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Antihypoxic Effects of Neuroglobin in Hypoxia-Preconditioned Mice and SH-SY5Y Cells

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Key Words
Neuroglobin · Hypoxic preconditioning · Mice

Abstract
This work aims at investigating the neuroprotective effects of neuroglobin (Ngb) in vivo and in vitro. RT-PCR and Western blotting were used to examine Ngb mRNA and protein levels in the mouse cortex after acute and repeated exposure to hypoxia. The cDNAs of mouse Ngb were cloned and transfected into SH-SY5Y cells to examine Ngb function in vitro. Expression of Ngb and mRNA was upregulated in the cortex of mice preconditioned by repetitive exposure to hypoxia. Tolerance to hypoxia of Ngb-transformed SH-SY5Y cells was enhanced. These results suggest that Ngb might be involved in hypoxic preconditioning which protects neurons from hypoxic injury.

Introduction

Ischemic/hypoxic preconditioning (I/HPC), a prominent neuroprotection against subsequent severe ischemia/hypoxia following sublethal exposure to hypoxia, has been extensively studied [1–4]. This protective effect of I/HPC was first observed in the heart and later in the brain of the gerbil [4, 5]. It is believed to trigger endogenous cellular adaptation. Due to this special protective effect, I/HPC is believed to be a potential target for a clinical therapeutic approach to cerebral ischemic/hypoxic damage. However, the precise molecular mechanism of I/HPC is unclear. To investigate the molecular mechanism of I/HPC in the brain, we developed a unique autohypoxia-induced HPC mouse model that mimics clinical asphyxia [1, 6]. Using this model, we have demonstrated that during the development of HPC, there are changes in protein expression and activity [7, 8]. One prominent feature of cellular adaptation to hypoxia is the increased expression of new proteins contributing to the neuroprotection induced by hypoxia [9]. In addition, there was evidence that inhibition of protein synthesis eliminated the effects of HPC on brain neuroprotection [10].

The newly discovered Ngb is a vertebrate globin primarily localized in nerve cells that can bind oxygen reversibly [11]. Expression of Ngb increases in response to oxygen deprivation which in turn can protect neurons from hypoxia in vitro and in vivo [12, 13]. Ngb is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum in the animal brain and prominently affected in hypoxia or ischemia [14, 15]. Until now, the biological functions of Ngb have re-

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mained a mystery, but is suspected to have various roles in oxygen transport and sensing, binding and scavenging of nitric oxide, binding with CO [16], and in signal transduction [17, 18]. Many studies have reported the changes of Ngb under different hypoxic conditions in vivo and in vitro. However, little is known about changes in Ngb expression in the brain after acute and repeated hypoxia stimulation. This study focused on exploring the possible involvement of Ngb in neuronal responses during HPC using our auto-hypoxia-induced HPC mouse model and demonstrating its antihypoxic effect in vitro.

Materials and Methods

Animal Model Reproduction

Male adult BALB/C mice (body weight 16.0–22.0 g) were randomly divided into three groups: blank control group with no exposure to hypoxia (H_0), the hypoxia control group exposed to hypoxia once (H_1), and the hypoxia preconditioning group exposed to 4 runs of hypoxia (H_4). The experimental conditions and procedures were approved by the Local Institutional Animal Care and Use Committee, and followed the US National Institutes of Health principles for laboratory animal care. The procedure of the hypoxic experiment was performed as previously described [1, 19]. Briefly, the mouse was placed into a 125-ml jar with fresh air, and the jar was sealed with a rubber plug. The mouse was removed from the jar as soon as the first gasping breath appeared, then it was switched to another fresh-air-containing jar of similar volume. This procedure was performed once (group H_1) or repeated four times (group H_4).

RNA Isolation and Semiquantitative Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was prepared from mouse cortex using the RNAeasy minikit (Qiagen, German Town, Md., USA). Total RNA (1 μg) was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA). One microliter of the resulting cDNAs was subjected to amplification in a total volume of 20 μl containing 10× buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 1 U Taq polymerase (Takara, Shiga, Japan), and a pair of specific primers (0.2 μmol/l each). Primers for Ngb and β-actin were designed using the Primer Premier 5.0 software, and both sequences were obtained from GenBank: β-actin forward primer: 5′-GAACGGTGAAGGCGACAG-3′, β-actin reverse primer: 5′-TTGGGAGGGTGAGGGACT-3′; Ngb forward, 5′-CTCTGGAACATGGCACTGTC-3′,Ngbreverse,5′-GCACTGGCTCGTCTCTTACT-3′. The PCR temperature for Ngb was as follows: cycles at 92°C for 30 s, at 55°C for 30 s, and at 72°C for 45 s, total 29 cycles, followed by a final extension period at 72°C for 7 min. The PCR temperature for β-actin was: cycles at 92°C for 25 s, at 55°C for 30 s, and at 72°C for 25 s, total 20 cycles, followed by a final extension period at 72°C for 5 min. Polymerase chain reaction (PCR) products (9 μl) were separated by 1.0% agarose gel electrophoresis and stained with ethidium bromide. The reverse transcription (RT)-PCR products were 323 bp for Ngb and 175 bp for β-actin.

Western Blotting Analysis

Total protein was isolated from mouse cortex or transfected SH-SY5Y cells according to a previously described protocol [20, 21]. The protein concentrations were determined using the bicinchoninic acid (BCA) method. Protein (40 μg) was separated by SDS-PAGE (12%) at 30 mA for 2.5 h and then blotted onto a nitrocellulose membrane. The membrane was then incubated for 1 h in blocking buffer (Tris-buffered saline containing 10% skimmed milk powder) at room temperature. Next, the membrane was incubated for 16 h at 4°C using a goat anti-neuroglobin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or a mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, Mo., USA). Then the membrane was incubated with horseradish-peroxidase-labeled secondary antibodies at room temperature for 1 h. After each incubation, the membrane was thoroughly washed 3 times with Tris-buffered saline containing 0.05% Tween 20. Protein signals were detected by an electrochemiluminescence detection system (Pierce Biotechnology, Rockford, Ill., USA), in which the membrane was exposed to the detection solution for 5 min.

Cloning of the Ngb and Generation of Expression Vectors

The full-length cDNA encoding the mouse Ngb were cloned according to the laboratory method of Burmester et al. [11]. The stop code of the mouse Ngb cDNA was deleted by PCR and the mouse Ngb cDNA without the stop code was subcloned into a pEGFP-N1 expression vector (Clontech, Palo Alto, Calif., USA) and named pEGFP-Ngb.

Cell Culture and Exposure to Hypoxia

Cells from the human neuroblastoma cell lines SH-SY5Y were cultured in Dulbecco's modified Eagle's medium (Clontech, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA), L-glutamine and antibiotics, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Ngb could be detected in SH-SY5Y cell lines [22]. For exposure to hypoxia, cell culture plates were incubated in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, and 5% CO_2. The duration of hypoxia was 0, 2, 4, 6, and 8 h.

Cell Transfection and Detection of Cell Survival

SH-SY5Y cells were plated in 35-m plastic dishes and maintained as described previously [23, 24]. Full-length mouse Ngb cDNA was subcloned into pEGFP-N1 plasmid with green fluorescent protein (GFP). The recombinant plasmid (pEGFP-Ngb) or vector alone (pEGFP-N1) was transfected into SH-SY5Y cells by using Lipofect AMINETM Reagent (Invitrogen Life Technologies, USA), by detecting GFP to confirm the results after 16 h. Transfection efficiency of recombinant plasmid and vector was approximately 25%. The injuries of cells without plasmid were detected by Hoechst 33342 and propidium iodide (PI, staining apoptotic and necrotic cells; live cells stain blue, necrotic or terminally apoptotic cells stain red; early apoptotic cells should not stain red) dyeing after hypoxic exposure at different time points in order to explore the optimal operation time point. Cultures were incubated simultaneously with 5 μM Hoechst 33342 and 5 μg/ml PI (both from Sigma) for 15 min in a water bath at 37°C. The cell survival ratio with pEGFP-Ngb or pEGFP-N1 was measured as the ratio of counted cells carrying PI-unstained nuclei to counted cells which express GFP. The survival of cells without

transfected plasmid was measured as the ratio of cells carrying PI-unstained nuclei to all cells counted (each about 2,000 cells). The reaction was visualized using a Leica inverted fluorescent microscope (Germany).

Caspase-3/7 Activity Assay

Apoptotic activity was measured using Caspase-Glo 3/7 Assay kits (Promega, Madison, Wisc., USA). SH-SY5Y cells were seeded into 24-well opaque microtiter plates at a density of 4×10^4/well. After 24 h, the cells were transfected with pEGFP–Ngb or pEGFP-N1 and then exposed to hypoxia just as described previously. Then caspase activity was measured with TD-20/20 Luminometer (Turner Designs, Sunnyvale, Calif., USA) using the Caspase-Glo 3/7 assay kit according to the manufacturer's protocol.

Quantification and Statistics

The optical density (OD) of bands of PCR products and Western blot were obtained by the Gel-Doc system and analyzed with SigmaGel software (Jandel Scientific, San Rafael, Calif., USA). The data of RT-PCR and Western blot were normalized to β-actin and presented as relative abundance. All data are expressed as mean ± SD. Statistical analysis was performed by means of ANOVA and the Tukey test using SPSS 10.0 software (SPSS Inc., Chicago, Ill., USA). $p < 0.05$ was considered to be statistically significant.

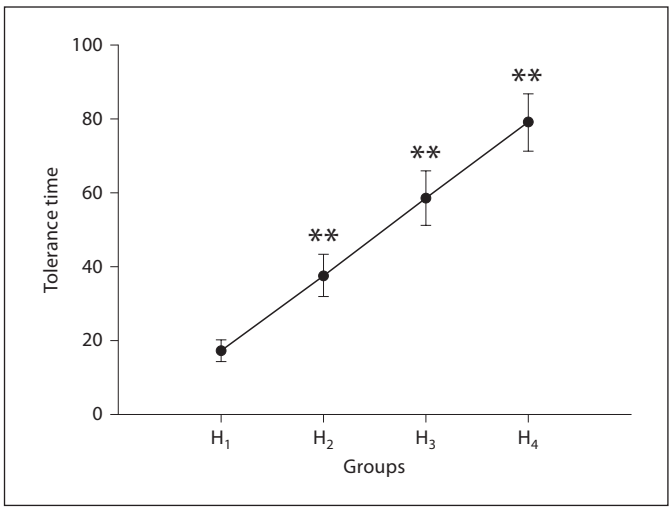


Fig. 1. Tolerance time in the different exposure runs (n = 30; ** $p < 0.01$ as compared with the preceding runs).

Results

Tolerance of Preconditioning Mice to Hypoxia

The increment of tolerance to hypoxia in each run was approximately in an arithmetic progression. The tolerance time increased significantly with each run. The average tolerance time of runs 1, 2, 3, and 4 was 17.2 ± 2.9, 37.4 ± 5.7, 58.5 ± 7.3, and 78.7 ± 7.9 min, respectively (fig. 1), which was in accordance with our previous findings [1, 19].

Expression of Ngb mRNA and Protein in Cerebral Cells

The Ngb mRNA was analyzed by RT-PCR immediately at the end of each exposure to hypoxia. The relative abundance value of Ngb in each group was calculated by the OD ratio of Ngb to β-actin. The relative abundance values of group H_1 (1.19 ± 0.16) were significantly higher than those of group H_0 (0.81 ± 0.09; $p < 0.05$). Furthermore, after repetitive exposure to hypoxia, the Ngb values increased more at the mRNA level in group H_4 (1.48 ± 0.31). A significant difference in the relative quantities of Ngb among the three groups was detected ($p < 0.05$). The results for the RT-PCR products of Ngb and β-actin are shown in figure 2a, and the relative abundance ratio of Ngb to β-actin is shown in figure 2b.

Ngb protein was detected in the three groups by Western blot (fig. 2c) using a goat polyclonal antibody (Santa Cruz Biotechnology). A faint band of about 17 kDa was seen in group H_0 while a more distinct band of Ngb was seen at the corresponding location in groups H_1 and H_4. The relative abundance value of Ngb in each group was calculated by the OD ratio of Ngb to β-actin. The relative abundance value of Ngb in group H_0 is 0.25 ± 0.09. The relative abundance value of Ngb protein increased in group H_1 (0.66 ± 0.17) and markedly increased in group H_4 (2.40 ± 0.45). A significant difference in the relative abundance value of Ngb was seen among the three groups (<0.01, n = 6) (fig. 2d).

Tolerance of Ngb-Transformed SH-SY5Y Cells to Hypoxia

When normal SH-SY5Y cells were exposed to 1% oxygen, the number of dead cells was shown to increase by Hoechst and PI staining as the exposure duration lasted for 6 h. Recombinant pEGFP-Ngb and vector pEGFP-N1 were then, respectively, transfected into cell SH-SY5Y. No apparent PI-stained and Hoechst-scattering cells were seen in most pEGFP-Ngb transformed SH-SY5Y cells after the Ngb-transformed SH-SY5Y cells were exposed to 1% oxygen for 6 h: 67.3% of them remained alive during and after exposure to hypoxia, whereas 83.2% of cells transfected with pEGFP-N1 were dead (fig. 3a–c). The cells transfected with pEGFP-Ngb or pEGFP-N1 showed different Ngb expression levels and different caspase-3/7 activity. Caspase activity decreased with increasing Ngb expression (fig. 4, 5).

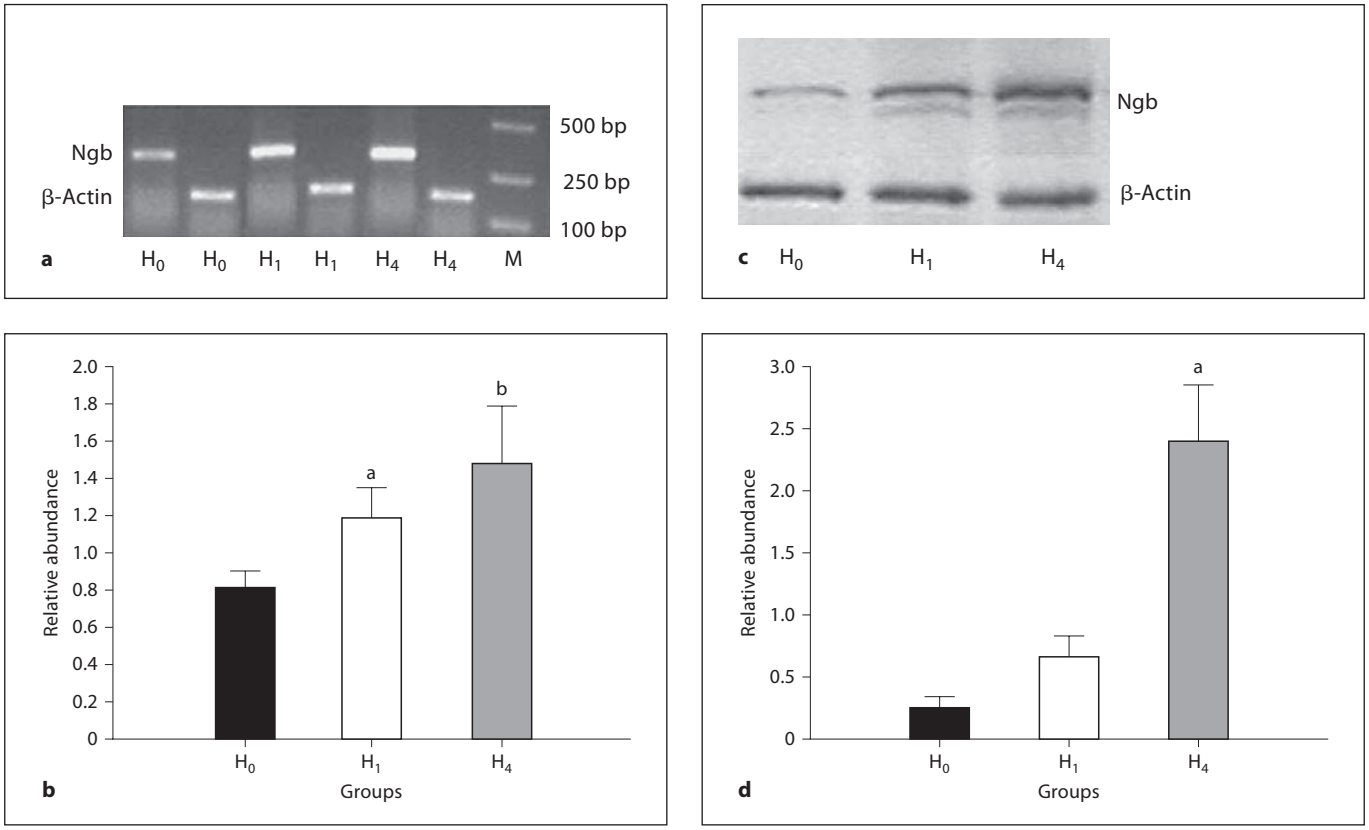
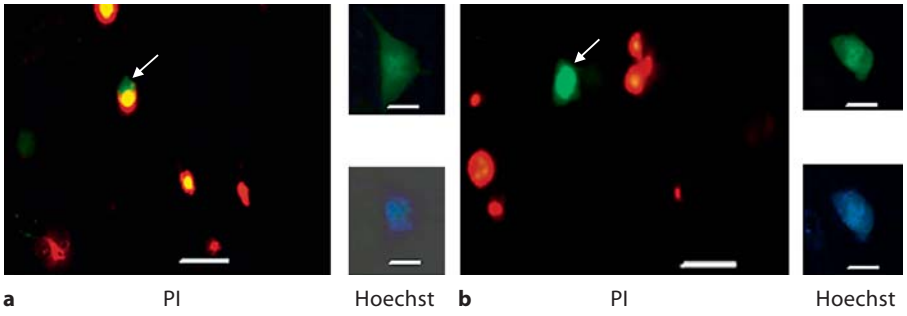


Fig. 2. Expression of Ngb in the cerebral cortex. **a** RT-PCR analysis of Ngb mRNA expression in mouse cerebral cortex; β-actin was used as control (n = 6). **b** Ratio of Ngb mRNA to β-actin mRNA in the H_0, H_1, and H_4 groups (n = 6, [a] $p < 0.05$ vs. group H_0; [b] $p < 0.05$ vs. groups H_0 and H_1). **c** Western blot analysis of Ngb protein expression in mouse cerebral cortex. **d** Ratio of Ngb protein to β-actin protein in the H_0, H_1, and H_4 groups (n = 6, [a] $p < 0.01$ vs. groups H_0 and H_1.

Discussion

Oxygen is essential for life processes such as oxidative phosphorylation, with oxygen serving as an electron acceptor and producing ATP. It maintains intracellular environmental homeostasis through ATP-dependent pumping systems. Lacking oxygen, neurons are injured mainly because of ATP insufficiency [25]. I/HPC (or HPC) may protect neurons against damage under hypoxic conditions. Endogenous cellular adaptation triggered by HPC through an unclear mechanism is believed to be responsible for it. One prominent feature of cellular adaptation to hypoxia is increased expression of the new proteins contributing to neuroprotection [9]. Some of these proteins may protect neurons against hypoxia injury by decreasing the demand for oxygen and increasing oxygen supply. Lowering metabolic demand for oxygen is generally considered effective [26]. Our previous studies showed that gas/energy metabolism in the brain could be decreased by acute and repeated exposure to hypoxia in mice [19]. During exposure, some oxygen/supply/enhancing molecules which were upregulated such as VEGF can improve hypoxia tolerance [7]. Sun et al. [12] reported that Ngb expression is increased by neuronal hypoxia in vitro, and in vivo focal cerebral ischemia and neuronal survival after hypoxia reduced by Ngb expression are inhibited by an antisense oligodeoxynucleotide, but enhanced by Ngb overexpression. Thus, Ngb was induced by neuronal hypoxia and cerebral ischemia and could protect neurons against hypoxia. Similarly, transgenic mice overexpressing Ngb in multiple body tissues were resistant to cerebral and myocardial ischemia [27]. We hypothesized that Ngb might play a neuroprotective role in HPC mice because the protein was mainly expressed in neuronal tissue (brain and retina), has a relatively high affinity for oxygen (P_{50} about 1 mm Hg at 37°C), and



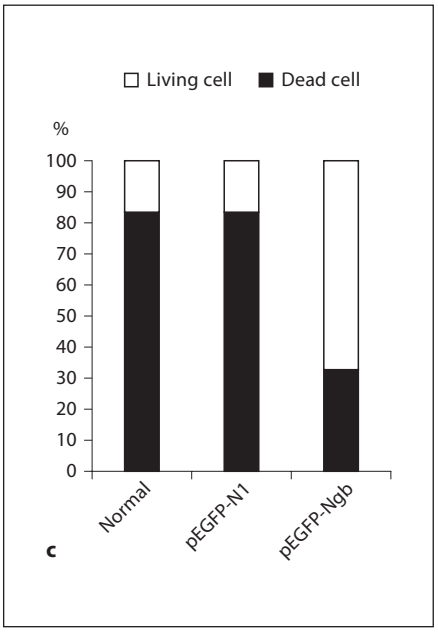


Fig. 3. Effect of Ngb overexpression on cell death. **a** pEGFP-N1- and **b** pEGFP-Ngb-transfected SH-SY5Y cells exposed to 1% oxygen for 6 h. The arrow in **b** shows a living pEGFP-Ngb-transfected cell stained by Hoechst, but not PI, and the arrow in **a** shows a dead pEGFP-N1-transfected cell stained by both Hoechst and PI (PI: bar = 75 μm, Hoechst: bar = 25 μm). **c** Percentage of living and dead SH-SY5Y cells transfected with pEGFP-Ngb, or pEGFP-N1, or without plasmid. After 6 h of hypoxic injury, cells were stained with PI/Hoechst, and the survival ratio of cells transfected with pEGFP-Ngb and pEGFP-N1 was measured as the ratio of cells carrying PI-unstained nuclei and GFP to cells with GFP; the survival ratio of cells not transfected (control) was measured as the ratio of cells with PI-unstained nuclei to all cells (each about 2,000 cells).

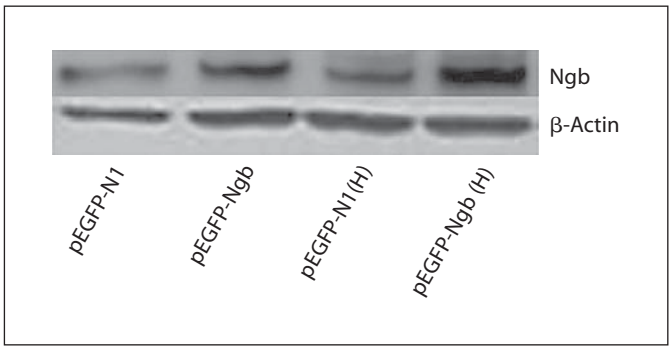


Fig. 4. Western blot analysis of the expression of Ngb protein in SH-SY5Y cells transfected with pEGFP-N1 or pEGFP-Ngb. Cells were exposed to hypoxia (1% oxygen for 6 h, H) or not.

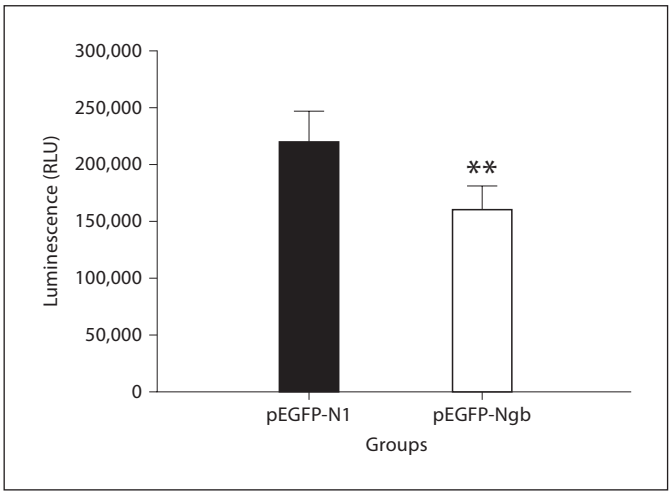


Fig. 5. Effect of Ngb overexpression (pEGFP-Ngb) on caspase 3/7 activity SH-SY5Y cells under hypoxia (1% oxygen for 6 h). RLU = Relative light units. n = 8; ** $p < 0.05$ vs. pEGFP-N1 group.

could prevent hypoxia/ischemia injury in neurons in vivo and in vitro [12, 27, 28].

Ngb is a member of the globin superfamily and is mainly expressed in the vertebrate brain and retina. Many researchers have reported increases in Ngb that protected the brain against hypoxia or ischemia, but the physiological role of Ngb has not been well understood, even though several putative functional roles have been proposed. Ngb in the brain, similar to that of the retina, may bind oxygen and facilitate its delivery to mitochondria [29]. However, Ngb is present at a fairly low concentration in the brain (less than 0.01% of the protein content), compared with myoglobin in the heart (about 0.2 mM) [30]. Because of the relatively high concentration of myoglobin in the heart and its role in facilitating the storage, transport, and intracellular diffusion of O_2, the ma-

jor function of Ngb in the brain was assumed to be different from that of myoglobin because of its very low concentration in the brain [18]. However, Reuss et al. [15] showed that Ngb mRNA was expressed in neuronal somata whose volume makes up only a small part of the total brain volume, and these authors suggested that local concentrations of Ngb within nerve cells can be quite high. Therefore, Ngb may greatly facilitate storage, transport, and intracellular diffusion of oxygen. As a heme protein, Ngb may bind with and scavenge nitric oxide. Ngb may be a hypoxia sensor or, as suggested recently, a sensor of the ratio of oxygen and nitric oxide levels [31]. Ngb may also be involved in MAPK pathways of phosphotidylinositol 3-kinase activation by interacting with G protein-α and promoting cell survival in the brain under conditions of oxidative stress such as ischemia and reperfusion [17].

Some studies have investigated whether Ngb is upregulated in response to ischemia or hypoxia [12, 32–34]. Ngb mRNA and protein can be detected under normoxic and hypoxic conditions in the brain, and Ngb mRNA and protein were up-regulated under hypoxic conditions [12, 32]. In contrast with these data, other studies showed that Ngb mRNA was not regulated by exposure to hypoxia [33]. This discrepancy could be explained by the different hypoxic stimuli causing divergent effects on the Ngb gene. Indeed, this explanation was supported by the research report of Hundahl et al. [34], which showed that Ngb is upregulated only in the acute phase of hypoxia. To our knowledge, the change in Ngb in HPC mice has not been reported under acute repeated hypoxic conditions. We found that tolerance to hypoxia increased in mice acutely and repetitively exposed to autoprogressive hypoxia with increased Ngb mRNA and protein levels. This finding may indicate a functional role of Ngb in contributing to tolerance to hypoxia and to its protective effect during the acute and repeated hypoxia.

It remains uncertain whether Ngb is actually controlled by the major regulator of cellular hypoxia response known as hypoxia-inducible factor-1 (HIF-1). It has been suggested that potential hypoxia responsive elements (HREs) were found upstream of the Ngb gene in mice [12]. We found that Ngb mRNA and protein expressed in the H_0, H_1 and H_4 groups were increased in group H_1 and markedly increased in group H4. Our previous study revealed that HIF-1α protein level and HIF-1 DNA binding activities increased in group H_1 mice and markedly increased in group H_4 mice as well [7]. Changes in Ngb mRNA and protein level paralleling HIF-1α protein level and HIF-1 DNA binding activity could be explained by the 5′-untranslated region of Ngb containing one or several copies of HREs. However, a comparative study of mouse and human Ngb genes did not show any conservation of HREs that would permit induction by HIF-1 [14]. Transcription factors other than HIF-1 may also regulate Ngb under conditions of hypoxia in our model. Fordel et al. [35] speculated that HIF-2 might play an important role in inducing Ngb expression.

To further test the protective effect of Ngb in acute hypoxia, we transfected full-length mouse pEGFP-Ngb expression vectors into human SH-SY5Y neuroblastoma cells, which are often used as a model in hypoxia and anoxia experiments [36]. Cells overexpressing Ngb showed increased tolerance to severe hypoxia reflected by decreased cell death and caspase-3/7 activity. Fordel et al. [21] showed that under anoxic conditions SH-SY5Y cells grew very similarly to those under normoxia, and that oxygen and glucose deprivation was lethal to SH-SY5Y cells. By contrast, we observed significant death of SH-SY5Y cells after short exposure (6 h) to 1% oxygen without glucose deprivation. The discrepancy may indicate that a tiny difference in experimental conditions can lead to significant differences in results and underscores the limitations of in vitro models. Although there exists a difference in experimental treatment, the results reported here were similar to those obtained by Fordel et al. [21], who also demonstrated an increased ratio of live cells expressing Ngb after a period of oxygen and glucose deprivation (16/24 h). Although our study largely supports that Ngb could increase tolerance to acute hypoxia, other authors have found no significant changes in Ngb expression in the adult murine brain in response to chronic 10% and acute 7.6% oxygen exposure [33, 34]. Therefore, Ngb might have diverse functional roles under different conditions of hypoxia. Future use of other biologic technologies, including transgenic strategies [27], would be useful to further define the function of Ngb in the brain in hypoxia/ischemia insults. Our results suggest the existence of endogenous neuroprotective mechanisms of Ngb. Understanding how Ngb confers neuronal protection might help improve the treatment of hypoxic/ischemic diseases.

Acknowledgments

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