoxia-induced RNV model of RMVECs. Moreover, the time course of the 15-LOX-1 change was negatively correlated with the proliferation of RMVECs. These findings suggested that 15-LOX-1 and PPAR-r may act as a negative regulator of retinal angiogenesis, and decreased 15-LOX-1 and PPAR-r levels led to RNV. However, the regulatory mechanism of 15-LOX-1 and PPAR-r expressions is presently unclear.

To evaluate the effect of 15-LOX-1 overexpression on RMVECs in the hypoxia-induced RNV model *in vitro*, we transferred *Ad-15-LOX-1* into the RMVECs in the hypoxia environment. The CCK-8 results showed that the proliferative capacities of RMVECs in 15-LOX-1 group were significantly inhibited compared with hypoxia group. Our study presented the first evidence suggesting that overexpression of the 15-LOX-1 in the RMVECs of hypoxia-induced RNV model inhibited the proliferation of RMVECs which led to angiogenesis and then resulted in RNV ultimately. These observations further indicate that the effect of 15-LOX-1 overexpression is an anti-angiogenic factor in the RNV.

It is well-known that VEGF plays an important role in the pathogenesis of neovascularization. VEGF is a potent endothelial cell mitogen that stimulates endothelial cell over-proliferation, migration, tube formation, and ultimately leads to pathological neovascularization. But few researches have elucidated the relationship between VEGF and 15-LOX-1 on RNV. Our analysis showed that in a hypoxic condition, the expressions of VEGF-A, VEGF-R₂ and eNOs mRNA decreased significantly in 15-LOX-1 group compared with hypoxia group. Our previous study displayed hypoxia triggers the induction of VEGF-A expression in RMVECs, which, through an autocrine mechanism, binds to the up-regulated receptor VEGFR₂ and the messenger eNOs, to promote the cells proliferation and ultimately lead to angiogenesis which results in retinal pathological

neovascularization. We hypothesized that the 15-LOX-1-mediated prevention of growth factor expression block these signaling cascades by inhibiting hypoxia-induced increasing in VEGF-A and VEGFR₂ expression as well as increasing in eNOs.

The 15-LOX-1 is an endogenous ligand of PPAR- α [31]. Interestingly, it has been shown in rabbit eye model that PPAR- α binding to VEGFR₂ promoter induced VEGFR₂ expression, but ligand binding to PPAR- α actually resulted in an inhibition of VEGFR₂ expression [32].

We hypothesized that 15-LOX-1 binding to PPAR- α in the RMVECs of hypoxia-induced RNV model could inhibit the hypoxia-induced expression of VEGFR₂. Thus, PPAR- α effect on VEGFR₂ could be an additional mechanism whereby 15-LOX-1 inhibits the hypoxia-induced angiogenesis. In conclusion, we have shown that 15-LOX-1 and PPAR- α may act as a negative regulator of retinal angiogenesis, and decreased 15-LOX-1 and PPAR- α levels leading to RNV. And the effect of 15-LOX-1 overexpression is an anti-angiogenic factor in hypoxia-induced RNV. Overexpression 15-LOX-1 in RMVECs of hypoxia-induced RNV blocks signaling cascades by inhibiting hypoxia-induced increases in VEGF-A and VEGFR₂ expression as well as increases in eNOs. PPAR- α effect on VEGFR₂ could be an additional mechanism whereby 15-LOX-1 prevents the hypoxia-induced angiogenesis.

Ranibizumab (trade name Lucentis) is the most effective treatment currently available for more damaging forms of RNV [33]. However, Ranibizumab has to be given intravitreally once a month because of short half-life (approximate 9 days) [34]. Patients have to undergo multiple rounds of intravitreal injection leading to complications like endophthalmitis, retinal detachment, and traumatic cataracts. Gene therapy may become more promising with comparable stable effect. Our study presented here showing that 15-LOX-1 gene transferred into RMVECs could also prevent hypoxia-induced RNV. For future gene therapy application in clinic, we need to make sure that the 15-LOX-1 gene introduced into RMVECs is functional and stable.

Understanding the mechanism of 15-LOX-1 pathway in inhibiting the proliferation of RMVECs may lead to developing better therapeutic approaches for the treatment of ocular neovascularization diseases.

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