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Original Article

VEGF receptor-2 protects cone photoreceptors under hypoxic conditions

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Abstract: Purpose: Hypoxia is one of the important links in the pathological changes of diabetic retinopathy (DR). We attempt to investigate the protective effect of vascular endothelial growth factor (VEGFR2) on retinal cone photoreceptors under hypoxia condition. Methods: Eight-week-old cone-specific VEGFR2-knockout (KO) mice and wild-type (WT) C57BL/6 mice received intravenous injection of 8 mmol/L cobalt chloride in the right eyes for hypoxia condition and PBS in the left eyes for control, with 6 mice in each group. On the next day electroretinogram (ERG) was conducted to evaluate photoreceptor function, and immunofluorescence colocalization was used to determine the density and morphology of cone photoreceptors. Results: Hypoxia KO and WT group manifested noticeable drop of a-wave and b-wave amplitude in scotopic ERG and are significant different in contrast with PBS controls ($P < 0.01$). However, no significant difference is detected when we compared hypoxia KO with WT group ($P > 0.05$). Similarly, the difference is significant in photopic ERG when we compared a-wave and b-wave amplitude of hypoxia KO and WT group with PBS controls ($P < 0.01$). Nevertheless, KO group showed much more significant decrease compared with WT group ($P < 0.05$, $P < 0.01$) which means cone function is impaired in KO animal. Morphology examination showed that density of cones in KO group decreased significantly and have a shorten appearance and disordered arrangement. The difference was statistically significant. Conclusion: Overall, our data indicate that VEGFR2 has a protective effect on cone photoreceptors under hypoxia condition.

Keywords: Hypoxia, cone photoreceptors, vascular endothelial growth factor receptor-2

Introduction

Hypoxia is one of the important links in the pathological changes of diabetic retinopathy (DR) [1], which results in impairment or even death of neurons because of massive oxygen consumption required by normal metabolism of retinal nerve cells. As primary neurons of the visual pathway, cone photoreceptors are responsible for daylight vision and colour vision; thus, visual function is seriously affected after impairment of retinal neurons; investigation on 'protective umbrella' of cone photoreceptors is of great significance in the preservation of visual function of DR patients. The development and progression of DR is always associated with high expression of vascular endothelial growth factor (VEGF) [2]. VEGF not only stimulates neovascularisation in tumour metastasis but also protects motor neurons [3]. However, limited reports focused on the effect of VEGF on the type of sensory neuron as retinal photoreceptor. VEGF must combine with VEGF receptor (VEGFR) to play its role. VEGFR mainly includes VEGFR1 and VEGFR2; inhibition of VEGFR2 can result in blockage of signalling pathway of VEGF, whereas inhibition of VEGFR1 leads to very little effect [4, 5]. Therefore, VEGFR2 is selected as the research target in the current study to establish hypoxia model using classic intravitreal injection method with cobalt chloride, as well as to observe and compare the changes in structures and functions of cone photoreceptors in cone-specific VEGFR2 knockout (KO) and wild-type (WT) mice. The effects of VEGFR2 on cone photoreceptors under hypoxic conditions were also investigated.

Subjects and methods

Animals and grouping

All animal experiments adhered 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 Nanchang University. Six clean-class, eight-week-old, 20 grams to 30 grams cone-specific VEGFR2 KO mice were donated by Professor Yun Le from the University of Oklahoma, Health Sciences Centre, United States, and six WT C57BL/6 mice were provided by the Institute of Zoology, Nanchang University. For all WT mice, 8 mmol/L cobalt chloride ($CoCl_2$, Sigma, St. Louis, MO) was injected via right eye as hypoxia group of WT ($CoCl_2$-WT); phosphate buffer solution (PBS) was injected via left eye as self-control group of WT (PBS-WT). For KO mice, 8 mmol/L $CoCl_2$ was injected via right eye as hypoxia group of KO ($CoCl_2$-KO), and PBS was injected via left eye as self-control group of KO (PBS-KO).

Hypoxia animal model

Mice were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg; VEDCO, St. Joseph, MO) and ketamine (60 mg/kg; Bioniche Teoranta, Inverin Co., Galway, Ireland), iodophor disinfection was performed around the eye, and a needle was inserted using micro-injector (Hamilton, Switzerland) towards the direction of the optic nerve at 1 mm outside of the corneal limbus under microscope. The medicine was slowly injected forward after the needle tip is shown in the pupil area. Up to 1 μL of 8 mmol/L $CoCl_2$ was injected into the vitreous cavity of the right eye, whereas 1 μL of PBS was injected into the vitreous cavity of the left eye.

Electroretinography

The mice were kept overnight in a dark chamber after pupil dilation by using compound tropicamide eye drops (Santen Pharmaceutical Co., Japan). Intraperitoneal anaesthesia was induced the next day. The mice were then placed on a heating board. The reference and grounding electrodes were inserted into the palate and tail. Platinum corneal electrode was placed at the cornea of both eyes, and sodium hyaluronate gel (Bausch & Lomb Freda Pharmaceutical Co., China) was applied for lubrication. The above operation was completed under lighting of dim red light in a dark chamber. Set-up illumination intensities of 0.0004, 0.04, 4, 400 and 2000 $(cd \cdot s)/m^2$ were recorded by Espion ERG Diagnosys machine (Diagnosys, Littleton, MA) of dark adaptation to reflect the function of rod cells. Subsequently, the light was turned on for 10 min light adaptation. The ERG response of light adaptation was recorded under illumination intensity of 2000 $(cd \cdot s)/m^2$ to reflect the function of cone photoreceptors.

Immunofluorescence

Mice eyeballs were removed and fixed in 4% paraformaldehyde for 1 hour. The cornea and lens were then removed and fixed again in 4% paraformaldehyde for 15 minutes. Samples were washed in PBS and dehydrated with gradient ethanol. Xylene I and xylene II were added to perform clearing. Wax embedding was performed after wax impregnation. The samples were cut into slices (5 μm) and then dried. The paraffin sections were added with xylene I and xylene II for de-waxing. Gradient ethanol was added after rinsing in absolute alcohol. Heat-induced antigen retrieval was performed for 20 min at 98°C in 0.01 mol/L citrate buffer solution. The samples were rinsed thrice after natural cooling. Incubation was performed in wet box for 1 h after dropping goat serum. Then Peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA) and polyclonal antibodies against VEGFR2 (R&D Systems parent company, Minneapolis, MN) were used for primary antibody incubation. PBS was used to replace primary antibody in the negative control group. Alexa Fluor secondary antibody (Invitrogen, Carlsbad, CA) was used for secondary detection and incubated at room temperature for 1 hour after washing the samples thrice. On the next day, 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA)-containing anti-quenching agent was added to seal the slices. The samples were observed under a fluorescence microscope (Olympus, Central Valley, PA), and the amount of positive cells in the inner segments of cone photoreceptors was counted.

Statistical analysis

SPSS 17.0 for Windows 7 software was used for statistical analysis. The results were represented as mean ± SD or mean ± SEM. Dunnett's t-test was used for multiple comparisons between groups. P value < 0.05 was considered significant.

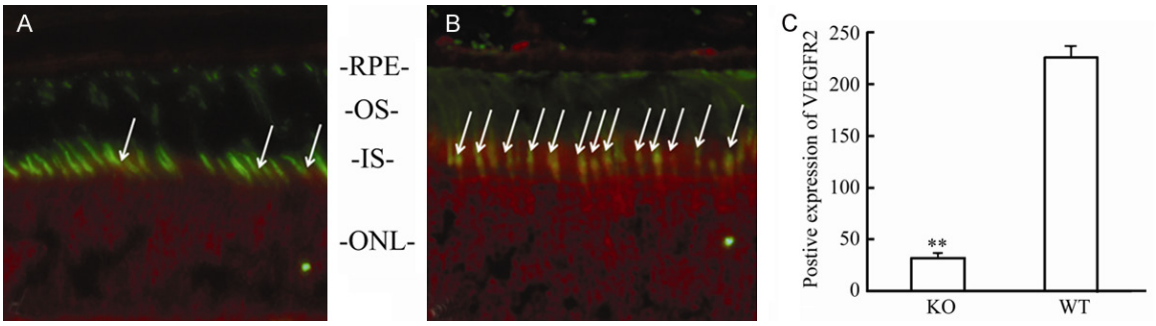

Figure 1. Retinal VEGFR2 expression in KO and WT mice under normal condition. A, B: Immmuno-colocalization image of cone-specific VEGFR2 knockout (KO) mice and wildtype (WT) mice. The white arrow signified the expression of VEGFR2 in cone inner segment. Green: Peanut agglutinin (PNA); Red: Vascular endothelial growth factor receptor 2 (VEGFR2). RPE: Retinal Pigmented Epithelium; IS: Inner segment; OS: Outer segment; ONL: Outer nuclear layer; Original magnification: ×400. C: Statistical analysis results, **P < 0.01 vs WT.

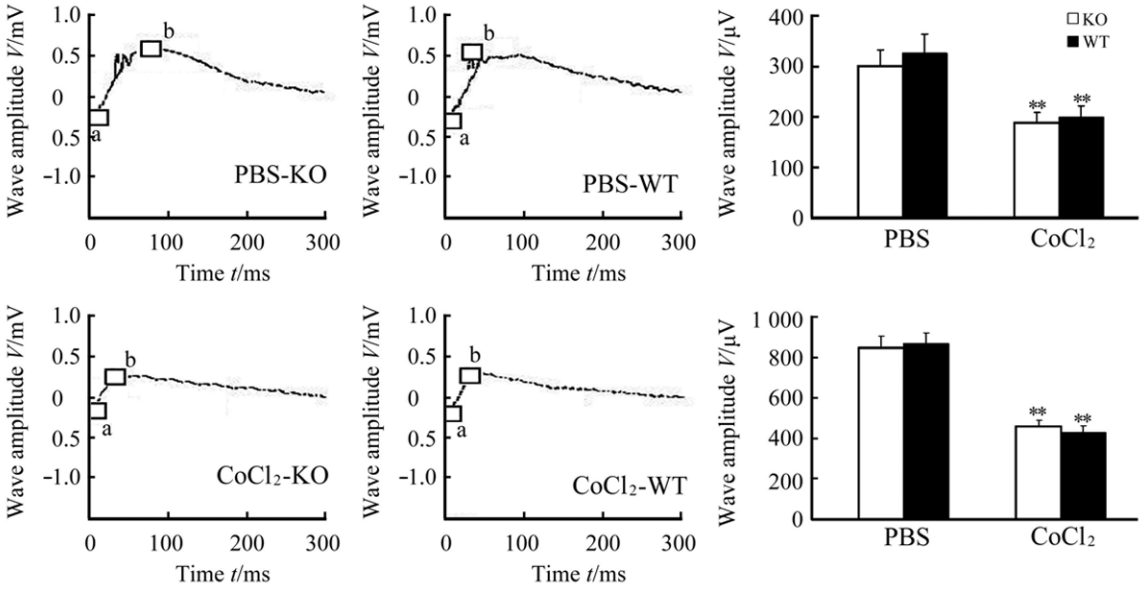

Figure 2. Scotopic ERG of KO and WT mice under normal and hypoxia condition.

Results

Expression of VEGFR2 in retina of KO and WT mice under normal conditions

VEGFR2 was significantly expressed in the inner segments of the cone photoreceptors of WT mice (**Figure 1A**). However, only slight VEGFR2 expression was found in the inner segments of the cone photoreceptors of KO mice (**Figure 1B**). The amount of VEGFR2 expression in cone photoreceptors of KO mice was about 15.3% of that in WT mice, and the difference was statistically significant ($P = 0.0021$, $P < 0.01$, **Figure 1C**).

Changes of ERG in KO and WT mice under hypoxic conditions

Under hypoxic conditions, the amplitudes of *a* and *b* waves of dark adaptation ERG in both KO and WT mice were significantly decreased compared with that of each PBS eyeball in the respective control group ($P < 0.01$). However, no statistical significance was presented when the results of KO mice were compared with those of WT mice ($P > 0.05$, **Figure 2**).

OD: Oculus Dexter (the right eye); OS: Oculus Sinister (the left eye). N = 6, mean ± SEM, **P < 0.01 vs. PBS. The amplitude of *a* and *b* waves

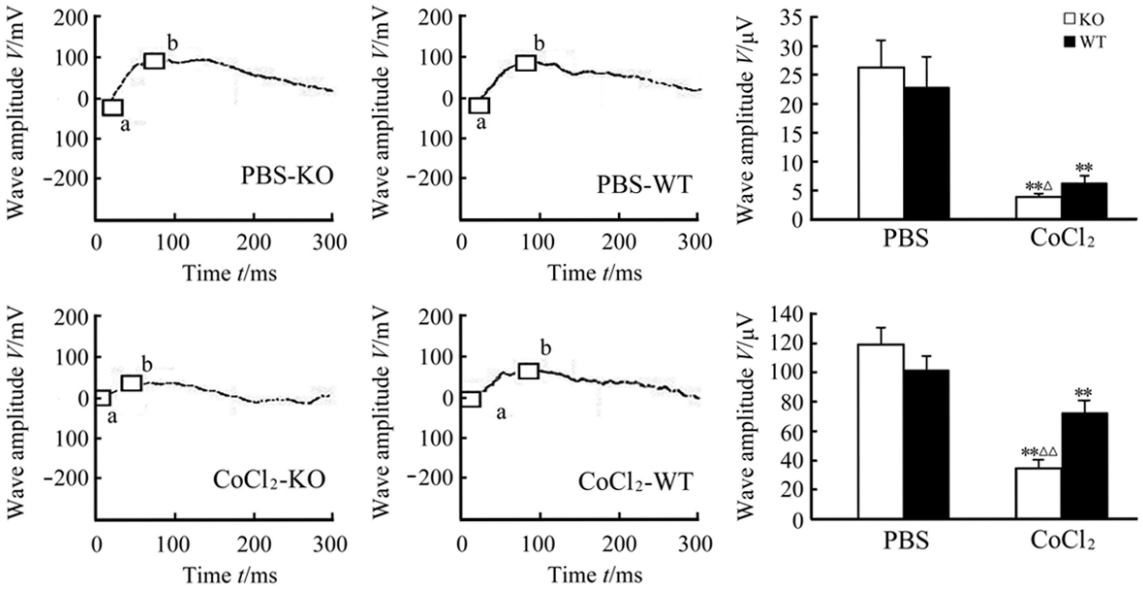

Figure 3. Photopic ERG of KO and WT mice under normal and hypoxia condition. N = 6, mean ± SEM, **P < 0.01 vs PBS; ΔP < 0.05, ΔΔP < 0.01 vs WT.

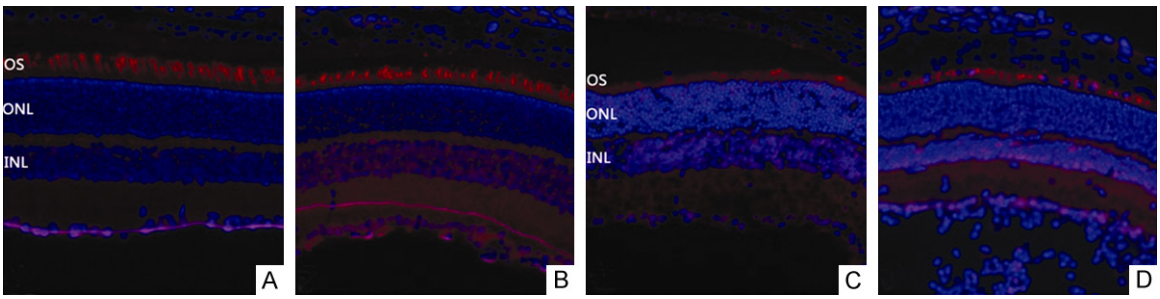

Figure 4. Cone photoreceptor morphology changes in KO and WT mice under normal and hypoxia condition. A: KO-PBS; B: WT-PBS; C: KO-$CoCl_2$; D: WT-$CoCl_2$. OS: Outer segment; ONL: Outer nuclear layer; INL: Inner nuclear layer. Red: Peanut agglutinin (PNA); Blue: 4',6-diamidino-2-phenylindole (DAPI). Immmuno-colocalization, Original magnification: ×400.

of light adaptation ERG in both KO and WT mice were significantly decreased compared with that of each PBS eyeball in the respective control group ($P < 0.01$). The results of KO mice were more significantly decreased than those of WT mice, and the difference was statistically significant ($P < 0.05$, $P < 0.01$, **Figure 3**).

Changes of morphology and density in cone photoreceptors of KO and WT mice under hypoxic conditions

Under hypoxic conditions, changes in cone photoreceptors of both KO and WT mice were presented. Compared with the density of each PBS eyeball in the respective control group, density of the outer segment in the cone photoreceptors was decreased, appeared shorter in morphology and loosely arranged in disorder. The above changes in KO mice were more significant than those in WT mice (**Figure 4**).

Discussion

DR is one of the primary blindness-causing eye diseases. Increased blood circulation resistance, decreased blood flow rate and poor perfusion have resulted in ischemia and hypoxia in retina tissues, leading to impairment of retinal neurons [6]. Therefore, investigation on pathological changes of retinal tissues under hypoxic conditions contributes to the study on pathogenesis of DR. The most commonly used chemical drug for simulating hypoxic environment is

$CoCl_2$, of which the principle lies in preventing intracellular respiratory chain from transferring to cause cellular chemical hypoxia [7, 8]. Intravitreal injection with $CoCl_2$ is a newly emerging in vivo experimental research method derived from the above principle to simulate hypoxia in retinal tissues, of which the period is shorter, and the operation is more convenient than that of establishment of diabetic model by intraperitoneal injection with streptozotocin. Foreign scholars have tested different concentrations of $CoCl_2$ during intravitreal injection in mice to investigate the optimal concentration for inducing hypoxia, and their results indicated that 3 mmol/L concentration resulted in the most viable effect [7]. However, 8 mmol/L concentration created the optimal effect to induce acute hypoxic environment in mice. Considering our experiment was designed to observe the impairment of cone photoreceptors under hypoxic conditions, and the structure damage in hypoxic environment for retinal tissues created by simulation with $CoCl_2$ appeared the earliest in the photoreceptor layer, the experiment was performed on alternate days.

VEGF plays an important role in diabetic microangiopathy [9]. Inhibition of VEGF activity by intravitreal injection of VEGF antibody has achieved a certain curative effect in clinical practice [10]. However, importance was gradually attached to neurodegeneration in the course of DR in recent years; DR treatment should not only aim at microangiopathy because neuroprotective therapy is also important [11]. Considering the neuroprotective effect of VEGF, VEGF antibody may reduce the protective effect and may damage optic nerves and retina. Further investigation on the neuroprotective effect of VEGF may provide experimental basis for the development of new DR drugs in the future. As previously mentioned, VEGFR may become one of the research targets because it needs to be combined with VEGFR to be activated. VEGFR2 is an important member of the VEGFR family; López [12] indicated that VEGFR2 could create protective effect on new brain cells via interaction with angiotensin receptor 2 in newborn rats under hypoxic environment simulated with high concentration of nitrogen. However, few reports currently exist about the effects of VEGFR2 on photoreceptors in retina in both local and foreign literature. In this experiment, KO mice are used to observe the protective effect of VEGFR2 on cone photoreceptors of photoreceptors in retina under hypoxic conditions.

ERG is a technology used for recording the potential produced after the retina is stimulated by flash, which is often used in the study of retinal function because the potential can be recorded non-invasively from the surface of the cornea [13]. ERG includes reactions arising from stimulation of weak light in dark adaptation (originated from rod photoreceptors) and reactions arising from stimulation of white standard flash in light adaptation (originated from cone photoreceptors). No significant changes of ERG in mice are observed under normal conditions, which indicate that VEGFR2 KO does not affect the cone cell function under normal conditions. However, compared with WT mice after intravitreal injection of $CoCl_2$, no significant difference was found in the decrease of ERG wave amplitude during dark adaptation. By contrast, the ERG wave amplitude significantly decreased during light adaptation in KO mice compared with that in WT mice, indicating significant reduction of cone cell function in KO mice. We performed immunofluorescence co-localisation using marker of cone cell-PNA to observe changes of the structure after completion of functional evaluation. The results demonstrate that significant changes in cone photoreceptors of retina were presented under hypoxic environment caused by intravitreal injection of $CoCl_2$. The density of outer segment was decreased, appeared shorter in morphology and was loosely arranged in disorder. The above changes in KO mice were more significant than those in WT mice.

In summary, under acute hypoxic environment induced by $CoCl_2$, impairment of cone cell function in KO mice is more significant than that in WT mice, and the absence of VEGFR2 decreases both functions and anatomic structures of cone photoreceptors. Under hypoxia and stress conditions, VEGFR2 may exhibit a protective effect on cone photoreceptors, but the underlying mechanisms require further research.

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Disclosure of conflict of interest

None.

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