·Basic Research·

Sequential changes of HIF-1α protein and mRNA in hypoxic bovine retinal microvessel endothelial cells

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Abstract

• AIM: To investigate the sequential changes of HIF-1α protein and mRNA in hypoxic bovine retinal microvessel endothelial cells.

• METHODS: The bovine retinal microvessel endothelial cells were cultured in normoxic and $CoCl_2$-induced hypoxic conditions respectively. Expressions of HIF-1α protein were measured with immunohistochemical staining, and RT-PCR was used to determine the HIF-1α mRNA.

• RESULTS: HIF-1α began to increase 1 hour after hypoxia, and reached the peak at 4 hour. After 16 hours, it declined significantly. Compared with the normoxic group, the expression of HIF-1α protein in the hypoxic groups had significant difference ($P<0.01$), and HIF-1α mRNA expression was unchanged under hypoxia.

• CONCLUSION: HIF-1α participates in the hypoxic procedures in retinal microvessel endothelial cells, and hypoxia induce time-dependent changes of HIF-1 α protein expression, which is not modulated on the transcription level. Analysis of HIF-1 α expression revealed a temporal and spatial changes with regard to the hyperoxic repression, indicating that HIF-1 may play a major role in the development of retinopathy of prematurity(ROP) and other ischemic retinal disorders such as diabetic retinopathy(DR).

• KEYWORDS: HIF-1α ; retina; microvessel endothelial cells

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INTRODUCTION

Retinal neovascularization (RNV) is the most common pathway of retinopathy of prematurity (ROP), diabetic retinopathy (DR) and retina venous occlusion, which eventually leads to visual loss. Numerous clinical and experimental observations have indicated that ischemia or hypoxia triggers RNV through an excessive production of one or more angiogenic factors [1]. Identification of these factors is an important step toward understanding the mechanism of pathological angiogenesis and development of specific treatments for diseases involving angiogenesis. Among the multiple factors known to be responsible for RNV, many groups have reported the importance of vascular endothelial growth factor (VEGF) [2]. The transcription factors HIF-1α (hypoxia-inducible factor-1α) play important roles in embryonic vascularization, and activate the expression of genes such as VEGF, erythropoietin (EPO) and a series of glycolytic enzymes in response to ischemic or hypoxic conditions. The recent investigation has suggested a role for HIF-1 and VEGF in hypoxia driving angiogenesis in a wide variety of human tissues. Interestingly, little is known regarding the role of HIF-1 in retina. HIF-1 is a heterodimer of basic-helix-loop-helix PAS domain proteins, HIF-1α and HIF-1β. HIF-1α is the oxygen-regulated component that determines HIF-1 activity. In this study, we investigated the sequential changes of HIF-1α and its mRNA in hypoxic bovine retinal microvessel endothelial cells exposed to cobaltous chloride ($CoCl_2$).

MATERIALS AND METHODS

Bovine Retinal Endothelial Cell Culture Bovine eyeballs were purchased from a local slaughterhouse. The retinas were removed, cut into small pieces, and incubated at 37℃ for 1-2 hours in the F_{12} containing 0.2g/L of collagenase I. The cells were obtained by

centrifuging after washing 3 times with phosphate-buffered saline (PBS). After centrifugation, the pellet was resuspended and the cell suspension was mechanically dispersed using borosilicate glass pipettes, passed through 80μm mesh size nylon sieves and plated onto gelatin-coated culture dishes. Cells were then incubated in which contained F_{12} and endothelial growth medium (EGM) (1:1) mixture 200mL/L fetal bovine serum (FBS) at 37℃ in 50mL/L CO_2 humidified atmosphere, with frequent medium changes to remove cell debris. After 15a, subcultures were seeded onto uncoated culture dishes and characterized by morphology and immunohistochemistry (positive staining for factor VIII). Cells between the third and tenth passage were used for the experimental procedures.

Experimental Groups The experimental samples were divided into 2 groups: ① Normoxia Group, cultured in normoxic conditions (200mL/L O_2, 50mL/L CO_2, and 750mL/L N_2); ② Hypoxia Group, cells were incubated with 125μmmol/L $CoCl_2$ for 1, 2, 4, 8, and 16 hours.

Immunohistochemistry Techniques Following hypoxic or normoxic exposure, cells were immediately fixed in a solution of 10℃ methanol for 5 minutes. Fixed cells were washed with PBS, (0.2mol/L) and blocked in a 30mL/L solution of bovine serum albumin. Cells were then incubated with the primary antibody for HIF-1α (1:100, Transduction Laboratories) for 2 hours at 37℃ followed by overnight incubation at 4℃ . Secondary labeling was achieved by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibodies (1:5) at 37℃ for 60 minutes. PBS was evaluated by substituting rabbit serum for the primary antibodies. All measurements were made using a ×400 objective. Specific staining was calculated as staining intensity of cultures incubated with primary antibodies minus background (maximum staining intensity in cultures treated with rabbit serum). Fluorescence intensity (the sum of grayscale levels for all pixels in the field above background) was measured for the entire field of view. Immunofluorescence intensity values measured in 5 different fields of view for each coverslip was averaged as a single 'n'.

RT -PCR Total RNA from BREC was prepared by Trizol(Gibco, Life Tech, CA, USA). cDNA was synthesized with 1μL of total RNA, 1μL random hexadeoxynucleotide primer 0.2g/L in 20μL of a solution containing RT. Then cDNA was diluted 1:5 with water and stored at -20℃ until use. PCR reactions were carried out in a total volume of 50μL containing 10 ×PCR buffer 5μL, 25mmol/L $MgCl_2$ 3μL, 2 mmol/ L dNTP 5μL, 1μmol/L each primer 0.5μL, cDNA 2μL, Taq ExTaq polymerase 1U. The PCR condition consisted of 2 minutes at 94℃ followed by 28 cycles of 15 seconds at 95℃ , 30 seconds at 55℃ and 30 seconds at 72℃ , followed by 72℃ for 5 minutes. PCR products were subjected to electrophoresis in 12g/L agarose gel, visualized by UV, and quantified using NIH Image. The PCR primer sequences of HIF-1α and β-actin which was used as an internal control were as follows: sense primer for HIF-1α, 5' GGTATTATT CAGCACGAC 3' and antisense primer for HIF-1α, 5'GAGTTTCAGAGGCAGGTA 3' corresponding to 756 bp; sense primer for β-actin, 5'GCGGCATTCACGAAA CTA 3' and antisenseprimer for β- actin, 5'CACCTTCA CCGTTCCAGT 3' corresponding to 278 bp.

Statistical Analysis All data were presented as mean±SD. Statistical analysis was performed using the analysis of variance and *t*-test. $P<0.05$ was taken as being significant.

RESULTS

HIF-1α Expression In normoxic condition, the expression of HIF-1α was very low. HIF -1α began to increase 1 hour after hypoxia, and reached the peak at 4 hour. After 16 hours, it declined significantly. Compared with the normoxic group, the expression of HIF-1α in all the hypoxic groups had significant difference ($P<0.01$).

HIF -1α mRNA Expression RT-PCR revealed that HIF-1α mRNA was expressed in the hypoxic group and normoxic group. There was no significant difference between the groups.

DISCUSSION

Maintaining oxygen homeostasis is a primary requirement constraining the development, growth and internal organization of all large animals. The transcriptional complex HIF-1 has emerged recently as a key regulator of these processes, mediating a wide range of cellular and physiological responses necessary to adapt to changes in oxygen tension [3]. HIF-1 is composed of 2 subunits: HIF-1α and HIF-1β. The expression of HIF-1α is extremely sensitive to the onset of cellular hypoxia, making it one of the earliest effectors of the response to

hypoxia. HIF-1β, constitutively expressed[4], on the other hand, is a high affinity protein that binds to HIF-1α in the cytosol and transports HIF-1α into the nucleus, where HIF-1α can exert its effect. HIF-1α binds to a specific hypoxia-responsive element (HRE) in the regulatory regions of many target genes, initiating gene transcription and increasing the level of mRNA expression. HIF-1 has now been shown to activate the transcription of a wide variety of genes. These include genes encoding proteins involved in haematopoiesis, angiogenesis, energy metabolism, catecholamine synthesis and iron metabolism, indicating that HIF-1 activation may be involved in the regulation of vascular growth and cellular metabolism. Therefore, it might be predicted that an understanding of the patterns of HIF regulation in the retina would provide important insights for RNV.

In the study, we demonstrated only very low levels of expression of HIF-1α protein in normoxic cells. Upon initiation of the hypoxic response by stimulation of cells with $CoCl_2$, however, HIF-1α protein levels were dramatically up-regulated, suggesting that HIF-1α expression, and possibly function, is regulated by oxygen deprivation.

Previously, it has been suggested that in response to hypoxia (or $CoCl_2$), both mRNA and protein levels of HIF-1α are induced. However, contrary to this model of regulation, others have detected significant levels of constitutively expressed HIF-1α mRNA in a number of cell lines and human tissues, and these steady-state levels are not altered by exposure to hypoxia [5, 6]. In our efforts to understand the mechanism of regulation of HIF-1α, we found that HIF-1α mRNA expression was unchanged by hypoxia. It means that the activity of HIF-1α can be regulated at the posttranscriptional level. In cells replete with oxygen, HIF-1α subunits are unstable, being rapidly destroyed by the ubiquitin-proteasome pathway (half-life <5 minutes). While in hypoxic condition, the degradation can be blocked[7].

In summary, analysis of HIF-1α expression revealed a temporal and spatial changes with regard to the hyperoxic repression, indicating that HIF-1 may play a major role in the development of ROP and other ischemic retinal disorders such as DR. We can suggest that inhibition of HIF-1 activity may be of therapeutic utility in these conditions.

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