Application of recombinant peroxisome proliferator-activated receptor-γ coactivator-1α mediates neovascularization in the retina

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Abstract. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is able to induce the expression of vascular endothelial growth factor (VEGF), promoting the formation of new blood vessels in skeletal muscle. The aim of the current study was to determine whether PGC-1α is able to regulate angiogenesis in human retinal vascular endothelial cells (hRVECs) *in vitro* and in retinas *in vivo*. hRVECs treated with recombinant PGC-1α were incubated for 24 h and then placed into a normoxic (20% O_2) or hypoxic (1% O_2) environment for a further 16 h. Following this, VEGF mRNA and protein levels were significantly increased. Cellular proliferation was enhanced by treatment with recombinant PGC-1α in normoxic and hypoxic conditions. At 24 h following recombinant PGC-1α treatment, hRVECs were plated into Matrigel-coated plates and cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions for a further 24 h. Recombinant PGC-1α-treated cells were observed to form significantly greater numbers of tubes. In a C57BL/6J mouse model of ischemic retinopathy, mice received an intravitreal injection of recombinant PGC-1α, resulting in a significant increase in VEGF mRNA and protein levels in the retina. Retinal neovascular tufts and neovascular nuclei were investigated by angiographic and cross-sectional analysis and were observed to be significantly increased in the PGC-1α group compared with the control group. These results indicate that PGC-1α is able to induce angiogenesis in hRVECs and retinas, and suggests that PGC-1α is a potential anti-angiogenic target in retinal neovascularization.

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Introduction

The peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1 (PGC-1) family of coactivators is an extensively regulated group of proteins that are highly responsive to a variety of environmental cues, including temperature, nutritional status and physical activity. This family of coactivators serves a crucial role in integrating signaling pathways and adapts them to best suit the varying cellular and systemic environment. The first identified member of the PGC-1 family was PGC-1α, which was initially identified as a PPARγ-interacting protein from brown fat (1). PGC-1α is a transcription cofactor that contains binding sites for a number of nuclear hormone receptors. It regulates oxidative reactions and mitochondrial energy metabolism in various cells and tissues (1). PGC-1α is expressed at high levels in numerous human and rodent tissues, including brown fat, skeletal muscle, heart, kidney, liver and brain, and additionally in vascular endothelial cells (1-4).

Under ischemic and hypoxic conditions, the expression and transcriptional regulation activity of PGC-1α is enhanced in rat cardiac myocytes (5,6) and brain cells (7,8), rabbit renal tubular cells (9), and human skeletal muscle cells (10,11). A previous study demonstrated that PGC-1α is able to regulate an angiogenic program including vascular endothelial growth factor (VEGF) and additional angiogenic factors, in cultured muscle cells and skeletal muscle *in vivo* (12). Transgenic expression of PGC-1α in skeletal muscle markedly increased microvascular density and was protective in a model of skeletal muscle ischemia (12). PGC-1α may represent a novel therapeutic target to aid in improving the treatment of ischemic and hypoxic diseases (13).

Neoangiogenesis is a common pathophysiological feature of numerous diseases. Ischemia and hypoxia induce the formation of new blood vessels by altering the balance between angiogenesis-promoting factors and angiogenesis-inhibiting factors. Nascent blood vessels grow from existing ones by sprouting. In certain diseases, new blood vessels frequently have abnormal vessel walls, which may lead to complications. Retinal neovascularization (NV) occurs in various ocular disorders including proliferative diabetic retinopathy, retinopathy of prematurity and secondary neovascular glaucoma, which are a major cause of blindness worldwide (14).

Considering that PGC-1α is able to mediate angiogenesis in skeletal muscle, it has been suggested that it may do so in

additional tissues, such as the retina. The retina is a highly metabolic neural tissue with the highest oxygen consumption per unit weight of any human tissue (15). PGC-1α may serve a role in retinal neovascularization as a regulator of angiogenesis. In order to investigate this hypothesis, the current study used human retinal vascular endothelial cells (hRVECs) and a mouse model of oxygen-induced ischemic retinopathy (OIR) to explore the potential role of PGC-1α in mediating retinal neovascularization *in vitro* and *in vivo*. In the current study, hRVECs and OIR mice were treated with recombinant PGC-1α and the effect on VEGF expression, cell proliferation, endothelial cell tube formation and retinal neovascularization was investigated.

Materials and methods

Cell culture and treatment. Human retinal vascular endothelial cells (HUM-CELL-0112; Wuhan PriCells Biomedical Technology Co., Ltd., Wuhan, China) were cultured in 6-well plates with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a 5% CO_2, 20% O_2 environment. Subsequently, 24 h following plating and once cells had reached the logarithmic growth phase, media was replaced and the cells were divided into the following groups: Normoxia PGC-1α, normoxia control, hypoxia PGC-1α and hypoxia control. A total of 5 μl (0.25 μg/μl) recombinant PGC-1α (Abnova Corporation, Taipei, Taiwan, R.O.C.) was added to each well of cells in the normoxia PGC-1α and hypoxia PGC-1α groups. A total of 5 μl phosphate-buffered saline (PBS) was added to the cells in the normoxia control and hypoxia control groups. At 24 h following treatment, the cells were left in the normoxic conditions or placed into a hypoxic environment (1% O_2, 5% CO_2 and 94% N_2) and cultured for a further 16 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to measure VEGF mRNA expression in hRVECs. RNA was extracted from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 1 μg template was reverse-transcribed using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). The primers used in the qPCR were as follows: Human VEGF (124 bp), forward 5'-CTGTCT AATGCCCTGGAGCC-3' and reverse 5'-ACGCGAGTCTGT GTTTTTGC-3'; and human β-actin (169 bp), forward 5'-TCT GGCACCACACCTTCTAC-3' and reverse 5'-GATAGCACA GCCTGGATAGC-3'. The PCR reaction conditions were as follows: Denaturation at 94˚C for 3 min followed by 40 cycles of 94˚C for 30 sec, 59˚C for 30 sec, and 72˚C for 45 sec. The $2^{-\Delta\Delta Cq}$ method was used to analyze the relative changes in gene expression from the RT-PCR data.

Measurement of VEGF protein expression in hRVECs using ELISA. The amount of VEGF secreted into the cell culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (USCN, Houston, TX, USA). The standard wells were filled with 100 μl VEGF standard at various concentrations, blank wells were filled with 100 μl buffer and sample wells were filled with 100 μl of sample. The microplates were then covered and incubated at 37˚C for 2 h. Each well was filled with 100 μl working solution A and the microplates were covered again and incubated at 37˚C for 1 h. Subsequently, 100 μl working solution B was added to each well and the microplates were covered and incubated at 37˚C for 30 min. A total of 90 μl substrate solution was then added to each well and the microplates were covered and incubated at 37˚C for 20 min in the dark. Following this, each well was filled with 50 μl stop solution and the optical density at 450 nm was measured using a plate reader (Infinite 200 Pro; Tecan, Männedorf, Switzerland).

Cell proliferation assay. hRVECs were seeded in 96-well tissue culture plates and incubated for 24 h. Following this, cells were starved in M199 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 2% FBS in the absence of endothelial cell growth supplements for a further 16 h. Following starvation, cells were treated with recombinant PGC-1α or PBS for 24 h, followed by culturing under normoxic or hypoxic conditions for a further 16 h. Cell proliferation was determined using a Cell Proliferation ELISA, bromodeoxyuridine (BrdU) kit (Roche Diagnostics, Indianapolis, IN, USA.) based on the colorimetric detection of the incorporation of BrdU, following the manufacturer's instructions.

Tube formation assay. At 24 h following treatment with recombinant PGC-1α or PBS, the media was replaced with endothelial cell basal medium and the cells were added to plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). At 30-60 min prior to plating the cells, 100 μl Matrigel was placed at the bottom of each well in a 96-well chamber, avoiding bubbles. The plate was incubated at 37˚C for 30-60 min to allow the Matrigel to solidify. Subsequently, cells were dissociated and counted, and 100-200 μl cells/well were added on top of the Matrigel. Cells were placed under normoxic (20% O_2) or hypoxic (1% O_2) conditions and cultured for a further 24 h. The tubes formed were observed and images were captured using an optical microscope (DM5000B; Leica Microsystems, Wetzlar, Germany). The number of tubes in each well were quantified by a blinded observer.

Animal model. C57BL/6J mice were used in the current study and were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. OIR was generated in C57BL/6J mice (16), as previously described by Smith *et al* (17). The present study was approved by the ethics committee of Xiangya Hospital, Central South University (Changsha, China). Mice, obtained from the Experimental Animal Center, Chinese Academy of Sciences, were selected at postnatal day 7 (P7) to optimize the balance in retinal development between hyaloid regression and incomplete retinal vascularization. P7 mice and their mothers were placed in an oxygen chamber and exposed to an oxygen concentration of 75±2% as monitored by an oxygen analyzer (CY-7B; Mei Cheng Electrical Analysis Instrument Factory, Jiande, China) for 5 days. The mice were exposed to 12 h cyclical broad spectrum light. The room temperature was maintained at 23±2˚C. On P12, the mice were removed to room air from P12 to P17 or P21, when the retinas were assessed for the maximum neovascular response. Age-matched C57BL/6J

mice maintained in room air were used as the controls. The mice were randomly divided into normal, OIR control and OIR PGC-1α groups. A total of 114 C57BL/6J mice were used for *in vivo* studies.

Intravitreal injection of recombinant PGC-1α. OIR mice were anesthetized intraperitoneally with 1% pentobarbital sodium (30 mg/kg body weight) and given an intravitreal injection of recombinant PGC-1α or PBS. The tip of a 10 mm 34-gauge steel needle, mounted on a 5 μl Hamilton syringe, was pushed through the sclera, 1 mm posterior to the corneoscleral limbus, into the vitreous body (18). OIR mice received an intravitreal injection of 1 μl (0.25 μg/μl) recombinant PGC-1α (OIR PGC-1α group) or 1 μl PBS (OIR control group) at P11, and were returned to room air at P12. Mice in the normal group were not subjected to an intravitreal injection.

Angiography using fluorescein-dextran. At P17, 10 mice from each group were sacrificed through overdose of 1% pentobarbital sodium at 30 mg/kg body weight and were then perfused through the left ventricle with 1 ml PBS, containing 50 mg of $2x10^6$ molecular weight fluorescein-dextran (Sigma-Aldrich, St. Louis, MO, USA). Eyes were enucleated and fixed in 4% formaldehyde for 10 min. The retina was dissected free of the lens and cornea, and placed in 4% formaldehyde for 5 min. The peripheral retina was then cut in four places and flat-mounted with glycerol/PBS (50/50).

Cross-sectional analysis of NV. At P17, 10 mice from each group were sacrificed and the eyes were enucleated and fixed in 4% formaldehyde for 24 h, prior to embedding in paraffin. Serial sections (5 μm) of whole eyes were cut sagittally through the cornea and parallel to the optic nerve, and stained with hematoxylin and eosin (Boster Systems, Inc., Wuhan, China). A total of 10 nonserial sections were analyzed per eye. Sections including the optic nerve were excluded, and the nuclei of new vessels extending from the retina into the vitreous were counted.

RT-qPCR analysis of the expression of VEGF in the retina. Total RNA was prepared from each group of mouse retinas at P17, to measure the expression of VEGF mRNA. Each RNA sample was obtained from two retinas. In brief, retinas were lysed in TRIzol reagent and RNA was extracted and purified, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1 μg template was reverse-transcribed using the RevertAid First Strand cDNA Synthesis kit. The primers used in qPCR were as follows: Mouse VEGF (240 bp), forward 5'-CATCTTCAAGCCGTC CTGT-3' and reverse 5'-GAGGAAAGGGAAAGGGTCA-3'; and mouse β-actin (203 bp), forward 5'-TTCCTTCTTGGG TATGGAAT-3' and reverse 5'-GAGCAATGATCTTGA TCTTC-3'. The PCR reaction conditions were as follows: Denaturation at 95˚C for 5 min followed by 40 cycles of 94˚C for 20 sec, 60˚C for 20 sec and 72˚C for 20 sec. The $2^{-\Delta\Delta Cq}$ method was used to analyze the relative changes in gene expression from the RT-PCR data.

Western blot analysis of VEGF protein expression in the retina. Proteins were prepared from each group of mouse retinas at P17 to measure the expression of VEGF. Each protein sample was obtained from four retinas from the same group. The retinas were homogenized in ice-cold lysis buffer, containing 20 mM HEPES (pH 7.5), 1% Triton X-100, 1 mM EDTA and 0.1 mol/L NaCl), and the lysates were centrifuged at 15,000 x g for 15 min at 4˚C. The supernatant was collected and the protein concentration was determined with Bradford assay. For each sample, 100 μg protein was fractionated using 10% SDS-PAGE (Pierce Biotechnology, Inc., Rockford, IL, USA), and transferred onto polyvinylidene membranes (Pierce Biotechnology, Inc). The membranes were gently agitated in the blocking solution (5% skim milk) for 1 h at room temperature and then incubated overnight with primary antibodies at 4˚C with agitation. The primary antibodies used were as follows: 1:200 polyclonal goat anti-mouse VEGF164 (cat. no. AF-493-NA; Novus Biologicals LLC, Littleton, CO, USA) and 1:1,000 polyclonal rabbit anti-mouse β-actin (cat. no. sc-130656; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were washed with PBS, incubated with the secondary antibodies for 1 h at room temperature and washed again. Antibodies bound to the membranes were detected using enhanced chemiluminescence (Pierce Biotechnology, Inc.) and exposed to X-ray films (Kodak, Rochester, NY, USA) in the dark. The intensity of the protein bands was analyzed using BandScan software, version 5.0 (Glyko, Inc., Novato, CA, USA). The relative levels of VEGF were calculated as VEGF band intensity/β-actin band intensity.

Statistical analysis. One-way analysis of variance was used for comparisons across all groups, and pair-wise comparisons between groups were conducted using the Fisher's least significant difference test. SPSS software version 19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Data from two groups were compared by paired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Alterations in VEGF mRNA expression in hRVECs. RT-qPCR was used to measure the levels of VEGF mRNA (Fig. 1). The expression of VEGF mRNA was significantly upregulated in the cells in the normoxia PGC-1α group compared with the cells in the normoxia control group ($P<0.01$). Cells in the hypoxia control group expressed significantly greater levels of VEGF mRNA compared with the cells in the normoxia control group. The cells in the hypoxia PGC-1α group expressed significantly greater levels of VEGF mRNA compared with the cells in the hypoxia control group ($P<0.01$).

Alterations in VEGF protein levels in hRVECs. ELISA was used to measure the VEGF protein levels (Fig. 2). The levels of VEGF protein were significantly greater in the cells in the normoxia PGC-1α group compared with the cells in the normoxia control group ($P<0.01$). Cells in the hypoxia control group expressed significantly greater levels of VEGF protein compared with cells in the normoxia control group. Cells in the hypoxia PGC-1α group expressed significantly greater levels of VEGF protein compared with the cells in the hypoxia control group ($P<0.01$).

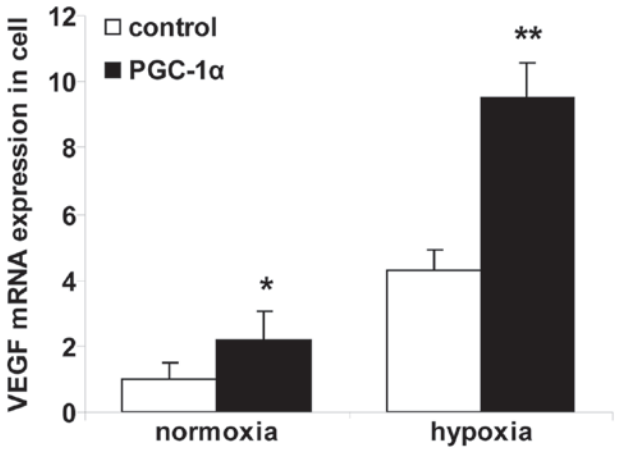

Figure 1. Alterations in VEGF mRNA expression in hRVECs. The effect of recombinant PGC-1α on VEGF mRNA expression levels in hRVECs at 16 h of normoxia and hypoxia was measured by reverse transcription-polymerase chain reaction. The VEGF mRNA expression levels in the PGC-1α-treated groups were significantly upregulated compared with the control groups during normoxia and hypoxia. *P<0.01 vs. the normoxia control group. **P<0.01 vs. the hypoxia control group. VEGF, vascular endothelial growth factor; hRVECs, human retinal vascular endothelial cells; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.

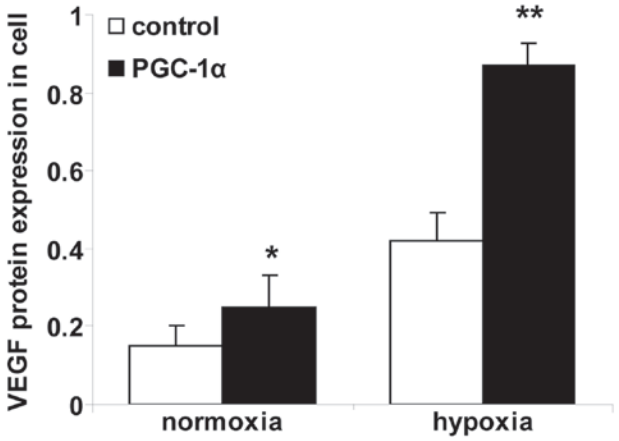

Figure 2. Alterations in the protein levels of VEGF in hRVECs. Recombinant PGC-1α-mediated upregulation of VEGF protein expression in hRVECs at 16 h of normoxia and hypoxia was measured by an enzyme-linked immunosorbent assay. The VEGF expression levels in the PGC-1α groups were significantly upregulated compared with the control groups under normoxia and hypoxia. *P<0.01 vs. the normoxia control group. **P<0.01 vs. the hypoxia control group. VEGF, vascular endothelial growth factor; hRVECs, human retinal vascular endothelial cells; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α.

Recombinant PGC-1α promotes cell proliferation in hRVECs. Endothelial cell proliferation is an essential step in angiogenesis. To investigate the angiogenic activity of PGC-1α, the effect of PCG-1α on cell proliferation in hRVECs was analyzed (Fig. 3). The percentage of BrdU-labeled cells was significantly increased in the normoxia PGC-1α group compared with the normoxia control group (P<0.01). Cell proliferation in the hypoxia control group was enhanced significantly compared with the cells in the normoxia control group. The percentage of BrdU-labeled cells was significantly increased in the hypoxia PGC-1α group compared with the hypoxia control group (P<0.01). Recombinant PGC-1α significantly promoted cell proliferation in hRVECs under normoxic and hypoxic conditions.

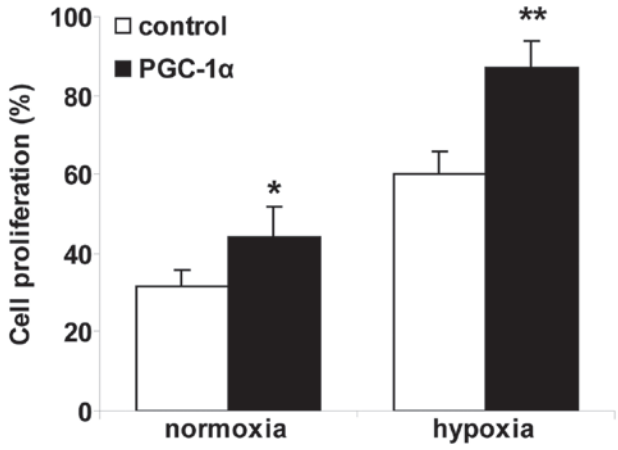

Figure 3. Recombinant PGC-1α promotes cellular proliferation in hRVECs. Effect of recombinant PGC-1α on the proliferation of hRVECs at 16 h of normoxia or hypoxia. The percentage of bromodeoxyuridine-labeled cells in the PGC-1α groups was significantly increased compared with the control groups under normoxia and hypoxia. *P<0.01 vs. the normoxia control group. **P<0.01 vs. the hypoxia control group. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; hRVECs, human retinal vascular endothelial cells.

Recombinant PGC-1α promotes cell tube formation in hRVECs. Following the culture of hRVECs in Matrigel-coated plates, cells began forming tubes at 6 h, with the tubes appearing to be stabilized by 24 h. Recombinant PGC-1α induced significantly greater tube formation compared with PBS (18.2±2.8 and 13.5±1.2 tubes/well, respectively; P<0.01) under normoxic condition in hRVECs. Similar results were obtained from the hypoxia PGC-1α group and the hypoxia control group (43.6±2.5 and 27.1±3.7 tubes/well, respectively; P<0.01). Cells in the hypoxia control group exhibited markedly enhanced tube formation compared with the cells in the normoxia control group. Taken together, these results suggest that PGC-1α is able to directly upregulate angiogenic activity in hRVECs (Fig. 4).

Effects of recombinant PGC-1α on retinal NV. Following the exposure of P7 mice to hyperoxia, the initial response of the retinal vasculature was reversible central vasoconstriction followed by nonperfusion. Exposure of P7 mice to 5 days of 75% oxygen followed by a return to room air lead to relative ischemia in the central retina. As a result of hypoxia, the larger central radial vessels became tortuous and engorged. NV at the junction between the vascularized and nonvascularized retina then occurred., determined using retinal flat-mount angiography using fluorescein dextran.

Following the observation that recombinant PGC-1α induced VEGF expression and promoted cell proliferation and tube formation *in vitro*, it was investigated whether recombinant PGC-1α is able to induce NV *in vivo*. No mice used in the current study developed signs of infection or retinal detachment. The patterns of vascular development and NV were observed in retinal flat-mounts following fluorescein-dextran perfusion (Fig. 5). The retinas of P17 mice from the normal

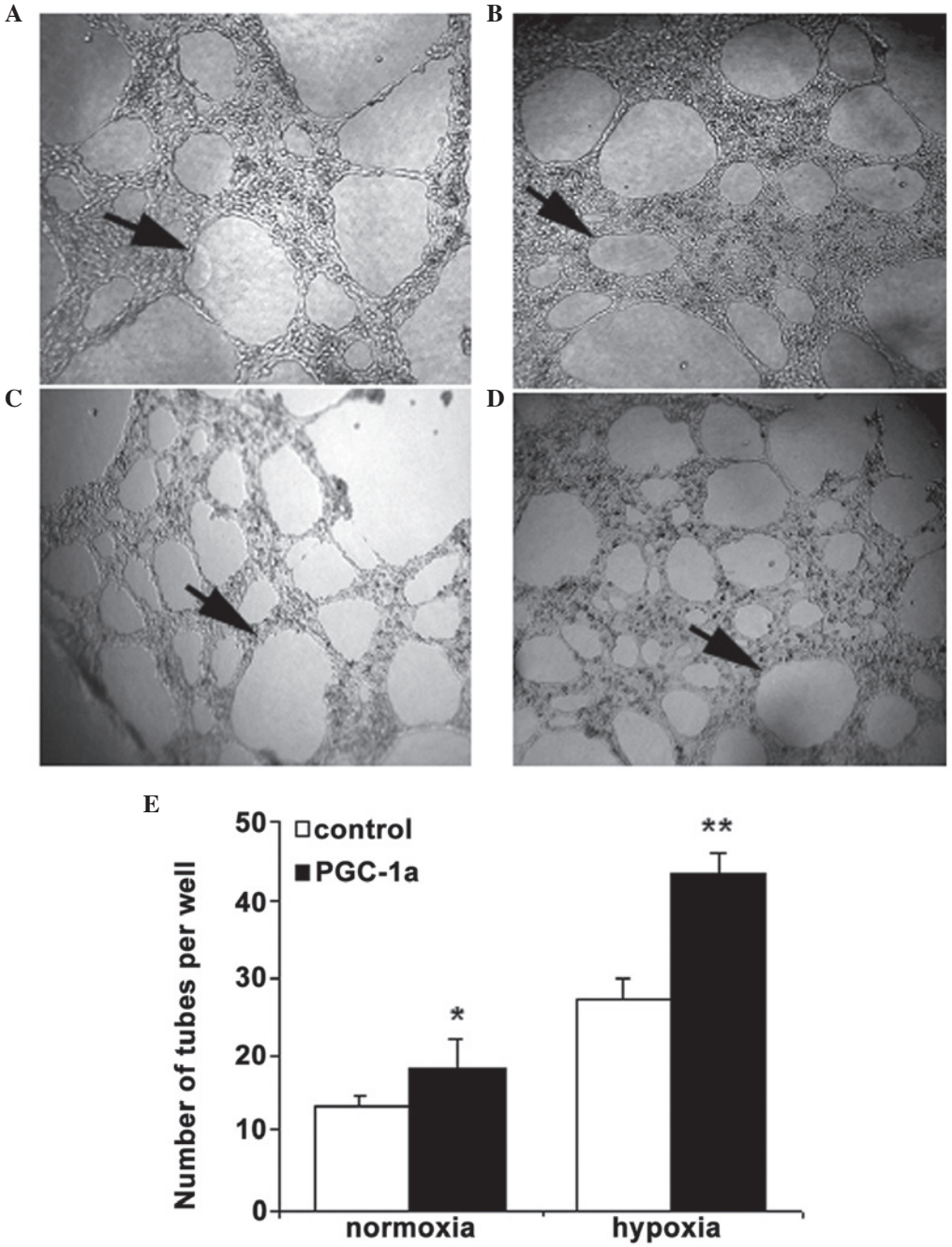

Figure 4. Recombinant PGC-1α promotes cell tube formation in hRVECs. Images of tubular structures were quantified by counting the number of tubes (indicated by arrows) per well in cultured hRVECs. (A) Normoxia control group. (B) Normoxia PGC-1α group. (C) Hypoxia control group. (D) Hypoxia PGC-1α group. (E) Quantification of the number of tubes. Recombinant PGC-1α significantly induced tube formation in cells compared with the control groups under normoxia and hypoxia. Cells in the hypoxia control group formed significantly greater numbers of tubes compared with the cells in the normoxia control group. *P<0.01 vs. the normoxia control group. **P<0.01 vs. the hypoxia control group. Magnification, x20. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; hRVECs, human retinal vascular endothelial cells.

group exhibited superficial and deep vascular layers that extended from the optic nerve to the periphery. The vessels formed a fine radial branching pattern in the superficial retinal layer and a polygonal reticular pattern in the deep retinal layer (Fig. 5A). The retinal vascular pattern in mice exposed to hyperoxia was characterized by a central nonperfused region and neovascular tufts (Fig. 5B). At P17, the results indicated that in the OIR PGC-1α group, the neovascular tufts were increased compared with the OIR control group, and fluorescein leakage was additionally aggravated (Fig. 5C).

To further investigate the effect of recombinant PGC-1α, examination of 5 μm paraffin-processed cross-sections of mouse eyes was conducted. Neovascular tufts are defined as vascular cells extending beyond the internal limiting membrane into the vitreous. The degree of NV was quantified in cross-sections by counting the number of vascular cell nuclei on the vitreal side of the internal limiting membrane. There were no neovascular nuclei in the normal group (Fig. 6A). The mean number of nuclei per cross-section in OIR PGC-1α group (28.2±2.9) was significantly increased

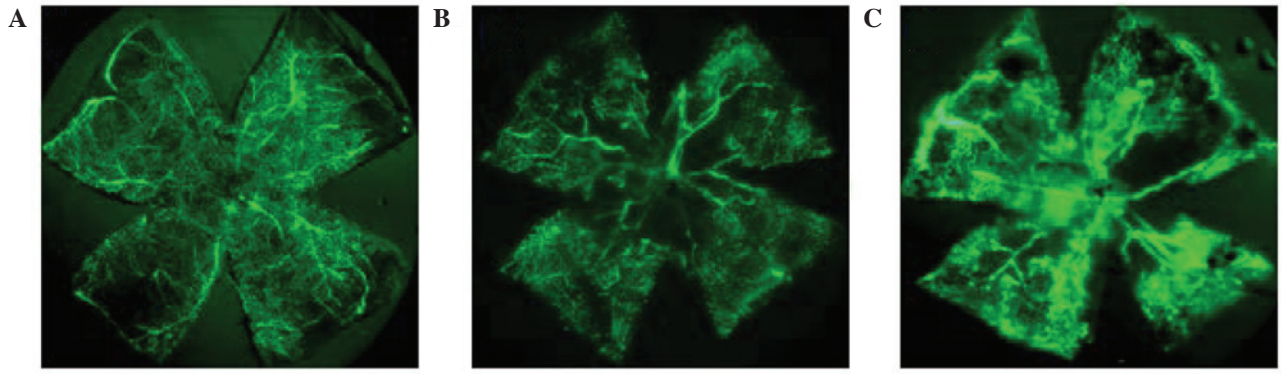

Figure 5. Effect of recombinant PGC-1α on retinal neovascularization in angiographic analysis. Fluorescein-dextran perfused flat-mounts of P17 C57BL/6J mice. (A) Normal group, retinal vessels formed a fine radial branching pattern that extended from the optic nerve to the periphery. (B) OIR control group, a central nonperfused region and neovascular tufts were observed. (C) OIR PGC-1α group, neovascular tufts were increased compared with the OIR control group, and fluorescein leakage was additionally aggravated. Magnification, x40. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; P, postnatal day; OIR, oxygen-induced ischemic retinopathy.

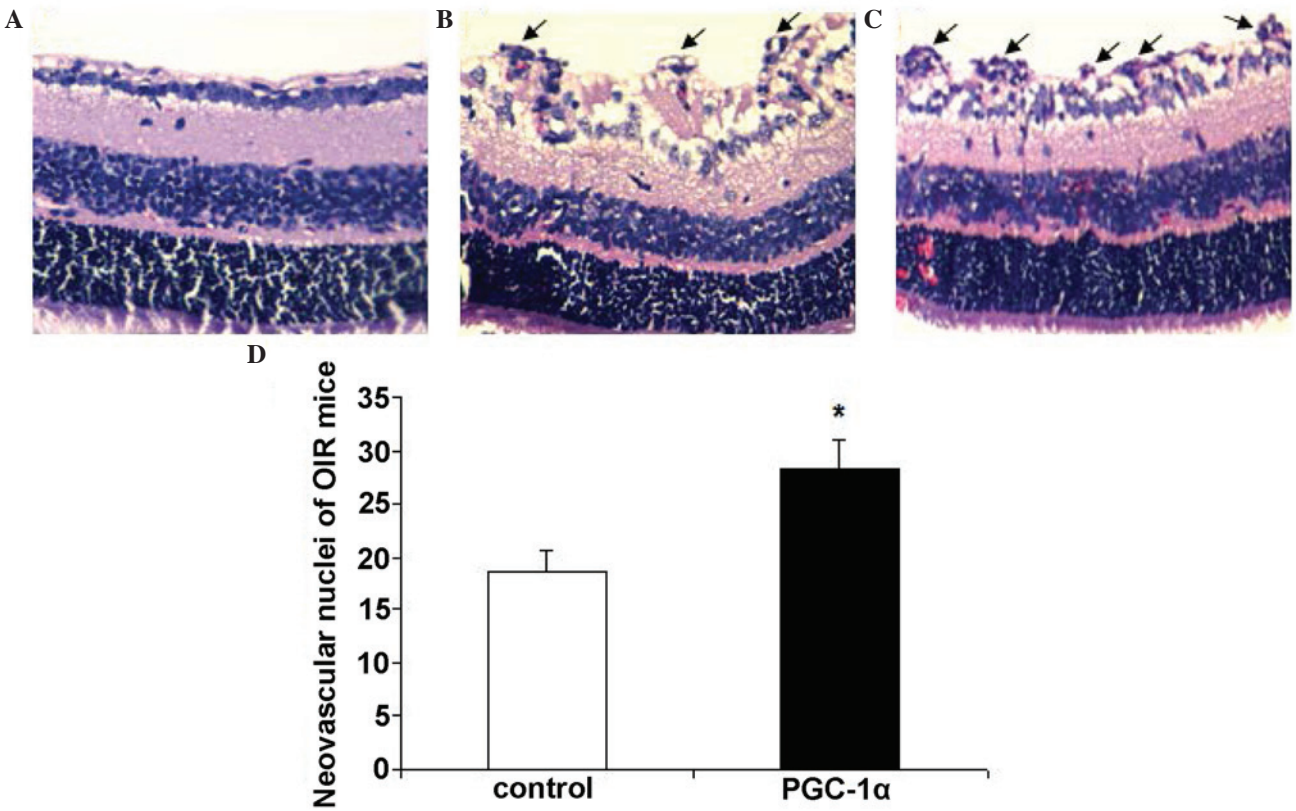

Figure 6. Effect of recombinant PGC-1α on retinal neovascularization in cross-sectional analysis. Effect of recombinant PGC-1α on the induction of retinal neovessels (arrows) in cross-sections from C57BL/6J mice (P17). Images of sections from (A) normal group, (B) OIR control group and (C) OIR PGC-1α group. Magnification, x400. (D) Histogram presenting the number of vascular cell nuclei on the vitreal side of the internal limiting membrane from each group. Neovessels were increased significantly in the OIR PGC-1α group compared with the OIR control group. *P<0.01 vs. the OIR control group. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; OIR, oxygen-induced ischemic retinopathy.

compared with the OIR control group (18.6±2.1); (P<0.01; Fig. 6).

Regulation of the mRNA expression of VEGF in the retina. The results of the RT-qPCR analysis demonstrated that the level of VEGF mRNA in the OIR control group retinas was significantly upregulated, compared with that in the normal group (P<0.01). Following the treatment with recombinant PGC-1α, the retinas in the OIR PGC-1α group were observed to have significantly higher mRNA expression levels of VEGF, compared with the levels in the retinas in the OIR control group (P<0.01, Fig. 7).

Western blot analysis in the retina. Retinal VEGF levels were measured by western blot analysis. The VEGF protein levels in the OIR control group retinas was significantly upregulated compared with the normal group (P<0.01; Fig. 8). The protein expression levels of VEGF were significantly increased in the

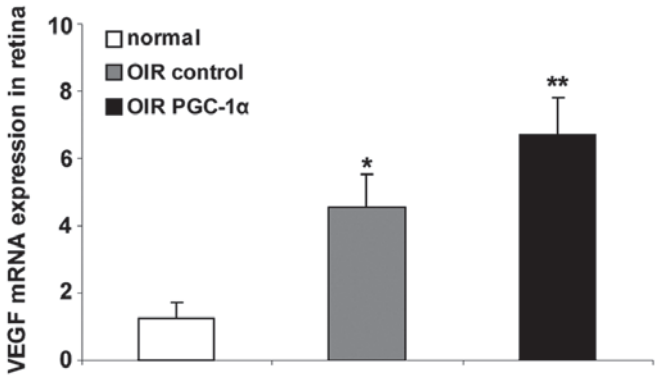

Figure 7. Regulation of VEGF mRNA expression in the retina. Effect of recombinant PGC-1α on the expression levels of VEGF mRNA in C57BL/6J mice (P17) was measured by reverse transcription-quantitative polymerase chain reaction. The VEGF mRNA levels of the OIR control group retinas were significantly upregulated compared with the normal group. Following treatment with recombinant PGC-1α, retinas in the OIR PGC-1α group expressed significantly greater levels of VEGF mRNA compared with the retinas in the OIR control group. *P<0.01 vs. the normal group. **P<0.01 vs. the OIR control group. VEGF, vascular endothelial growth factor; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; OIR, oxygen-induced ischemic retinopathy.

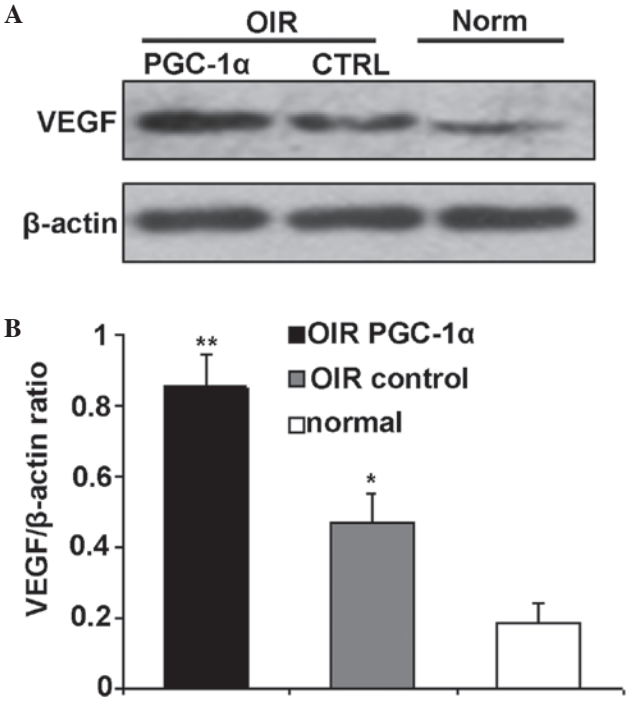

Figure 8. Regulation of VEGF protein expression in the retina. (A) Recombinant PGC-1α-mediated upregulation of VEGF protein expression in C57BL/6J mice (P17) was measured by western blotting. (B) The VEGF protein levels in the OIR control group retinas were significantly upregulated compared with the normal group. Following treatment with recombinant PGC-1α, the retinas in the OIR PGC-1α group expressed significantly greater levels of VEGF compared with the retinas in the OIR control group. *P<0.01 vs. the normal group. **P<0.01 vs. the OIR control group. VEGF, vascular endothelial growth factor; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; OIR, oxygen-induced ischemic retinopathy; norm, control group.

OIR PGC-1α group compared with the OIR control group (P<0.01; Fig. 8).

Discussion

Retinal NV is the abnormal proliferation and migration of new blood vessels from pre-existing vessels in the retina. It occurs in several disease processes including proliferative diabetic retinopathy, retinopathy of prematurity and secondary neovascular glaucoma, and is a major cause of blindness. Neoangiogenesis is the formation of new blood vessels from pre-existing vessels, usually small veins, by sprouting, and occurs in wound healing and additional pathological conditions. In the current study, a mouse model of OIR was used to recapitulate certain aspects of neovascularization in ocular diseases in humans. OIR is characterized by vessel loss followed by vascular regrowth and hypoxia-induced neovascularization (17).

During angiogenesis, vascular endothelial cells secrete platelet-derived growth factor to stimulate the proliferation of mesenchymal cells, promoting the maturation of the vascular wall (19). Vascular endothelial cells form a barrier between the vascular wall and the blood, and injuries to endothelial cells lead to numerous vascular diseases (19). In wound healing, inflammation and cancer, endothelial cells proliferate and form new blood vessels. The abnormal growth of new vessels results in vessels which are very fragile, that leak and are susceptible to hemorrhage. The resultant hemorrhage or accumulation of blood in ocular cavities, such as in the vitreous, leads to the further blockade of light transportation and a reduction in visual acuity. For these reasons, retinal vascular endothelial cells represent an important research tool in the *in vitro* investigation of angiogenesis-associated ocular diseases.

PGC-1α is a transcriptional coactivator identified as an upstream regulator of lipid catabolism, mitochondrial number and function (20). Consistent with its emerging role as a central regulator of energy metabolism, PGC-1α is abundantly expressed in tissues with high metabolic rates. PGC-1α is a potent modulator of oxidative metabolism in numerous settings (21). In particular, PGC-1α regulates oxidative phosphorylation, mitochondrial biogenesis and respiration (22,23). Numerous previous studies have suggested that ischemia and hypoxia significantly induce the expression of PGC-1α (5-11,24,25). Arany *et al* (12) reported that PGC-1α was able to upregulate the expression of angiogenic factors such as VEGF, and promote the formation of new blood vessels in skeletal muscle. In the current study, hRVECs were cultured in a hypoxic environment to simulate hypoxia *in vivo*. hRVECs cultured in the hypoxic environment grew well and exhibited no overt cell death, suggesting that hypoxia did not affect the growth or survival of endothelial cells. The expression of VEGF was significantly upregulated at the mRNA and protein levels under hypoxic conditions in hRVECs and in OIR mice, confirming that hypoxia is able to upregulate the expression of VEGF *in vitro* and *in vivo*.

In order to investigate the regulatory effects of PGC-1α on VEGF *in vitro* and *in vivo*, hRVECs and OIR mice were treated with recombinant PGC-1α. Following treatment with recombinant PGC-1α, VEGF mRNA and protein expression levels were significantly increased, indicating that PGC-1α regulates VEGF expression in hRVECs and mice. VEGF is a specific mitogen for endothelial cells and is a key inducer of angiogenesis. It induces the migration and proliferation of endothelial cells

and increases the permeability of the endothelium (26). VEGF acts directly on vascular endothelial cells to increase vascular permeability, leading to the extravasation of fibrin, which forms a fibrin gel with fibronectin and serves as a temporary matrix for the migration and invasion of fibroblasts, endothelial cells and additional cells. These cells are subsequently incorporated into the vasculature (27). In the current study, the cell proliferation assay demonstrated that hypoxia increased the proliferation of hRVECs via the stimulation of VEGF expression. Following treatment with recombinant PGC-1α, VEGF expression in hRVECs was upregulated, followed by an enhancement of cell proliferation. Therefore, recombinant PGC-1α may promote cellular proliferation in hRVECs.

To further investigate the molecular mechanisms of recombinant PGC-1α responsible for the reinforcement of angiogenic activity in hRVECs, the potential effect of PGC-1α on angiogenic activity *in vitro* was investigated by tube formation assays. This assay mimics numerous key steps of the angiogenic process, including endothelial cell adhesion, migration, differentiation and growth (28). In the current study, Matrigel coating provided the necessary extracellular matrix for human endothelial cells to form a network of tubes. The endothelial cells began to form tubes at 6 h following addition to the Matrigel-coated plates, with the tubes becoming stable by 24 h. The endothelial cells formed tubes in normoxic and hypoxic environments, however greater numbers of tubes formed in the hypoxic environment, suggesting that hypoxia is able to promote tube formation. A previous study indicated that PGC-1α promotes blood vessel formation by increasing the expression of VEGF (12), therefore, the current study investigated whether PGC-1α is able to increase tube formation. Cells treated with recombinant PGC-1α under normoxic and hypoxic conditions formed greater numbers of tubes compared with their corresponding control PBS treated cells, suggesting that recombinant PGC-1α upregulates VEGF, thereby increasing tube formation. Therefore, the *in vitro* experiments suggest that recombinant PGC-1α is able to promote angiogenesis.

In a previous study, PGC-1α-knockout mice subjected to oxygen-induced retinopathy exhibited reduced expression of VEGFA and were protected against pathological revascularization (29). As demonstrated in the present study, recombinant PGC-1α was able to induce cell proliferation and tube formation in hRVECs, which indicates its potential for promoting retinal neovascularization. This was demonstrated by overexpression of PGC-1α *in vivo* in the mouse model of OIR with retinal neovascularization. From the *in vivo* experiments, the current study indicated that the intravitreal injection of recombinant PGC-1α is able to increase the neovascular tufts in the retina, and specific protein levels were consistently greater in PGC-1α-treated eyes. Furthermore, treatment with recombinant PGC-1α exacerbated retinopathy by increasing the invasion of new vessels beyond the inner-limiting membrane of the retina according to the analysis of eye sections, and aggravating fluorescein leakage according to the angiography of retinas. The data presented here demonstrates that recombinant PGC-1α is able to increase the expression of VEGF through the PGC-1α-VEGF pathway, thereby promoting retinal neovascularization.

In conclusion, recombinant PGC-1α is able to increase the expression of VEGF in hRVECs and retinas, thereby promoting cellular proliferation, tube formation and retinal neovascularization, suggesting an important role of PGC-1α in regulating vascular growth. This indicates that PGC-1α may be considered as a potential anti-angiogenic target in retinal neovascularization.

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