

Original Article

Celecoxib attenuates retinal angiogenesis in a mouse model of oxygen-induced retinopathy

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Abstract: This study aimed to investigate the anti-angiogenic effects of Celecoxib on the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible transcription factor 1 α (HIF-1 α) in a mouse model for oxygen-induced retinopathy (OIR). The OIR mice were exposed to 75% oxygen from postnatal day 7 (P7) to P12, after which the mice were randomly assigned to two groups (Celecoxib and vehicle) and were brought to room air for additional five days. Celecoxib or vehicle was administered from P12 to P17. Age-matched mice maintained in room air from birth to P17 were administered vehicle from P12 to P17 (RA group). Blood vessel profiles in the retina were used to count by histologic methods. Retina protein and mRNA of VEGF and HIF-1 α were assessed by immunohistochemistry, western-blot and RT-PCR. Compared with the RA group, the OIR mice exhibited over-expression in VEGF and HIF-1 α mRNA and protein. In addition, they had a positive and spatial correlation. Celecoxib-treated OIR mice reduced the retinal neovascular tufts and the levels of VEGF and HIF-1 α . These data suggest that Celecoxib inhibits retinal pathogenic angiogenesis through down-regulating HIF-1 α expression which suppressing VEGF transcription. Celecoxib could potentially serve as a potent pharmaceutical agent to inhibit retinal angiogenesis.

Keywords: Hypoxia-inducible factor-1 α , vascular endothelial growth factor, retinal neovascularization, a selective cyclooxygenase-2 inhibitor

Introduction

Pathological ocular angiogenesis, or ocular neovascularization (NV), is a central feature of retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion and neovascular age-related macular degeneration, and is the leading cause of irreversible blindness in developed countries [1]. It is clear that ischemia-induced hypoxia is a central etiological factor in retinal NV. In response to hypoxia, various pro-angiogenic growth factors are produced, among which the VEGF is the major one promoting retinal NV [2]. Hypoxia has been shown to increase VEGF expression at the level of gene transcription, mRNA stability, translation, and protein secretion [3]. VEGF transcription is largely regulated by hypoxia-inducible transcription factor 1 (HIF-1), which consists of a hypoxically inducible subunit HIF-1 α and constitutively expressed subunit HIF-1 β . In normoxia, the HIF-1 α proteins are degraded causing no detectable HIF-

1 α protein. During hypoxia, however, HIF-1 α becomes stabilized and dimerizes with HIF-1 β to form a complex which becomes transcriptionally active [4]. VEGF binds with VEGF receptors (VEGFR-1 and VEGFR-2) expressed on the endothelial cells, initiating signal transduction cascades and leading to angiogenic endothelial cell behaviors [5]. Increased levels of VEGF and HIF-1 α were detected in the vitreous humor and in fibrovascular tissues from eyes with PDR [6-9]. VEGF and HIF-1 α were indicated to provide targets for therapeutic intervention on retinal neovascularization [10].

The cyclooxygenase (COX) pathway is also found to be vital in ocular NV [11]. COX enzymes catalyze the biosynthesis of prostanoids (prostaglandins and thromboxanes) from arachidonic acid. There are at least two COX enzymes. COX-1, the constitutively active isoform of COX, is a housekeeping enzyme and expressed in most tissues. The inducible isoform, COX-2, is

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induced under inflammatory conditions and cytokines, tumor promoters and plays a key role in regulating angiogenesis through the induction of prostanoid synthesis [12]. Studies suggest that COX-2-induced prostanoids exhibit angiogenic effects both up and downstream of growth factor production. Upstream, the prostanoids induce VEGF and bFGF [13]. Downstream, pro-angiogenic factors (e.g. hypoxia, VEGF) are believed to induce endothelial cell expression of COX-2 [14], and COX-2-derived prostanoids stimulated proliferation, migration and formation in human vein endothelial cells [15].

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as Aspirin and Naproxen inhibit the formation of both COX-1 and COX-2 and are commonly used for the treatment of pain and arthritis. Studies demonstrated that administration of high dose Aspirin reduced the incidence of retinopathy in patients with diabetes, retinal vascular leakage in diabetic rats and retinal vascular abnormalities in diabetic dogs [16-18], indicating benefits of COX inhibition in ameliorating diabetic retinopathy. However, there is evidence that NSAIDs produce serious gastrointestinal side effects due to the blockade of COX-1. COX-2 specific inhibitors were thus developed as anti-inflammatory and anti-angiogenic agents. Celecoxib, a highly selective COX-2 inhibitor, has been shown to effectively decrease tumor angiogenesis, reduce tumor growth in a variety of experimentally-induced tumors [19], and prevent pathological angiogenesis in the cornea and retina [20]. However, the exact mechanisms through which the COX-2 inhibition down-regulate pathological NV remain unclear. In the present study, we used the laboratory animal model of oxygen-induced retinopathy (OIR) that is a generally used method to observe the retinal microvascular complications in retinopathy of prematurity and diabetic retinopathy to test our hypothesis that Celecoxib is a preventive agent against pathological retinal NV by inhibiting the HIF-1 α and its downstream gene VEGF expression.

Materials and methods

Mouse model of OIR

C57BL/6J mice from the Laboratory Animal Center of China Medical University (Shenyang,

China) were obtained. All animal experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Medical University Ophthalmic Center. The methodology used for OIR model was consistent with that described by Smith et al [21]. In general, postnatal day 7 (P7) mice and their nursing mother were exposed to hyperoxia (75 \pm 3% oxygen) for 5 days (P7 to P 11) and then removed into normoxic condition for an additional 5 days (P12 to P17). The airtight incubator temperature was maintained at 23 \pm 2°C, and oxygen levels were monitored using a portable oxygen analyzer (Model CY-12C, Electrochemical Analytical Instruments, Ltd, Meicheng, Zhejiang, China).

Celecoxib preparation and administration

As described previously [22], Celecoxib (Sigma Chemical Co., St. Louis, MO) were suspended in vehicle to a 0.5-ml suspension (0.5% methylcellulose, 0.1% polysorbate 80 in water). From P12 to P17, the OIR mice were randomly assigned to two groups: Celecoxib (n = 24) and Vehicle (OIR) groups (n = 24), where 3 mg/kg/day celecoxib or equivalent volume of vehicle was administered through a gastric feeding tube. Age-matched mice maintained in room air from birth to P17 (RA group, n = 24) were treated with equivalent volume of vehicle daily from P12 to P17. The mice in all three groups were euthanized at P17 and their eyeballs or retinas were prepared for morphological study and biochemical assays.

Histological analysis of retinal sections and quantification of proliferative retinopathy

At P17, randomly chosen eight pups from each group were sacrificed and the eyes were enucleated, fixed in 4% paraformaldehyde for at least 24 h, and then embedded in paraffin. Sections (6 μ m), each 30 to 90 μ m apart, were cut from each eye parallel to the optic nerve, and stained with hematoxylin and eosin. Using an established technique [21, 23, 24], blood vessel profiles (BVPs) were counted in at least 10 sections from each eye by two independent observers blinded to treatment. Vascular cell nuclei were considered to be extending beyond the inner limiting membrane (ILM) into the vitreous. BVPs were counted in the ILM, ganglion

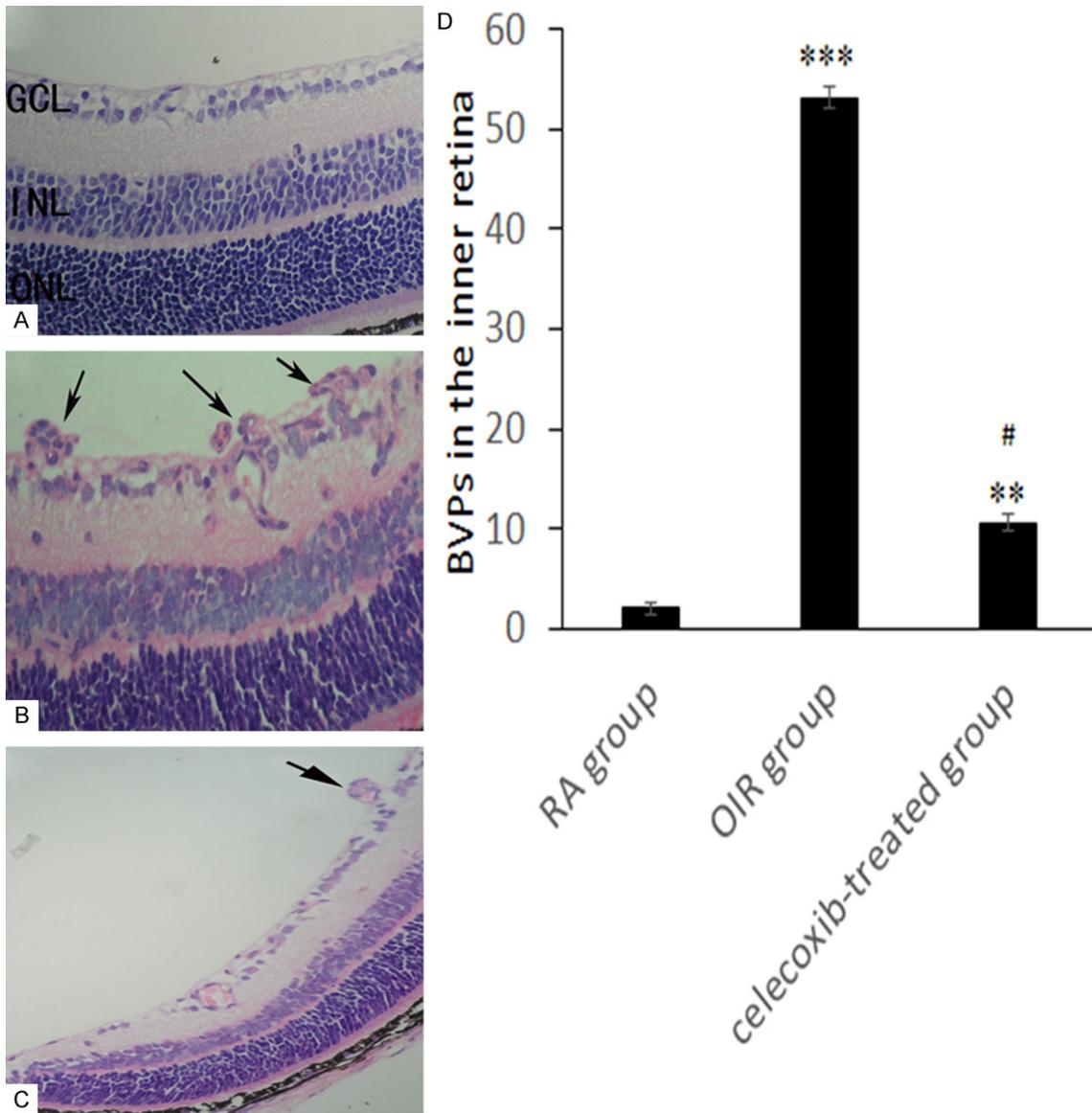


Figure 1. Hematoxylin and eosin stained cross-section from all the groups. Retinal NV was resolved by counting the number of extraretinal cell nuclei anterior to the ILM on P17. Neovascular tufts (BVPs) were proved by extending into the vitreous. A. No nuclei were detected in the RA group which extending to the vitreous, compare with OIR group. B. In contrast, there are many of pathologic neovascularizar tufts beyonding the ILM into the vitreous (arrows). C. The group treated with Celecoxib displays a significant reduction of the number of retinal neovascular cell nuclei, compared with OIR group, but more than that of the RA group (arrows). Magnification, $\times 200$. D. The average numbers of BVPs in the inner retina of the Celecoxib group mice were compared with OIR group and RA group mice using one-way ANOVA. Data are expressed as means \pm SD ($n = 8$). *** $P < 0.001$ compared with RA group; ** $P < 0.01$ compared with Celecoxib group; * $P < 0.05$ compared with RA group.

cell layer (GCL), and inner plexiform layer (IPL). A BVP was defined as an endothelial cell or a blood vessel with a lumen.

Retinal immunohistochemistry

Randomly chosen ten paraffin sections from each group were deparaffinized and rinsed with

3% hydrogen peroxide for 10 min and then incubated for 30 min at room temperature with blocking serum. The sections were subsequently incubated overnight at 4°C with primary antibodies (rabbit polyclonal antibodies; Santa Cruz, CA): HIF1- α (1:200 in PBS), VEGF (1:200 in PBS), respectively. After washing with 0.1 M PBS (three 2-min washes), the sections

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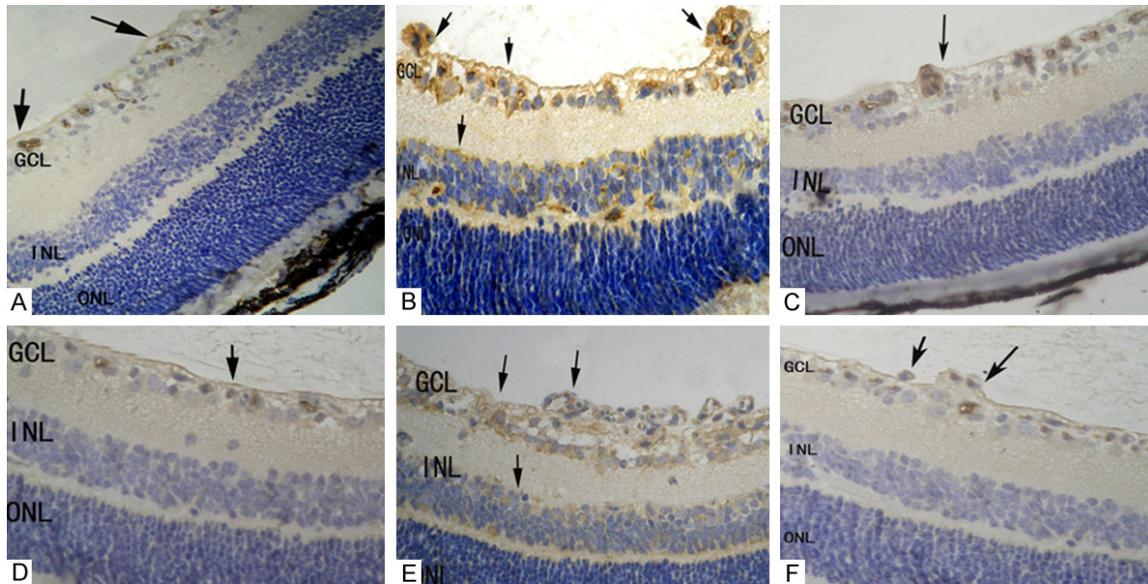


Figure 2. Immunohistochemical analysis for the protein expression of HIF-1a and VEGF in all the experimental groups. Low HIF-1a protein expressions were detected in GCL of the RA group retina (A, arrows). In contrast, there was strong HIF-1a protein expression in the inner nuclear layer (INL), the ganglion cell layer (GCL), as well as neovascularization breaking through the inner retinal layer in OIR mice (B, arrows); In the Celecoxib-treated OIR group, the HIF-1a immunolabeling was reduced, only showed in the cytoplasm of some ganglion cells or small amount extraretinal cell nuclei anterior to the ILM (C, arrows). The protein levels of VEGF were only weak present in the GCL in the RA groups (D, arrows), conversely, strong and many of VEGF staining were showed in the INL, GCL, as well as vascularized lesions (E, arrows). In the OIR treated with Celecoxib, the expression of VEGF was attenuated and was detected in the GCL and individual extraretinal cell nuclei (F, arrows). Magnification, $\times 200$.

were incubated with secondary antibody goat anti-rabbit Ig G (1:200 in PBS, Santa Cruz, CA) for 20 min at room temperature and were visualized by 0.05% diaminobenzidine (DAB) solution and stained with Mayer hematoxylin, and negative controls were taken by omitting the primary antibodies. Cells positive for HIF1- α and VEGF showed light yellow or dark brown coloration in the cytoplasm. The integrated areas of HIF1- α and VEGF staining were analyzed by using automatic microscope and image analysis system (LUZEX-F, Japan) and identified the integral optical density (IOD) values as an indicator of HIF1- α and VEGF expression.

Western blot analysis

Retinas were isolated from eight mice per group and were lysed in 200 μ L of lysis buffer with protease inhibitors (Sigma Chemical Co., St. Louis, MO). Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). 40 μ g of total protein extract from each sample were analyzed by SDS-PAGE and transferred onto a nitrocellulose membrane that was incubated with rabbit polyclonal anti-HIF1- α antibody (1:400, Santa Cruz,

CA) or polyclonal anti-VEGF antibody (1:300, Santa Cruz, CA) and β -actin monoclonal antibody (1:400 Santa Cruz, CA) overnight at 4°C followed by horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit antibody, 1:1500, Santa Cruz, CA). The proteins were detected with enhanced chemiluminescence-detection (Santa Cruz, CA). β -actin served as the internal standard. Experiments were repeated three times.

Reverse transcription-polymerase chain reaction

Total retinal RNA was isolated from all the three groups ($n = 8$) using Tri-Zol reagent (Life Technologies, Glasgow, UK) according to the manufacturer's protocol. RT-PCR assays were described previously [25]. Reverse transcription (RT) was performed with approximately 0.5 μ g of total RNA, reverse transcriptase (SuperScript II; Life Technologies, Gaithersburg, MD), and 5.0 μ M oligo-d (T) primer. Aliquots of cDNA were used for polymerase chain reaction (PCR) amplification with primers specific for VEGF, HIF1- α and β -actin. The primer sequenc-

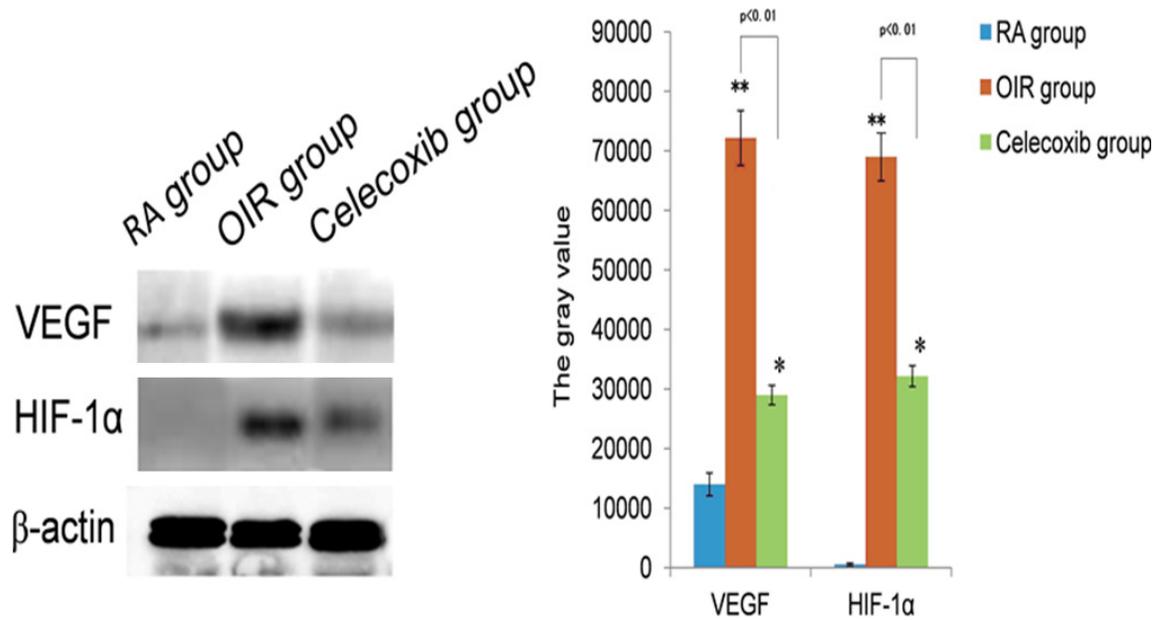


Figure 3. Western blot analysis for HIF-1α and VEGF protein levels in the three experimental groups. Both of the protein expression levels were increased greatly in the OIR group compared with the RA group (** $P < 0.01$). After the treated with Celecoxib in OIR group, the expression of these protein was significantly decreased compared with the OIR group ($P < 0.01$), but still much than in the RA group (* $P < 0.05$).

es were VEGF (127 bp): sense 5'-CTG TGC AGG CTG CTG TAA CG-3', antisense 5'-GAT CATT CGC TCT ATG TGG TGGC-3'; HIF1-α (460 bp): sense 5'-ACTTGATGTTCATCGTCCCTC-3', antisense 5'-CGGCGGAAGG AAAGAGT-3'; and β-actin (450 bp): sense 5'-TCT ACA ATG AGC TGC GTG TGG-3', antisense 5'-GGA ACC GCT CAT TGC CAA TG-3'. Standard PCR amplification was performed at 95°C for 2 min, 58°C for 1 min, and 72°C for 1 min for 30 cycles, 7 min at 72°C. The amplified products were visualized on 2% agarose gels. Calculation method: absorbance of target gene/β-actin absorbance value.

Statistical analysis

The results were expressed as mean ± standard deviation. Analysis of correlation between HIF1-α and VEGF expression was detected by linear correlation. One way ANOVA was used to analysis multiple variables and with student's *t* tests. A *P* value < 0.05 was considered to be statistically significant.

Results

Effects of celecoxib on preretinal neovascular nuclei

The degree of preretinal NV, vascular cell nuclei on the vitreal side of the internal limiting mem-

brane (ILM) was assessed quantitatively through counting at P17. In RA group, no nuclei of new vascular endothelial cells were broken through the inner retina (0.21 ± 0.06) (**Figure 1A**). In contrast, all mice in the OIR groups had large pathologic neovascular tufts that were adherent to the ILM (53.14 ± 1.06 , $P < 0.001$) (**Figure 1B**). The preretinal NV in the Celecoxib group was significantly reduced than that in the OIR group (10.65 ± 1.82 , $P < 0.01$), but more than that of the RA group ($P < 0.05$) (**Figure 1C**).

Immunohistochemistry for HIF1-α and VEGF protein expression

RA group retinas expressed low levels of HIF1-α and VEGF in ganglion cells layer (GCL) (IOD = 1.22 ± 0.50 , IOD = 2.36 ± 0.31 , respectively) (**Figure 2A, 2D**). In OIR group retinas, there was higher expression for HIF1-α and VEGF in the INL and GCL and neovascularization breaking through the inner retina compare with RA group (IOD = 8.42 ± 0.13 and IOD = 10.37 ± 0.17 respectively, $P < 0.05$) (**Figure 2B, 2E**). However, Celecoxib-treated OIR retinas displayed a significantly reduced expression in HIF1-α (IOD = 3.78 ± 0.56) and VEGF (IOD = 3.42 ± 0.23) compared with the OIR group ($P < 0.05$). HIF1-α and VEGF protein were detected in the cytoplasm of some ganglion cells and small amount

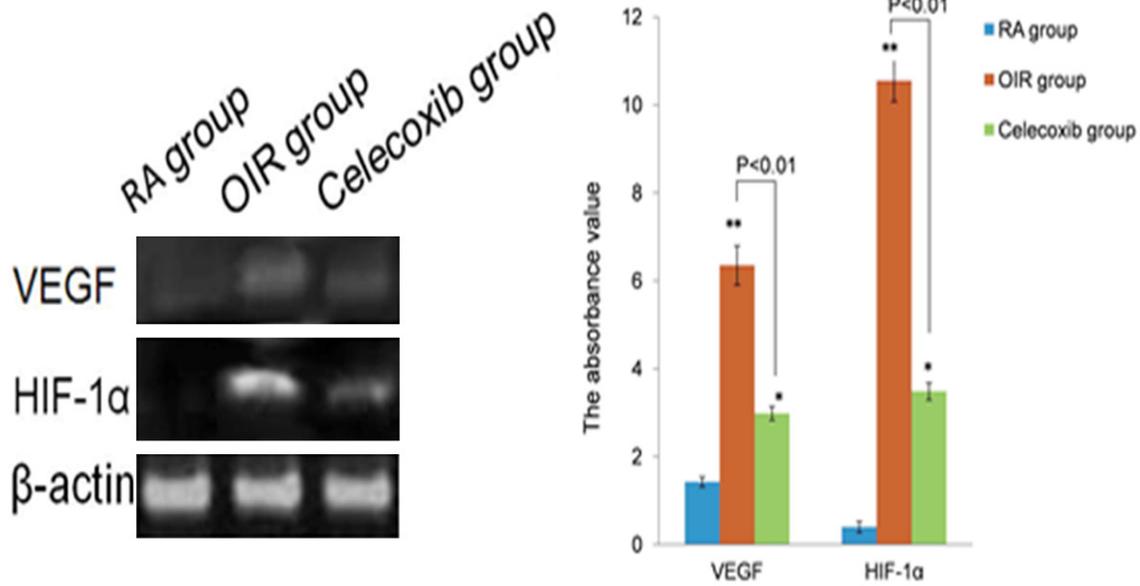


Figure 4. HIF-1 α and VEGF mRNA expression in all the groups were detected by RT-PCR. There was marked elevated of HIF-1 α and VEGF mRNA in ROP mice compared with those of the RA group (** $P < 0.01$). There was a significantly suppressed of HIF-1 α and VEGF mRNA in the retinas of ROP mice treated with Celecoxib, compared with those of the ROP mice. ($P < 0.01$), but was still more than the RA group ($*P < 0.05$).

extraretinal cell nuclei anterior to the ILM (Figure 2C, 2F).

Celecoxib Inhibits HIF1- α and VEGF protein levels

In the RA group, HIF1- α protein was not detected but the expression of VEGF was relatively strong. A significantly increased protein expression in HIF1- α and VEGF was seen in OIR group when compared with RA group ($P < 0.01$). In the Celecoxib-treated retina, the protein expression levels for HIF1- α and VEGF were markedly reduced compared to OIR ($P < 0.01$), but remained stronger than those in the RA group ($P < 0.05$) (Figure 3).

Effect of Celecoxib on HIF1- α and VEGF mRNA expression in the mouse OIR model

Overexpression of HIF1- α and VEGF was detected in the OIR group, treatment of Celecoxib significantly inhibited the mRNA expression of HIF1- α and VEGF. The level of HIF1- α mRNA expression in OIR group was higher than that in the RA group (3.94-fold, $P < 0.01$) and higher than those in the ROP group treated with Celecoxib (1.42-fold, $P < 0.01$). The VEGF mRNA expression was increased in OIR group than those in RA group (7.78-fold, $P < 0.01$) and high-

er than those in the ROP group treated with Celecoxib (1.51-fold, $P < 0.01$) (Figure 4).

Correlation analysis

The correlation between the levels of expression in HIF1- α , VEGF protein and their mRNA in the OIR group were statistically significant. ($P < 0.001$, $r = 0.725$ and $P < 0.001$, $r = 0.537$, respectively).

Discussion

OIR is a well-established animal model to investigate hypoxia-induced ocular NV. Using this method, the present study provided further evidence that hypoxia does up-regulate the HIF1- α and VEGF expressions. More importantly, our study for the first time demonstrated that the potent anti-angiogenic activity of celecoxib was associated with decreased expression of HIF1- α and VEGF on both protein and mRNA levels in retinal NV as assessed using western blot and RT-PCR. The suppression of retinal NV by celecoxib is, at least partially, the result of reduced VEGF and HIF1- α expression.

Today, various therapeutic strategies such as laser photocoagulation, photodynamic therapy, anti-VEGF therapy shows regression of the reti-

nal NV, severe undesirable side effects have been also reported such as the damage of retina and its photoreceptors, potential neuronal and glial toxicity, that do not cure the disease completely [26]. Thus, it is necessary to thoroughly understand mechanisms of NV in order to develop effective therapeutic targets. Clinical and experimental evidence suggests that retinal NV is often the result of ischemia-induced hypoxia. Several angiogenic growth factors and cytokines that stimulate angiogenesis participate in the pathogenesis of retinal neovascularization [27].

VEGF, an essential angiogenic factor, plays a crucial role in tumor angiogenesis and progression. Expression of the VEGF is up-regulated in the retina and vitreous of patients or the model animals with ischemic retinopathies. COX-2 expression has been found in various ocular tissues: cornea, iris, ciliary body, neuroretina and the retinal pigment epithelium, and its function is achieved through its prostanoid products which subsequently induce production of VEGF and other growth factors [28-30]. Cancer literature has shown that COX-2 and its prostanoid metabolites, in particular PGE₂, induce the expression of VEGF and bFGF thus promote angiogenesis during tumor growth [31]. In eyes, it has been found that hypoxia induces COX-2, prostanoid production, and VEGF synthesis in Müller cells, and that VEGF production is at least partially COX-2 dependent [32]. Using wild-type mouse Müller cells and COX-2 null mouse Müller cells, one study found that in response to hypoxia, the production of COX-2 and its prostanoid was increased in wild-type cells. VEGF levels were also found to be increased with a temporal sequence that lagged behind COX-2 induction and activity, suggesting that there is COX-2 dependent aspects of VEGF production, whereas COX-2 null mouse Müller cells treated with hypoxia produced significantly less VEGF. It was also revealed that prostanoid PGE₂ signaling through the EP₂ and/or EP₄ receptor and PKA, mediates the VEGF response of Müller cells [33]. As COX-2 and its prostanoids induce VEGF production, theoretically COX-2 inhibitors reduce VEGF production; therefore inhibit hypoxia-induced ocular NV. Several reports showed that Celecoxib, a highly selective COX-2 inhibitor, was a potent antiangiogenic agent in vitro and in vivo [34]. Oral Celecoxib inhibited angiogenesis by 79% in a rat model of bFGF-

induced corneal angiogenesis [35]. Celecoxib inhibited diabetes-induced retinal VEGF expression and vascular leakage in vivo [36]. Our study showed that the protein and mRNA levels of VEGF were increased in the ROP group and were significantly decreased in the ROP group treated with Celecoxib. This finding is consistent with studies demonstrating that treatment with Celecoxib decrease expression of VEGF in several other tissues.

As mentioned, HIF-1 α is a principal regulator responsible for the transcription of VEGF and other angiogenic factors. Our study showed that under hypoxic condition, the expression of HIF-1 α was increased along with over-expression in VEGF. Consistent with the research [38], it has been shown that tumor angiogenesis occurs partly by elevating the expression of VEGF, along with the up-regulation of HIF-1 α . Inhibition of COX-2 by Celecoxib caused a reduced expression in HIF-1 α and VEGF on both mRNA and protein levels. Retinal angiogenesis reduction was also showed. The findings suggest that, under hypoxic condition, COX-2 may play a vital part in promoting VEGF transcription by up-regulating HIF-1 α expression. The anti-angiogenic effect of Celecoxib is achieved, at least partially, by down-regulating HIF-1 α , thus reducing VEGF production. To our knowledge, this is the first study which demonstrated Celecoxib inhibited VEGF expression at transcriptional level through down-regulating HIF-1 α expression. The application of our findings is that Celecoxib may be a promising anti-angiogenic pharmacological agent for inhibition of retinal NV.

In our study, Celecoxib was effective in reducing retinal neovascularization through the inhibition of VEGF and HIF-1 α expression. However, suppression of retinal neovascularization was incomplete in ROP mice treated with Celecoxib, indicating that angiogenesis is a complex process, and there are other factors involved in these complex angiogenic cascades. A number of molecules that play role in hypoxia-induced angiogenesis remain to be further examined. The molecular mechanisms through which Celecoxib suppresses HIF-1 α expression also need further investigation.

In conclusion, our animal experiments suggest that Celecoxib inhibits retinal pathogenic angiogenesis through down-regulating HIF-1 α expres-

sion which subsequently suppressing VEGF production. Although the molecular mechanisms of how COX-2 inhibition suppresses HIF-1 α remains nuclear, our study provided evidence that Celecoxib can be a potentially promising potent anti-angiogenic agent for retinal NV.

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Disclosure of conflict of interest

None.

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