Biochemical and Biophysical Research Communications 460 (2015) 457–463

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Retinal hypoxia induces vascular endothelial growth factor through induction of estrogen-related receptor γ

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ARTICLE INFO

Article history:
Received 8 March 2015
Available online 19 March 2015

Keywords:
ERRγ
Ischemic retinopathy
VEGF

ABSTRACT

Ischemic retinopathies causing overexpression of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), are the most common cause of blindness. Thus, understanding the pathophysiology of targetable pathways that regulate retinal VEGF is of great interest. A conserved binding site for estrogen-related receptor γ (ERRγ) has been identified in the promoter of the *Vegfa* gene. ERRγ is a constitutively active orphan nuclear receptor and its expression is increased by hypoxic stimuli in metabolically active tissues. This study evaluated the role of ERRγ in the ischemic retina and the anti-VEGF potential of GSK5182, a selective inverse agonist of ERRγ. In an oxygen-induced retinopathy (OIR) mouse model, immunohistochemistry showed significantly increased ERRγ expression in the ganglion cell layer at postnatal day (P) 17. In a ganglion cell line (RGC-5), mRNA and protein levels of ERRγ were increased by desferrioxamine treatment and hypoxic conditions (1% O_2). Transient transfection of RGC-5 cells revealed that ERRγ regulated *Vegfa* expression and this was inhibited by GSK5182. Intravitreal injection of GSK5182 into the OIR model at P14 inhibited retinal *Vegfa* mRNA expression at P17. GSK5182 suppresses hypoxia-induced VEGF expression via ERRγ; therefore, ERRγ could be a treatment target for ischemic retinopathies.

1. Introduction

Ischemic retinopathy is common to the major causes of blindness, including proliferative diabetic retinopathy, venous occlusion, and retinopathy of prematurity. The hypoxia that follows retinal capillary obliteration drives deregulated growth of new blood vessels protruding out of retinal surfaces, without ameliorating retinal ischemia [1–3].

Under hypoxic conditions, dimerization of the stabilized hypoxia-inducible factor (HIF)-α subunit with the HIF-β subunit enables the transcription of a wide range of genes including those involved in cellular metabolism, hypoxia tolerance, and angiogenesis, such as vascular endothelial growth factor (VEGF) [4]. VEGF is a potent inducer of vasopermeability and macular edema [5], the leading cause of vision loss in working-age populations. Recently developed treatments using anti-VEGF agents result in the improvement of visual acuity in patients with diabetic macular edema. However, despite the clinically significant benefits of intravitreal injection of anti-VEGF agents, some patients show only a partial response to this therapy, with persistent diabetic macular edema and/or poor visual acuity [6]. Thus, understanding the pathophysiology of ischemic retinopathy and identifying potentially targetable pathways that regulate retinal VEGF is of great interest.

Abbreviations: DFO, desferrioxamine; ERRγ, estrogen-related receptor γ; GCL, ganglion cell layer; HIF, hypoxia-inducible factor; IHC, immunohistochemistry; INL, inner nuclear layer; OIR, oxygen-induced retinopathy; ONL, outer nuclear layer; P, postnatal day; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SEM, standard error of the mean; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.bbrc.2015.03.055
0006-291X/

A conserved binding site for estrogen-related receptor γ (ERRγ) has been identified in the promoter of the *Vegfa* gene [7–9]. ERRγ, like other members of the ERR subfamily, is a constitutively active orphan nuclear receptor. It is selectively expressed in metabolically active tissues such as skeletal muscle, liver, brain, and retina [10–13]. In liver cell lines, mRNA and protein levels of ERRγ are increased by hypoxia or desferrioxamine (DFO) treatment, and HIF-1α mediates the induction of ERRγ under hypoxic conditions [14]. The retina is a neural tissue with high metabolic activity and the highest oxygen consumption per unit weight of any human tissue [15]. However, the role of ERRγ in the retina, particularly in regulating VEGF in ischemic retinopathy, has not been studied.

GSK5182, a 4-hydroxytamoxifen analog, is a highly selective inverse agonist of ERRγ and does not interact with any other nuclear receptors, including ERRα, due to its non-covalent interactions with Y326 and N346 at the active site of ERRγ [16,17].

In this study, retinal ERRγ expression was increased in the oxygen-induced retinopathy (OIR) mouse model of ischemic retinopathy. Furthermore, VEGF expression was increased via ERRγ induction by hypoxic stimuli in a retinal cell line. Furthermore, the anti-VEGF potential of GSK5182 was demonstrated in the retinal cell line and OIR mouse model.

2. Material and methods

2.1. Chemicals and plasmids

GSK5182 (4-[(1Z)-1-{4-[2-(dimethylamino)ethoxy]phenyl}-5-hydroxy-2-phenylpent-1-en-1-yl]phenol) was synthesized as previously described [16]. GSK5182 was used in HCl salt form and dissolved in sterile-filtered 30% PEG-400 aqueous solution or dimethyl sulfoxide (Sigma, St. Louis, MO, USA). Desferrioxamine mesylate salt was purchased from Sigma. Human *Vegfa* promoter reporter constructs were provided by M.O. Lee (Seoul National University, Seoul, Korea). The pcDNA-ERRγ plasmid was provided by H.S. Choi (Chonnam National University, Gwangju, Korea).

2.2. Mouse model of OIR

C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME, USA) were used. Every procedure for nurturing and sacrificing mice strictly confirmed to the 'Agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research'. All experiments were approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-2012-80). All mice were kept in a 12 h light–dark cycle at ambient room temperature (19–22 °C). Mice were maintained on a standard diet for breeding (2018S rodent diet; Harlan Laboratories, IN, USA), with chow and water available ad libitum. For the OIR model, newborn pups at postnatal day (P) 7, along with their mother, were transferred to a chamber supplied with 75 ± 1% oxygen, as described previously [18], under continual monitoring with a ProOx 110 oxygen controller (Biospherix Ltd., Lacona, NY, USA) for 5 days. On P12, the mice were returned to the room air and induced subsequent relative retinal hypoxia to P17.

2.3. Retinal flat mounting

For retinal flat mounts, eyes from control mouse in normoxia and OIR mice at P17 were fixed in 4% paraformaldehyde (PFA) at 4 °C for 30 min prior to dissection and then removed the cornea, lens, and the vitreous for making the eye cups. After overnight fixation in 4% PFA, whole-mount retinas were washed in 1× phosphate-buffered saline (PBS) and incubated with an anti-PECAM-1 antibody (BD Biosciences, CA, USA) diluted in 1× PBS containing 1% bovine serum albumin and 0.1% Triton X-100. A Cy3-conjugated anti-rat antibody (Jackson ImmunoResearch, PA, USA) was used as the secondary antibody. Retinas were washed in 1× PBS containing 0.1% Triton X-100 and flat-mounted in ProLong® Gold antifade reagent (Life Technologies, NY, USA). Images were captured by confocal microscopy (Olympus IX81, Tokyo, Japan).

2.4. Tissue preparation for RNA analysis

Mice were anesthetized on P17 by intraperitoneal injection of pentobarbital at a concentration of 50 mg/kg body weight. After induction of deep anesthesia, eyes were carefully enucleated and the lens, vitreous, and optic nerve were removed. Thereafter, the eye cups were directly submerged in RNAlater (Ambion, Applied Biosystems, USA), incubated overnight at 4 °C in complete darkness, and frozen at −80 °C prior to RNA isolation.

2.5. Immunohistochemistry (IHC)

Eyes were enucleated at P17 and fixed in 4% PFA for 24 h at 4 °C. After fixation, the cornea, lens, and vitreous were carefully removed and the eye cup was embedded in paraffin. Specimens were sectioned in the sagittal plane at a thickness of 4 μm and adhered to glass slides. An anti-ERRγ antibody (R&D Systems, MN, USA) was used as the primary antibody. Hematoxylin was used as the counter stain. IHC data were quantified as the percentage of positively stained cells. A mean of 10 fields were examined at 400× magnification. Data are representative of three different experiments.

2.6. Cell culture and exposure of cells to hypoxia

The rat retinal ganglion cell line RGC-5 was a gift from Dr. N. Agarwal (University of North Texas Health Science Center, Fort Worth, TX, USA). RGC-5 cells were maintained in Dulbecco's Modified Eagle's Medium containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (all from Gibco, NY, USA). All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were pretreated with GSK5182 at the indicated concentration for 1 h and were then treated with DFO for 12 h. RGC-5 cells were placed in 1% oxygen (hypoxic) or room air (normoxic) conditions at 37 °C in a hypoxic chamber (Invivo$_2$ 400, Ruskin Technologies, UK).

2.7. Reverse transcription-PCR analysis

Total RNA was isolated using QIAzol reagent (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). The primers used for PCR were as follows: *Errγ*: forward, 5′-CTCAAGCAATAGTTGAAGGG-3′, and reverse, 5′-TGGCTCACAGCTCCTTCTGA-3′; mouse *Vegfa*: forward, 5′-TTACTGCTGTACCTCCACC-3′, and reverse, 5′-ACAGGACGGCTTGAAGATG-3′; rat *Vegfa*: forward, 5′-ATCATGCGGATCAAACCTCACCA-3′, and reverse, 5′-GAGCACTTTGGGTCCGGAGG-3′; *Actin*: forward, 5′-ATCCTGCGTCTGGACCTGGCT-3′, and reverse, 5′-CTGATCCACATCTGCTGGAAG-3′. Actin was used as an internal control to normalize the samples. Image J (NIH, USA) was used to analyze relative gene expression levels.

2.8. Western blot analysis

Cell lysates were prepared using lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl

fluoride, and 0.5% NP-40] containing a protease inhibitor cocktail (Sigma). The amount of protein was quantified using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA). Protein samples were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA). The membrane was incubated in blocking buffer. Anti-ERRγ (R&D Systems) and anti-HIF-1α (Novus Biologicals, CO, USA) primary antibodies were used. The primary anti-β-tubulin antibody (Abm, CA, USA) was used as an internal control. Immunoreactive proteins were visualized by chemiluminescence according to the manufacturer's instructions (UVITec, Cambridge, UK). The densities of the protein bands were quantified using Image J.

2.9. Generation of recombinant adenoviruses

ERRγ was inserted into the shuttle vector pAdTrack-CMV. The vector was then electroporated into BJ5183 cells containing the adenoviral vector Adeasy to generate recombinant adenoviral plasmids. Adenoviruses were amplified in HEK-293 cells and purified by CsCl (Sigma) gradient centrifugation. The virus titer was determined using the Adeno-X Rapid Titer Kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. The infection efficiency was visualized by GFP fluorescence after infection with a mock adenovirus or an adenovirus harboring ERRγ for 72 h.

2.10. In vitro transient transfection assay

RGC-5 cells were plated at a density of 1×10^4 cells per well in a 24-well plate and incubated for 24 h. The *Vegfa* promoter reporter construct (200 ng/well) and ERRγ plasmid (200 ng/well) were transiently transfected using Lipofectamine™ 2000 reagent (Invitrogen, NY, USA). The β-galactosidase plasmid (100 ng/well) was co-transfected as an internal control. The total amount of DNA was

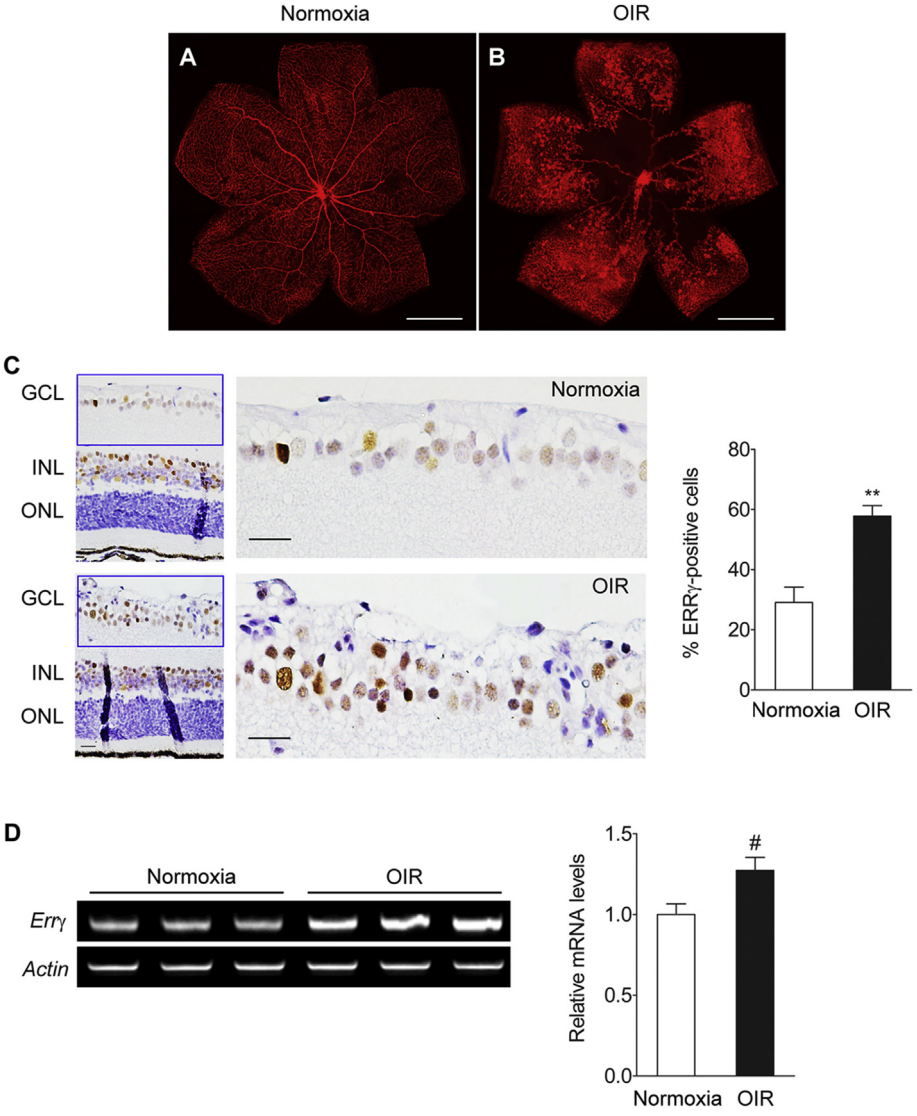

Fig. 1. Estrogen-related receptor γ (ERRγ) expression is increased in the retina of oxygen-induced retinopathy (OIR) mice. (A, B) PECMA-1-stained retinal flat mounts of a control mouse in normoxia (A) and an OIR mouse (B). Scale bars: 1000 μm. (C) Left: Immunohistochemical staining of ERRγ in vertical retinal sections of a control mouse in normoxia (upper) and an OIR mouse (lower) at postnatal day (P) 17. ERRγ expression in the ganglion cell layer (GCL) was higher in the OIR mouse than in the control mouse. Representative images are shown at a magnification of ×400. The boxed regions are shown at higher magnification on the right. Scale bars: 20 μm. INL: inner nuclear layer; ONL: outer nuclear layer. Right: Quantification of ERRγ-positive ganglion cells, presented as the percentage of total cells in the GCL. Data represent the means ± standard error of the mean (SEM). $^{**}P < 0.01$ vs. control. (D) mRNA analysis of *Errγ* in whole retinas of control and OIR mice at P17. Actin served as the internal control. The graph shows mRNA expression relative to that in control mice. Data are representative of three independent experiments. Values represent the means (±SEM) of three independent experiments. $^{\#}P < 0.05$ vs. control.

kept constant by adding the pcDNA3 empty vector. After 24 h, cells were treated with GSK5182 dissolved in dimethyl sulfoxide at the indicated concentration. Cells were harvested 48 h after transfection and luciferase activity was measured. Cell lysates were analyzed using a luciferase assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA). Luciferase activity was detected using an Orion L Microplate Luminometer (Berthold, Pforzheim, Germany) and normalized to β-galactosidase activity. Fold activity was calculated compared to that in cells transfected with the reporter gene alone.

2.11. Intraocular administration of GSK5182

To deliver GSK5182 into the retina of OIR mice, the indicated amount of reagent was injected into the vitreous cavity using a Nanoliter 2000 micro-injector (World Precision Instruments,

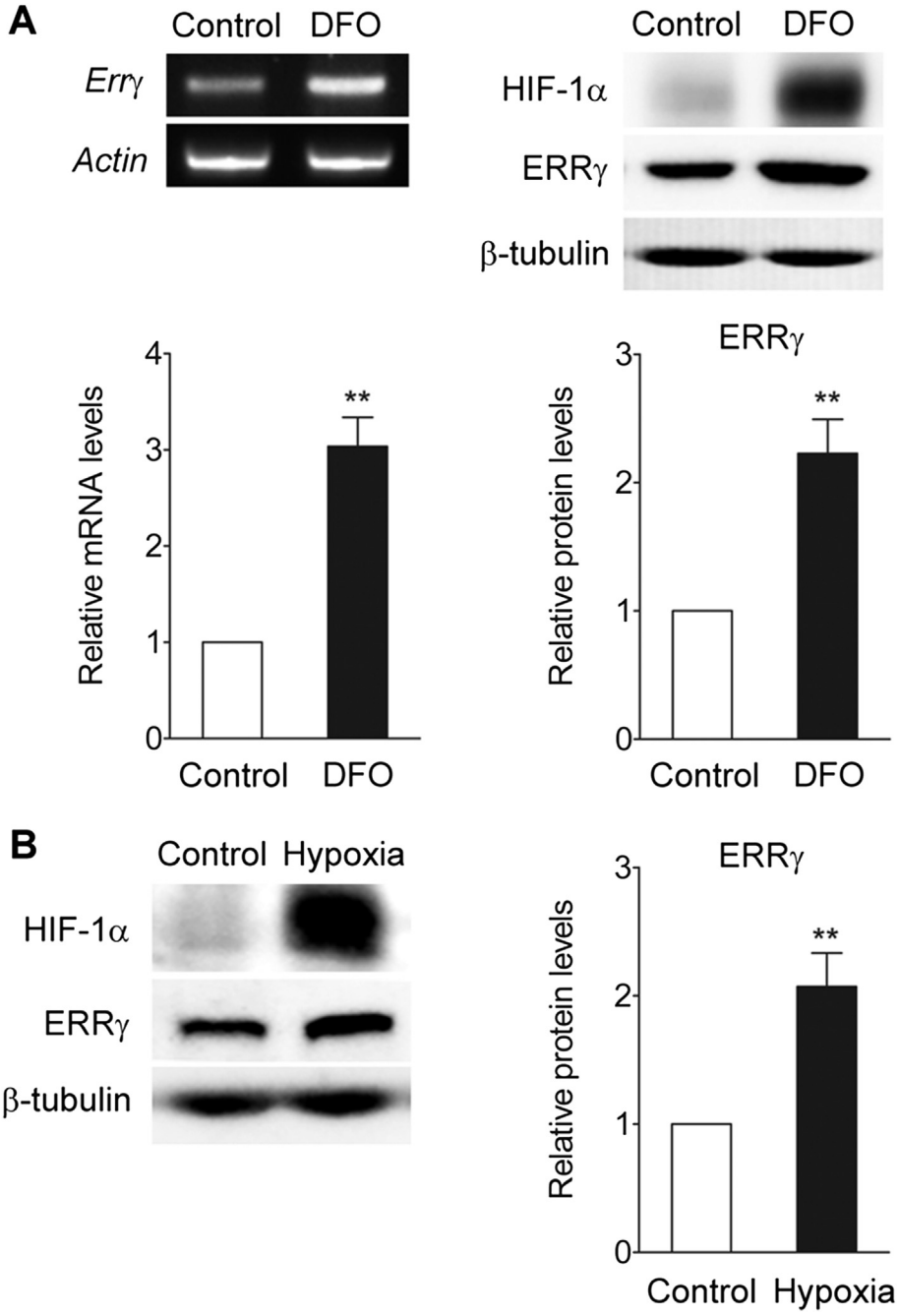

Fig. 2. Estrogen-related receptor γ (ERRγ) expression in retinal ganglion cells is increased by hypoxia. (A) Left: Representative reverse transcription-PCR analysis of the effect of desferrioxamine (DFO) treatment on mRNA expression of *Errγ*. RGC-5 cells were incubated with DFO (100 μM) for 12 h. Data represent the means ± standard error of the mean (SEM) of three independent measurements. Right: Representative Western blot analysis of the effect of DFO treatment on protein expression of ERRγ in RGC-5 cells. (B) Representative Western blot analysis of the effect of hypoxia (1% O_2) on protein expression of ERRγ in RGC-5 cells. RNA and protein band densities were quantified, with actin or β-tubulin as the internal control. Values represent the means (±SEM) of independent experiments (n = 3–4 per group). Graphs show mRNA/protein expression relative to that in control cells. $^{**}P < 0.01$ vs. control.

Sarasota, PL, USA) fitted with glass capillary pipettes under anesthesia. Intravitreal injection was performed at P14 by delivering 1 μl of GSK5182 (10 mM) into the right eye. The contralateral eye was injected with vehicle (30% PEG 400 prepared in distilled water) as a control.

2.12. Statistical analyses

Data are shown as the means ± standard error of the mean. Data were examined by a one-way analysis of variance to determine the significance of differences between groups. $P < 0.05$ was considered significant. All experiments were independently repeated three or more times.

3. Results and discussion

3.1. Increased ERRγ expression in the retinas of OIR mice

The retina is a neural tissue with high metabolic activity and the highest oxygen consumption per unit weight of any human tissue; therefore, it is not surprising that previous reverse transcription-PCR analysis revealed that ERRγ is expressed in the retina [12].

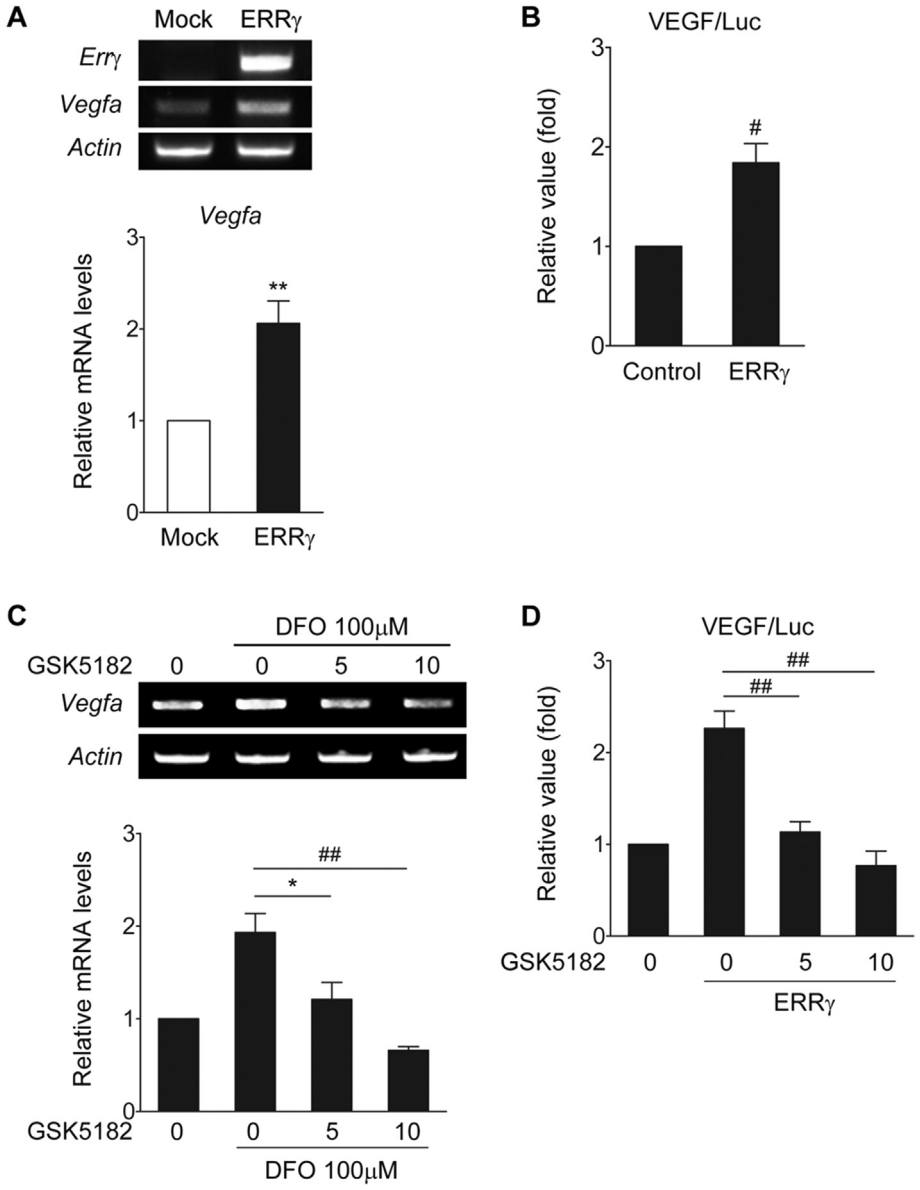

Fig. 3. Vascular endothelial growth factor A (VEGFA) expression is increased by estrogen-related receptor γ (ERRγ) overexpression and decreased by treatment with GSK5182, an inverse agonist of ERRγ. (A) Analysis of *Errγ* and *Vegfa* mRNA in RGC-5 cells. RGC-5 cells were infected with an adenovirus harboring ERRγ (ERRγ) or a mock adenovirus (Mock) for 72 h (multiplicity of infection = 100). mRNA expression was measured relative to that in Mock-infected cells, with actin as the internal control. Data represent the means ± standard error of the mean (SEM) of four independent measurements. $^{**}P < 0.01$ vs. control. (B) The effect of ERRγ on *Vegfa* promoter activity. Cells were transfected with a *Vegfa* promoter reporter construct and an ERRγ expression construct. Data represent the means ± SEM of independent measurements (n = 5). $^{\#}P < 0.005$ vs. control. (C) The effect of GSK5182 treatment on desferrioxamine (DFO)-induced *Vegfa* mRNA expression. Cells were treated with the indicated concentration of GSK5182 for 1 h and then with 100 μM DFO for 12 h mRNA levels were measured relative to those in non-treated cells, with actin as the internal control. Data represent the means ± SEM of five independent measurements. $^{*}P < 0.05$; $^{\#\#}P < 0.001$ vs. cells only treated with DFO. (D) The effect of GSK5182 treatment on ERRγ-induced *Vegfa* promoter activation. Cells were transfected with a *Vegfa* promoter reporter construct and an ERRγ expression construct. After 24 h, cells were treated with the indicated concentration of GSK5182 for a further 24 h. Data represent the means ± SEM of independent measurements (n = 5). $^{\#\#}P < 0.001$ vs. cells only treated with the ERRγ expression construct.

However, the specific retinal layer in which ERRγ is expressed and the role of ERRγ in the ischemic retina have not been investigated. Thus, the IHC of the retina was performed to compare the expression of ERRγ between control and OIR mice. To determine whether retinal ischemia was adequately induced in the OIR model, we examined the retinal vasculature of a control mouse in normoxia and an OIR mouse at P17. A central ischemic retina and neovascular tuft were observed in the flat-mounted retina of an OIR mouse at P17, but not in that of a control mouse (Fig. 1A and B).

In the retina of the control mouse, ERRγ expression was detected in the ganglion cell layer (GCL) and inner nuclear layer (Fig. 1C). ERRγ expression in the GCL was 2-fold higher in P17 OIR mice than in control mice. Analysis of *Errγ* mRNA was performed in whole retinas of control and OIR mice. *Errγ* mRNA expression was higher in OIR mice than in control mice (Fig. 1D). These results raise the possibility that ERRγ contributes to pathophysiology of the ischemic retinopathy.

3.2. ERRγ expression is upregulated by hypoxic stimuli

We next evaluated the role of ERRγ in the compensatory response of the retina, specifically the GCL, to hypoxia. To evaluate whether hypoxic conditions affect the expression of ERRγ, RGC-5 cells were treated with DFO, an iron chelator that stabilizes HIF-1α. Hypoxia was confirmed by increased HIF-1α protein levels. The mRNA and protein levels of ERRγ were increased by DFO treatment (Fig. 2A). Likewise, ERRγ expression was increased 2-fold in RGC-5 cells incubated in a hypoxic chamber (1% O_2) (Fig. 2B). These results show that hypoxic conditions upregulated ERRγ expression in RGC-5 cells. Liver cell lines also exhibit increased mRNA and protein levels of ERRγ upon exposure to hypoxia or DFO treatment [14], whereas skeletal muscle cells exhibit hypoxia-independent ERRγ induction [9]. These results indicate that the dependency of ERRγ induction on hypoxia differs among tissues.

Although questions have been raised regarding the true nature of RGC-5 cells, it is clear that these cells are derived from mouse, not rat [19]. Furthermore, RGC-5 cells express neuronal marker mRNAs such as microtubule-associated protein-2, microtubule-associated protein-1b, tau, and βIII-tubulin [20], and are a valuable tool for studying the responses of retinal ganglion cells to pathologic and protective stimuli.

3.3. ERRγ regulates Vegfa expression and this is inhibited by an ERRγ inverse agonist

A conserved binding site for ERRγ has been identified in the promoter of the *Vegfa* gene [7,8]. In the retina, VEGF is mainly expressed in Müller cells [21] and also in ganglion cells [22]. Our aforementioned data suggest that ERRγ regulates VEGF in hypoxic conditions in the retina, particularly in the GCL. To test this, RGC-5 cells were infected with an adenovirus expressing ERRγ or a mock control virus, and then the *Vegfa* mRNA level was measured. Overexpression of ERRγ in RGC-5 cells increased expression of *Vegfa* (Fig. 3A). To evaluate whether ERRγ directly regulates the expression of *Vegfa*, we demonstrated the activation of the *Vegfa* promoter by ERRγ using a luciferase assay system. *Vegfa* promoter activity increased in the presence of ERRγ (Fig. 3B). A previous study of ischemic skeletal muscle showed that the VEGFA protein level is higher in ERRγ transgenic plantaris than in wild-type muscle [9]. These results demonstrate that ERRγ can induce VEGFA in tissues which can be damaged by compromised blood supply as a complication of cardiovascular and metabolic diseases including heart failure, atherosclerosis, and diabetes.

To evaluate the direct effects of an ERRγ inverse agonist (GSK5182) on *Vegfa* overexpression, RGC-5 cells were treated with GSK5182 and DFO. DFO-induced *Vegfa* mRNA expression was reduced by GSK5182 (Fig. 3C). To test whether ERRγ-mediated activation of the *Vegfa* promoter is regulated by GSK5182, we measured luciferase activity. Consistent with the aforementioned data, GSK5182 treatment reduced ERRγ-induced activation of the *Vegfa* promoter (Fig. 3D). These results suggest that GSK5182 directly suppresses hypoxia-mediated VEGFA expression.

3.4. VEGFA inhibition by intraocular injection of an ERRγ inverse agonist into the OIR mouse model

To evaluate the effect of the ERRγ inverse agonist on VEGFA expression, GSK5182 was injected into the vitreous of the right eye of OIR mice at P14 and vehicle was injected into the vitreous of the contralateral eye. *Vegfa* mRNA levels were measured at P17. The *Vegfa* mRNA level was lower in retinas of eyes intravitreally injected with GSK5182 than in retinas of contralateral eyes intravitreally injected with vehicle (Fig. 4). These results indicate that intraocular administration of GSK5182 attenuates ischemia-induced VEGFA expression in the retina.

A variety of pro-angiogenic factors, such as VEGF, are implicated in the progression of ischemic retinopathies and retinal neovascularization. It was recently shown that intravitreal injection of anti-VEGF agents often improves the visual acuity of patients with ischemic retinopathies. However, some patients only show a partial response to this therapy [23], and anti-VEGF agents only offer temporary respite from vascular leakage at an extremely high cost. This emphasizes the need to identify alternative therapeutic targets.

The present study showed that ERRγ expression was increased in the RGC layer at P17 in OIR mice, a representative model of ischemic retinopathy. The mRNA and protein levels of ERRγ in RGC-5 cells were increased by hypoxic stimuli. Transient transfection

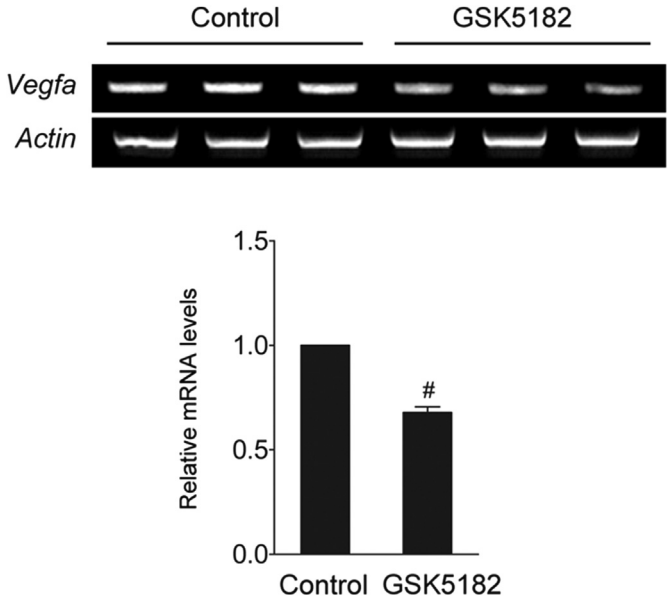

Fig. 4. Intraocular injection of the estrogen-related receptor γ (ERRγ) inverse agonist GSK5182 inhibits *vascular endothelial growth factor a* (*Vegfa*) expression in the oxygen-induced retinopathy (OIR) mouse model. OIR mice were injected with 1 μl of GSK5182 (10 mM) into the right vitreous cavity at postnatal day (P) 14. The same volume of vehicle was injected into the left vitreous cavity as a control. Reverse transcription-PCR analysis of *Vegfa* was performed with whole retinas of OIR mice at P17. The *Vegfa* mRNA level in GSK5182-injected retinas was measured relative to that in vehicle-injected retinas, with actin as the internal control. The graph shows the means + standard error of the mean of three experiments (n = 5). $^{\#}P < 0.005$ vs. control.

showed that ERRγ regulated *Vegfa* expression and this was inhibited by GSK5182, a selective inverse agonist of ERRγ. Furthermore, intravitreal injection of GSK5182 at P14 inhibited *Vegfa* mRNA expression in the OIR mouse model at P17. GSK5182 suppresses hypoxia-induced VEGFA expression via ERRγ; therefore, ERRγ could be a treatment target for ischemic retinopathies. Future studies should incorporate systemic administration perhaps using GSK5182 derivatives with increased biological stability, as intravitreal injection carries the associated risks of endophthalmitis, cataractogenesis, and glaucoma.

Conflict of interest

None.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A1A2055007) and by the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111345).

We appreciate Professor Akiyoshi Uemura at the Department of Retinal Vascular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan and Dr. Junyeop Lee at the Department of Ophthalmology, Asan medical center, University of Ulsan, College of Medicine, Seoul, South Korea for their generous advice on the OIR model and discussions about this article.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.055.

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