Study of Hypoxia-Induced Expression of HIF-1α in Retina Pigment Epithelium

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It was shown that the expression of HIF-1α in retinal pigment epithelium increased under hypoxic conditions. Eight hours after the start of hypoxic exposure, the expression of HIF-1α reached the peak and sustained after 24-hour hypoxia. However, the morphology of PRE cells began to change and the expression of HIF-1α decreased after long-term (48-hour) hypoxia. Hypoxia-induced increase in the level of HIF-1α in RPE. It can be an important step in choriodal neovascularization.

Key Words: *choriodal neovascularization*; *HIF-1α*; *hypoxia*; *retinal pigment epithelium*

Choriodal neovascularization (CNV) can be caused by many fundus diseases such as AMD and pathological myopia. However, the exact mechanism of CNV has not been examined clearly. It was found that cells of retinal pigment epithelium (RPE) play an important role in the development of CNV. Factors such as low perfusion of the choroidal, the release of ischemia-induced angiogenesis factors including VEGF and bFGF, and hypoxia can be important in the development of CNV [1]. Hypoxia-inducible factor-1α (HIF-1α) is the initial transcription factor induced by hypoxia in eukaryotic cell. It regulates a series of target genes such as VEGF, bFGF, and GLUT1, which have a close relationship with angiogenesis [5,6]. Studies of the reaction of RPE and HIF-1α expression under hypoxic conditions are regarded as the key to investigation of CNV formation.

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MATERIALS AND METHODS

Eight eyes without any eye diseases were donated from healthy male adults who died accidentally. RPE were isolated and cultured in 24 hours.

The agents used in this study were IMDM, trypsinase, calf serum (GIBCO), mouse anti-human cytokeratin monoclone antibody (Dako,), FITC-conjugated goat anti-mouse polyclone antibody (Dingguo), rabbit anti-human HIF-1α polyclone antibody, Cy3-conjugated goat anti-rabbit polyclone antibody, mouse anti-human GFAP monoclone antibody (Boshide), TRIZOL one-step RNA extraction kit, DNA retrieval kit, 100 b.p. DNA ladder (Dingguo), PCR kit, HeaIII incision enzyme, and DL2000 DNA Ladder (Takara).

The eye globe was scissored at the pars plana. The vitreous and the retina were removed under microscope. The retina was washed with D-Hank buffer and treated with 0.25% trypsin for one-hour digestion at 37°C. After neutralization with culture medium containing serum, RPE cells were scraped, suctioned, and twice centrifuged (1000 rpm, 10 min) in iris repository or obtuse measuring pipette. RPE cells (5×10^4 cells/ml) were inoculated in 100 ml culture flasks with IMDM containing 20% calf serum and cultured in a CO_2-incubator (5% CO_2

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and 95% O_2, 90% humidity) at 37°C. The culture medium was refreshed every 3 or 4 days till the cells were combined and reproduced. It took approximately 28 days from the primary culture to cell confluence. Then, 5×10^6 cells were harvested per bottle. Before reproduction, the cells were washed with D-Hanks buffer first, and then digested with 0.25% trypsin for one minute. The cells were rewashed with D-Hanks buffer and digested with 0.25% trypsin-EDTA for 1 min. Then, the cells were blown, isolated and reproduced with IMDM containing 20% calf serum. The reproduction period of RPE ranged from 15 to 24 days. The 3rd-6th generation cells were used for the experiment, because these cells were in a stable and good status.

The originally isolated RPE cells looked like black balls and became polygon-shaped after adherence. The cytoplasm was full of melanin pigment. The cell nucleus was transparent and round. The pigment was attenuated with the cell division. Hematoxylin and eosin staining showed that there was melanin pigment granula in RPE cells. Meanwhile, the cytokeratin was identified using immunofluorescence method. GFAP antigen staining was performed to distinguish RPE from fibroblasts.

When the bottom of the culture flask was full of the RPE cells, the bottle port was sealed with a rubber stopper. A pinhead (No. 16) was inserted through the rubber stopper and used as a vent of the culture flask. Meanwhile, the long pinhead used for lumbar anesthesia was also inserted through the stopper. Through the long pinhead, the flask was inflated with the mixed gas (0.5 kg/cm^2) of 5% CO_2 and 95% O_2 for 10 minutes. When the concentration of O_2 checked at the vent was less than 0.5%, the air in the culture flask approached to a relatively hypoxia status. Then, the flask was put in the CO_2 incubator. The cells were harvested after 6-, 8-, 16-, 24-, and 48-hour hypoxia, respectively.

HIF-1α contains an oxygen-dependent degradation domain (ODD) region, which can be degradated through ubiquitin-proteasome pathway under normal oxygen partial pressure [2,3]. ODD region between 401 and 603 amino acid residues is specific for HIF-1α. The corresponding gene sequence in this region was chosen in the experiments. In order to eliminate the interference from the genome, the chosen gene region strode over an intron with a length of 977 b.p.. The cloned target gene sequence is listed as follows:

5'-CAGAAGATACAAGTAGCCTCTTTGAC
AAACTTAAGAAGGAACCTGATGCTTT
AACTTTGCT*GGCC*CCAGCCGCTGGAGACACA
ATCATATCTTTAGATTTTGGCAGCAACGACA
CAGAAACTGATGACCAGCAACTTGAGGAAG
TACCATTATATAATGATGTAATGCTCCCCTC
ACCCAAGGAAAAATTACAGAATATAAATTTG
GCAATGTCTCCATTACCCA-3', where *GGCC* is the enzyme-cut site (located at 433 b.p.) of restriction enzyme HaeIII. The upstream primer is 5'-CAGAAGATACAAGTAGCCTC-3'. The downstream primer is 5'-TGGGTAATGGAGACATTG CC-3'. The β-actin upstream primer is 5'-GTGGGG CGCCCCAGGCACCA-3'. The β-actin downstream primer is 5'-CTTCCTTAATGTCACGCACGATT TC-3'. The length of the amplified target section of HIF-1α is 225 b.p. while the length of β-actin is 540 b.p.

A total of 5×10^6 RPE cells were taken at different hypoxia stages. Total RNA was extracted using Trizol one-step method and transcribed reversely to obtain cDNA. Then following this template, PCR was performed with the HIF-1α primer and β-actin primer mentioned above. The reaction conditions were listed as follows: 1) degeneration at 94°C for one minute; 2) annealing at 55°C for one minute; 3) extension at 72°C for one and a half minute; and 4) final extension at 72°C for 5 minutes.

The product was identified by 1% agarose gel electrophoresis. Using the cleaning retrieval kit, the target section of HIF-1α for the further identification was retrieved using the restriction enzyme HaeIII.

A pretreated glass slide was placed in the culture flask in advance. The reproduced RPE (4×10^4 cells/ml) were added. After 48 hours, the glass slide was overgrown with the RPE cells. Then, the hypoxia experiment could be performed. The glass slide fully covered with the cells was removed out of the flask using a tunnel spade, washed with PBS (3×5 min), and fixed with paraformaldehyde. The slide was washed with PBS for the second time and processed with 3% H_2O_2-methanol fixative for 30 minutes to block endogenous peroxidase. Then, the slide was washed with PBS (3×5 min) and incubated in the mixture of 0.25% Triton X-100 and 5% DMSO for 10 minutes to perforate the cell membrane. After washed with PBS (3×5 min), the slide was treated with 5% equine serum. The slide was put into a moist box at room temperature for 30 minutes in order to avoid non-specific absorption. Then, the slide was incubated with rabbit anti-human HIF-1α (1: 50) in a moist box at 37°C for 60 minutes. Finally, the slide was washed with PBS (3×5 min) and incubated with Cy3 conjugated goat anti-rabbit secondary antibodies at 37°C for 30 minutes. After washed with PBS (3×5 min), the slide was examined using laser confocal microscope.

In this study, the HeLa cells with HIF-1α expression were used as positive control. The RPE cells cultured under normal partial oxygen pressure were used as negative control.

RT-PCR electrophoresis gel digital images were captured using Kodak image sampling system and analyzed using Image tool 2.0 system. The ratio of the fluorescence grey scale of the electrophoresis strip of HIF-1α to that of β-actin was calculated.

Topcon confocal fluorescence microscope was used to observe and collect the immunofluorescence digital images. The background was processed using PHOTOSHOP 6.0 software. NOVA PRIME software was used to randomly choose three different regions of RPE cells in each image. The fluorescent quantitative analyses were carried out according to pixels and the fluorescence density. Two or three independent immunofluorescence experiments were selected to calculate the average fluorescence value. The mean immunofluorescence grey scale values of HIF-1α expression at different hypoxia stages were measured and the corresponding curves were drawn.

RESULTS

The RPE adhered firmly to the bottom of the flask. The reproduction period of RPE was approximately 15-24 days. The melanin granula decreased with increasing splitting frequency. Hematoxylin and eosin staining showed that RPE had a typical appearance of epithelium cell and the melanin granula was clearly seen in the cells (Fig. 1, *a*). The positive expression of keratin (Fig. 1, *b*) with immunofluorescence method and the negative staining of GFAP distinguished RPE from other cells such as fibroblasts.

The electrophoresis strips of HIF-1α and β-actin at different hypoxia stages were shown in Fig.

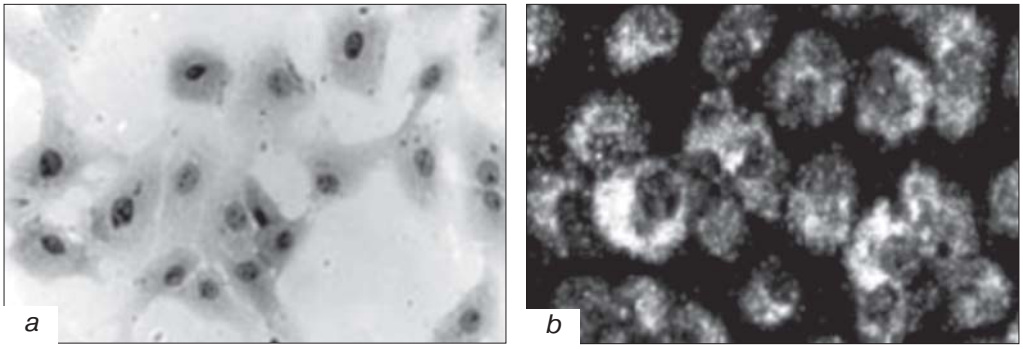

Fig. 1. PRE cells under normal conditions (*a*) and expression of keratoprotein in PRE cells (*b*). *a*) hematoxylin and eosin staining, ×400; *b*) immunofluorescence, ×400.

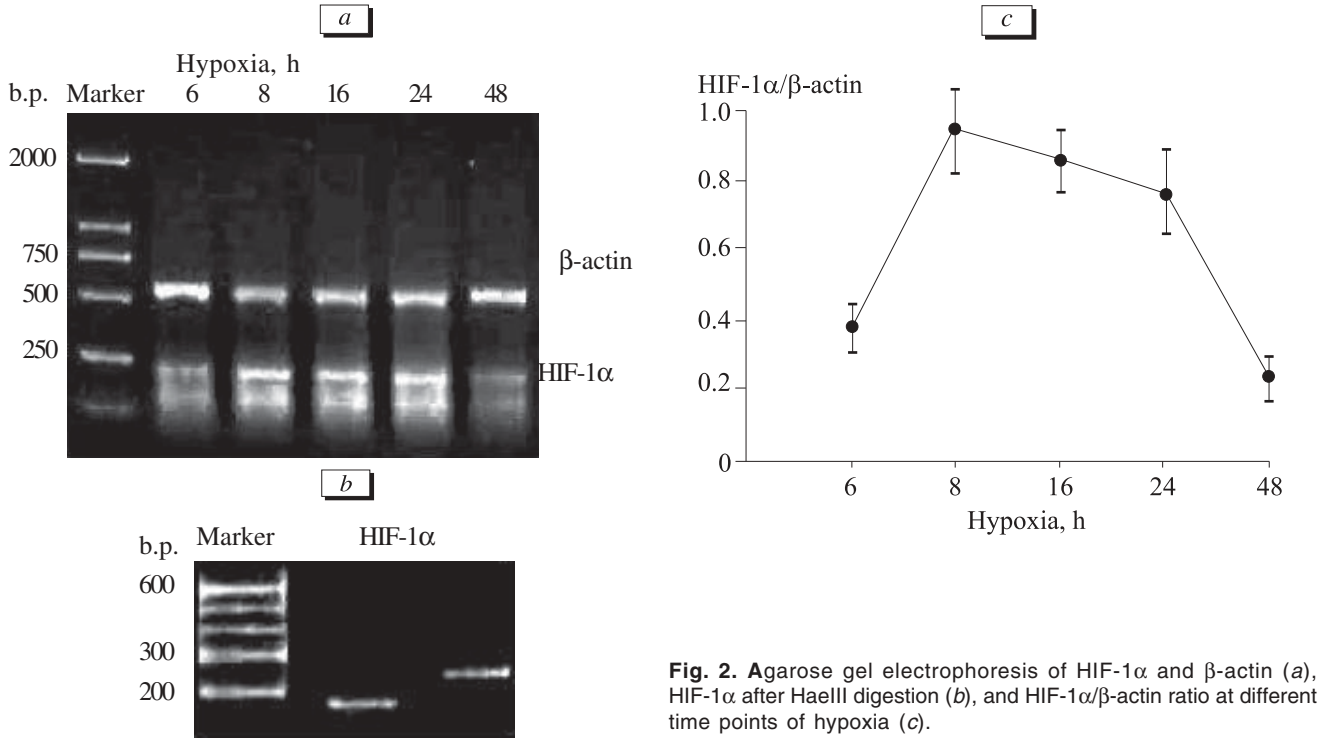

Fig. 2. Agarose gel electrophoresis of HIF-1α and β-actin (*a*), HIF-1α after HaeIII digestion (*b*), and HIF-1α/β-actin ratio at different time points of hypoxia (*c*).

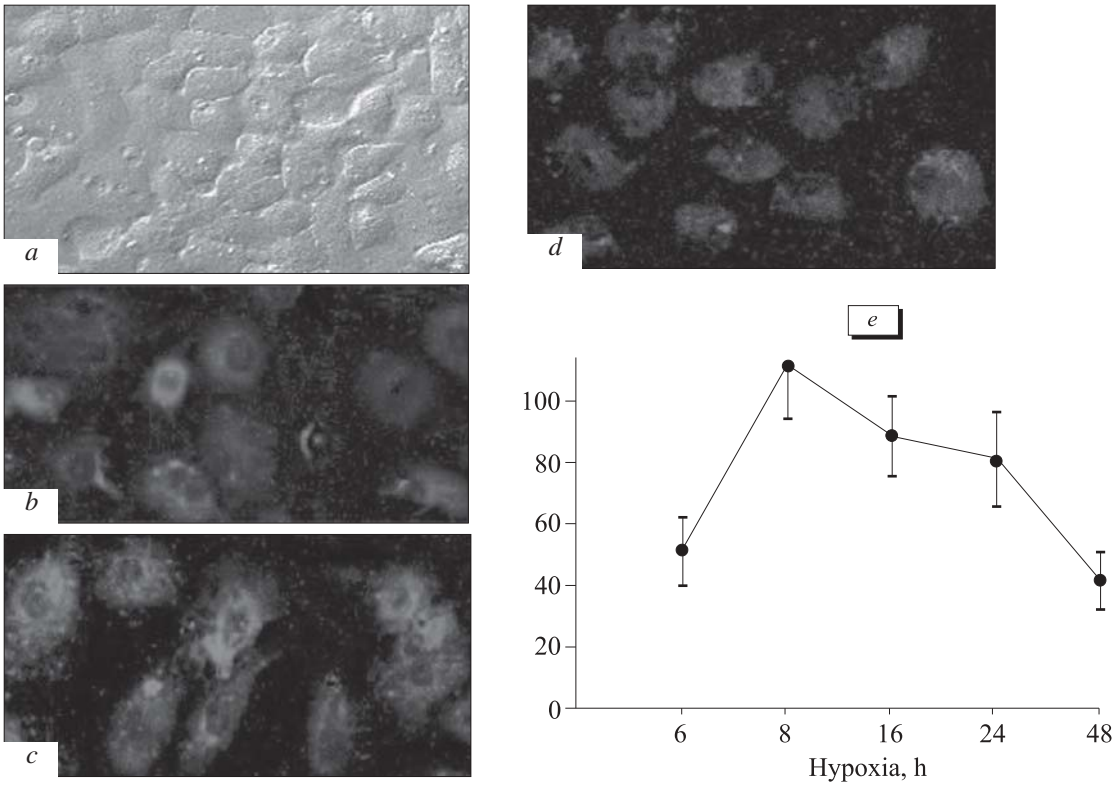

Fig. 3. PRE cells under normal oxygen pressure (*a*), expression of HIF-1α at different terms of hypoxia (*b-d*), and mean gray value of HIF-1α at different terms of hypoxia (*e*). *b*) 8 h; *c*) 16 h; *d*) 48 h of hypoxia; *a*) differential interference contrast, ×400; *b-d*) immunofluorescence; *b,d*) ×400; *c*) ×500.

2, *a*. The intrinsic gene of β-actin was located at 540 b.p. while the target section of HIF-1α was located at 225 b.p. Among the amplified HIF-1α genes, only one enzyme-cut site of the restriction enzyme HaeIII was determined at 433 b.p., which was presented as the sequence of 5'GGCC3'. The result of electrophoresis cut by HaeIII demonstrated two electrophoresis strips with a length of 162 b.p. and 66 b.p., respectively. This result further confirmed that the target part correctly amplified while the amplification of non-specific genes with the same length was prevented. HIF-1α was located at 225 b.p. and the enzyme-cut part was located at 162 b.p. (Fig. 2, *b*). However, the enzyme-cut part at 66 b.p. could not be shown simultaneously because of the limitation of the resolution of agarose gel electrophoresis.

The agarose gel electrophoresis result of HIF-1α and β-actin at different hypoxia stages was analyzed using Image tool 2.0 system. It was found that the expression of HIF-1α in RPE cultured under normal oxygen pressure was negative and greatly increased to reach the peak after 8-hour hypoxia and sustained the peak until 24-hour hypoxia (Fig. 2, *c*). After 48-hour hypoxia, the RPE cells were damaged and the expression of HIF-1α decreased.

Hypoxia significantly induced HIF-1α expression by RPE (Fig. 3). The expression of HIF-1α increased greatly after 8 hours of hypoxia. The HIF-1α expressed by RPE under normal oxygen pressure was negative (Fig. 3, *a*). Because they showed no fluorescence, these cells could only be observed using DIC cell stereogram. Fluorescence values of HIF-1α expression, which were calculated using NOVA PRIME software (Fig. 3, e) in the region of interest chosen randomly at different hypoxia stages were similar to the result measured by semiquantitative RT-PCR. However, the immunofluorescence method showed higher sensitivity in comparison with semiquantitative RT-PCR.

The results of this study demonstrated that human RPE cells were sensitive to hypoxia and hypoxia induced RPE transcription and HIF-1α expression. As the original transcription factor, HIF-1α could activate multiple target genes such as VEGF and bFGF, and stimulate angiogenesis or initiate a new pathology mechanism to respond to hypoxia.

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