·Basic Research·

Expression of neuroglobin in ocular hypertension induced acute hypoxic-ischemic retinal injury in rats

Shao-Yang Shi, Xue-Mei Feng, You Li, Xun Li, Xiao-Long Chen

Department of Ophthalmology, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China
Correspondence to: Xue-Mei Feng. Department of Ophthalmology, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China. fengxm_06@sina.com
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Abstract

• AIM: To investigate the expression of neuroglobin (Ngb) in the retina of rats with ocular hypertension induced acute retinal hypoxic-ischemic injury.

• METHODS: Seventy Wistar rats were divided into 7 groups randomly. The experimental model was induced by elevation of intraocular pressure *via* anterior chamber canula insertion in the left eyes and the fellow eyes were preserved as normal controls. The retinal tissues were taken at 1, 5, 10, 15, 20, 30 and 60 minutes after hypoxic-ischemia injury. Protein was extracted, and then analyzed by Western-blot method. SPSS was used for statistical analysis.

• RESULTS: The time-depended expressions of Ngb were observed. The level of Ngb increased rapidly at 1 minute after ischemia and reached to the peak at 5 minutes, which had significant difference from that of control group ($P<0.05$). It kept in high level during 5-15 minutes ($P<0.05$), then decreased after 20 minutes till 60 minutes. There were no significant differences between experimental and control group in the latter period ($P>0.05$).

• CONCLUSION: The expression of Ngb in retinal tissue increased rapidly after hypoxic-ischemic injury in rats, suggesting that Ngb may play an important role in the process of acute retinal hypoxic-ischemic injury.

• KEYWORDS: neuroglobin; hypoxia-ischemia; retina
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INTRODUCTION

Neuroglobin (Ngb), a member of heme-protein family, was firstly discovered as the third type of oxygen-carrying protein in human body by Burmester *et al* in 2000 [1]. It is named with Neuroglobin because it mainly expressed in the brain tissues, while Schmidt *et al* [2] reported that Ngb expressed in all neurons of mouse retinal nerve with an ultrahigh concentration which is 100 times of that in brain. Ngb can bind oxygen reversibly with a high affinity, and it plays a vital role in uptaking, transporting and utilizing oxygen in nervous system. Ngb concentrations differentiate in different regions in brain and retina, which are coincident with the different sensitivities of corresponding regions to hypoxia. Ngb plays an important role in the process of hypoxic preconditioning of nervous system. Increased expression of Ngb was observed in both *in vivo* and *in vitro* studies of hypoxia and/or ischemia in central nervous system. What's more, the lower the Ngb concentration is, the worse the tolerance of hypoxia is. Recent researches focus on the central nervous system, while studies on the expression of Ngb in retina are still at the initial stage. Retina is a high-differentiated nerve tissue and the continuation of brain tissue. Both of retina and brain are very sensitive to hypoxic-ischemia. Based on a rat model with acute retinal hypoxic-ischemia, we detected and analyzed the Ngb expression in retina at different time point *via* Western-blotting technique to study the relationship between Ngb and acute retinal hypoxic-ischemia injury.

MATERIALS AND METHODS

Experimental Animals Seventy healthy Wistar rats aged from 50 to 60 days (no limitation of gender, with body weight of 200-230g) were selected and fed with all-ingredient rats granule foods. Rats and foods were provided by Laboratory Animal Center of China Medical University. Rats were fed under temperature of 18-22℃, relative humidity of 55%-75%, light maintenance for 12 hours and free ingestion and drinking. Inclusive standards: no abnormal findings of the outer eyes; direct and indirect light reflexes of both eyes existed. After 3 days of adaptive domestication, 70 rats were randomly and evenly divided into 7 groups. The left eye of each rat was chosen to be operated as the hypoxic-ischemia model, while the right eye was maintained as self-control. All procedures involving animals were reviewed and approved by the Institutional

Animal Care and Use Committee.

Modeling and Sampling All rats were anesthetized with 10% chloral hydrate after weighing with a dosage of 0.3mL/100g *via* injection into left lower peritoneal cavity. Rats were fixed after anesthesia, then a $5^1/_2$ scalp acupuncture, which was conjugated with infusion tube for normal saline, was penetrated into the anterior chamber of the left eye along with corneal limbus at the temporal side. The dropping bottle was adjusted to a vertical height of 150cm from the animal eye to artificially increase intraocular pressure to 120mmHg, which was similar to the systolic blood pressure of general circulation in rats in order to completely block blood flow in retina [3,4]. The normal range of intraocular pressure of rats' eyes was 9-20mmHg. The average pressure was about 14.5±3.2mmHg. Subsequently, pale conjunctiva, blockage of retinal blood flow, retinal ischemia and disappearance of red reflex of fundus were observed by a direct ophthalmoscope (+8-+10D). The rats in 7 groups of were sacrificed at 1, 5, 10, 15, 20, 30 and 60 mintues after the high intraocular pressure, respectively. Finally, the eyes were immediately enucleated and the retinas were preserved in 1.5mL centrifuge tubes at -80℃.

Western Blot analysis Characterization of antigens was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting procedures that had been described previously[5]. The protein concentrations were determined with Keygen Lowry Protein Assay Kit (Keygen Biotech Co. Ltd, Nanjing, China). An equal amount of protein for each sample was heated at 100℃ for 5 minutes with an equivalent volume of double strength sample buffer and loaded onto 12% polyacrylamide gels. The proteins were electrotransferred, blocked and treated with rabbit polyclonal anti-Ngb Ab (1:1000, Santa Cruz Biotechnology, Inc, USA.). The blots were incubated and then exposed to the marker of diluted (1:2 000, Boster Biological Technology, Ltd, Wuhan, China) horseradish peroxidase-labelled secondary anti-rabbit antibody. In order to check equal loading, membranes were reprobed with an antibody against β-actin (1:10 000). Exposed Biomax films of bands represented the expression of Ngb protein. Density of bands was quantified using a GIS-2020 image-processing system (Technew Tech. Co. Ltd, Shanghai, China) and normalized to the intensity of the β-actin band.

Statistical Analysis All data were processed using SPSS software (version 15.0) with method of completely random design one-way analysis of variance (one-way ANOVA) and the LSD-*t* test was employed to compare the differences among groups. All the results were interpreted as mean ± standard deviation. In all analysis, $P<0.05$ was considered statistically significant.

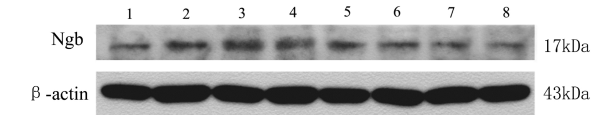

Figure 1 Neuroglobin and β−actin expression in acute retinal hypoxic−ischemic injury of rats 1: Normal control; 2: Hypoxic-ischemic injury 1 minute; 3: Hypoxic-ischemic injury 5 minutes; 4: Hypoxic-ischemic injury 10 minutes; 5: Hypoxic-ischemic injury 15 minutes; 6: Hypoxic-ischemic injury 20 minutes; 7: Hypoxic-ischemic injury 30 minutes; 8: Hypoxic-ischemic injury 60 minutes

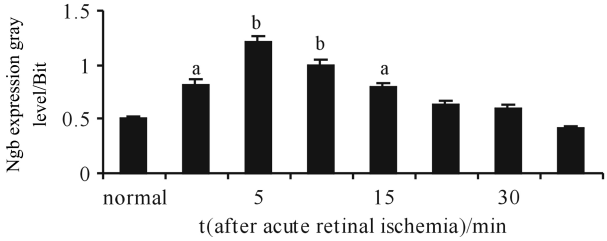

Figure 2 Ngb expression levels in retinal tissue of rat at different times of retinal ischemia The significance of the data was estimated with a Student's one-way ANOVA: $^aP<0.05$, $^bP<0.01$ *vs* control group; mean±SD, $n=10$

RESULTS

Variation on Ngb expression in retinal tissues The variations of Ngb expression in retinal tissues at different time point in rat models with intraocular hypertension induced acute retinal hypoxic-ischemic injury were shown in Figure 1. The expression of Ngb rapidly increased at 1 minute after ischemia ($P_{1min}=0.021$) and reached to the peak at the time point of 5 minutes ($P_{5min}=0.000$). Ngb mRNA expression began to decline at time points of 10 minutes and 15 minutes but still maintained at a high level ($P_{10min}=0.001$, $P_{15min}=0.036$). It quickly decreased at time points of 20 minutes and 30 minutes to a level higher than that of control group ($P_{20min}=0.327$, $P_{30min}=0.447$). At the time point of 60 minutes, Ngb expression was slightly lower than that of control group with no significant difference ($P_{60min}=0.611$) (Figure 2). The average expression levels of Ngb in each group at different time points in rats differentiated with statistical significance when compared with that of control group ($F=7.029$, $P=0.000$).

DISCUSSION

Retina is the highest oxygen-consuming tissue in body. Hypoxia can result in rapid and adverse effects on visual performance in man and other vertebrates. Several eye diseases and their progression are relative to hypoxia and ischemia in retina, *e.g.*, glaucoma, diabetic retinopathy, retinal artery occlusion and retinal vein occlusion. In the vascular retina of mouse and rat, oxygen is supplied by the outer choroidal, deep retinal and inner capillaries. Recent studies show that in this type of retina, mitochondria are

concentrated in inner segments of photoreceptor cells, outer and inner plexiform layers, and ganglion cell layer. These are the same regions in which oxygen consumption takes place and Ngb presents at high levels [6]. Ngb is mainly expressed in all neurons of the retina but not in the retinal pigment epithelium. It is also expressed in iris, ciliary and optic nerves in small amount but undetectable in other ocular tissues. The distribution of Ngb is correlated with the localizations of subcellular organs (mitochondria) and retinal relative oxygen demands, as most of the retinal oxygen is consumed in these locations[7]. Furthermore, Ngb expression is increased in retina from the eyes with chronic severe glaucoma, and it may protect against hypoxic, ischaemic or oxidative stress, which are thought to be pathological factors that affect the retina in glaucoma [8]. Our study demonstrated the variations of Ngb expression in rat retinal tissue at different time point in acute retinal ischemia injury model induced by elevation of intraocular pressure and found its corresponding change of Ngb expression. The expression levels of Ngb increased rapidly after ischemia in retina, which suggested that Ngb was very sensitive to hypoxic-ischemia and further indicated that Ngb might play an important role in adaptive protection for hypoxic-ischemia in retina. Under hypoxic-ischemia condition, the reactive increase of Ngb may accelerate oxygen transfer to the nerve tissues and within nerve tissues. Release of stored oxygen in Ngb may delay the death of nerve cells and enhance their tolerance to hypoxia to a certain extent. So it is speculated that the period from 1 to 15 minutes is the compensating period for hypoxia. Under retinal ischemic-hypoxia condition, Ngb may alleviate hypoxia degree through both ways of releasing binded-oxygen in it and increasing endogenous expression of it. However, the body compensation was limited and the expression of Ngb starts to decline from the time point of 20 minutes, but still with a higher level than that of normal control group. It decreased to a level below the normal level at 60 minutes, which it was speculated that severe hypoxia at this time was not capable of significantly affecting Ngb expression because continuous hypoxia had gradually been in the decompensation period and neurons begin undergoing irreversible changes. This result is consistent with the conclusions from Smith *et al* [9] and Johnson *et al* [10] that histological endurance time of rat retina on ischemia induced by increased intraocular pressure of 110mmHg is 15 minutes. Retinal hypoxic-ischemia injury is always a difficult clinical problem, which is now investigated mostly at the respects of morphological changes and metabolism changes after hypoxic-ischemia, such as the injury resulted from Ca^{2+} overloading in cells, the toxicity of irritative amino acids and oxidation of the free radicals. Researches on protection agent for retinal hypoxic-ischemia injury also focused on these respects, *e.g.*, calcium antagonists, irritative amino acid antagonists, free radical removing agent and antioxidants [11]. In addition, some cytokines have also been applied to the develop protection agent for retinal hypoxic-ischemia injury, including brain-derived neurotrophic factor (BDNF) [12], ciliary neurotrophic factor (CNTF)[13], basic fibroblast growth factor (bFGF) and heat shock protein 70 (Hsp70) [14] but with no satisfactory results. So the discovery of Ngb protein that plays an important role in oxygen transfer and storage provides a new direction for future researches on hypoxic and ischemic injuries, which may facilitates the interference of hypoxia and its development at very early stage using this oxygen carrier, and bring new hope for prevention and treatment of the common clinical intractable disease of retinal hypoxic-ischemia.

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