Neuroglobin expression in rats after traumatic brain injury

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Abstract
In this study, we used a rat model of severe closed traumatic brain injury to explore the relationship between neuroglobin, brain injury and neuronal apoptosis. Real-time PCR showed that neuroglobin mRNA expression rapidly increased in the rat cerebral cortex, and peaked at 30 minutes and 48 hours following traumatic brain injury. Immunohistochemical staining demonstrated that neuroglobin expression increased and remained high 2 hours to 5 days following injury. The rate of increase in the apoptosis-related Bax/Bcl-2 ratio greatly decreased between 30 minutes and 1 hour as well as between 48 and 72 hours post injury. Expression of neuroglobin and the anti-apoptotic factor Bcl-2 greatly increased, while that of the pro-apoptotic factor decreased, in the cerebral cortex post severe closed traumatic brain injury. It suggests that neuroglobin might protect neurons from apoptosis after traumatic injury by regulating Bax/Bcl-2 pathway.

Key Words
neuroglobin; traumatic brain injury; neuron; apoptosis; cerebral cortex; Bax; Bcl-2; neural regeneration

Research Highlights
Neuroglobin suppressed apoptosis and regulated the ratio of Bax/Bcl-2 protein in the cerebral cortex of rats with severe closed traumatic brain injury.

Abbreviation
TBI, traumatic brain injury

INTRODUCTION
In 2000, Burmester et al published the first report of a new vertebrate globin expressed in the brain, called neuroglobin. Since then, the protein has become a major focus of neuroscience because of its high oxygen affinity. It binds oxygen reversibly and plays an important role in oxygen homeostasis in neural tissues⁴⁻⁵. A great deal of experimental research on neuroglobin has focused on its role in neuronal hypoxic and ischemic injury. Studies have demonstrated that neuronal hypoxia and cerebral ischemia induce neuroglobin expression⁴⁻⁵.
Enhanced neuroglobin expression reduces hypoxic neuronal injury in vitro, while knocking down neuroglobin expression increases ischemic brain injury in vivo. However, relatively little is known about the changes in neuroglobin expression in neurons post traumatic brain injury (TBI), and the relationship between neuroglobin and neuronal apoptosis post brain trauma is also unclear. This study was designed to examine the dynamic changes in neuroglobin expression post TBI. We explore the relationship between neuroglobin expression and neuronal apoptosis. We anticipate that this study will provide insight into the mechanisms of neuroprotection and encourage the development of novel therapeutic strategies for the treatment of TBI.

RESULTS

Quantitative analysis of experimental animals

Of the 74 TBI model rats, 20 died from severe cerebral bleeding (n = 9), skull fracture (n = 4) or respiratory depression (n = 7). The 54 living rats were equally and randomly assigned to 9 experimental groups: TBI 30 minutes, 1, 2, 6, 12, 24, 48, 72 hours and 5 days. Additional six rats were selected as sham surgery group. A total of 60 rats were included in the final analysis.

Neuroglobin gene and protein expression increased in cerebral cortex of rats after TBI with dual peaks

Neuroglobin gene expression was assessed with quantitative reverse transcription-PCR. Neuroglobin mRNA expression increased quickly after TBI with dual peaks. 30 minutes post injury, the expression of neuroglobin mRNA increased noticeably, reaching its first peak. Then it decreased gradually until 6 hours post injury, recovering to the normal level. At 12 hours post injury, neuroglobin mRNA levels started increasing again and reached a second peak 48 hours post injury. Subsequently, mRNA levels decreased gradually, but remained high 5 days post injury. Statistical analyses showed significant differences in group comparison between all the TBI injury groups and the sham surgery group (P < 0.001; Figure 1).

Immunochemistry was used to evaluate neuroglobin protein expression in the cytoplasm and synapses of cells in the cerebral cortex. With semi-quantitative analyses, we clearly found that neuroglobin protein increased persistently in cells in the cerebral cortex post TBI (Figure 2). At 30 minutes post injury, the quantity of neuroglobin-positive cells increased. At 2 hours post injury, the number of neuroglobin-positive cells reached a peak. Thereafter, it decreased gradually. At 48 to 72 hours post injury, the number of neuroglobin-positive cells increased again (P < 0.001), and at 5 days post injury, the number of neuroglobin-positive cells was still higher than in the sham surgery group (P < 0.001).

Expression of Bcl-2 and Bax proteins in cells in the cerebral cortex of rats post TBI

Bax staining was observed in the cytoplasm of cells in the cortex. At 30 minutes post injury, the quantity of Bax-positive cells increased noticeably. At 2 hours post injury, the number of Bax-positive cells reached a peak (P < 0.001). Thereafter, it decreased gradually with a mild fluctuation 48 to 72 hours post injury (P < 0.001). Statistical analyses showed significant statistical difference between all the injury groups and the sham surgery group (Figure 3).

Strong staining for Bcl-2 was observed in the cytoplasm of neurons in the cerebral cortex. Immunochemical analyses also showed persistent increases in Bcl-2-positive neurons in the rat brain post TBI. At 30 minutes post injury, the quantity of Bcl-2-positive neurons increased. At 2 hours post injury, it reached a peak level. After that, the number of Bcl-2-positive neurons decreased gradually. At 24 hours post injury, the number of Bcl-2-positive neurons increased for the second time (P < 0.001). At 5 days post injury, the number of Bcl-2-positive neurons was comparable to that in the sham surgery group (P = 0.088; Figure 4).

As shown in Figure 5, the ratio of Bax/Bcl-2 post TBI exhibited biphasic changes. After injury, the Bax/Bcl-2 ratio increased sharply and reached its peak at 6 hours post injury. At 30 minutes to 1 hour post injury, the rate of increase in the Bax/Bcl-2 ratio decreased significantly. At 48 hours post injury, the Bax/Bcl-2 ratio increased again and reached a second peak at 72 hours post injury (P < 0.001). During this period (from 48 to 72 hours post injury), the rate of increase in the Bax/Bcl-2 ratio decreased again (P < 0.001).
Figure 2   Traumatic brain injury (TBI)-induced changes in neuroglobin (NGB) protein expression in the cerebral cortex (immunocytochemical staining).

Strong immunocytochemical staining for neuroglobin was observed in the cytoplasm and synapses of cells in the cerebral cortex (A, Scale bar: 25 µm; TBI 30 minutes). Compared with the sham surgery group (Sham) (B; scale bar: 50 µm), the number of neuroglobin-positive neurons was significantly increased in the rat cerebral cortex 30 minutes to 5 days after TBI, with a peak at 2 hours (C, Scale bar: 50 µm; TBI 2 hours) post TBI.

(D)  aP < 0.001, vs. sham. Data were expressed as mean ± SD, n = 6. Statistical analyses were performed using one-way analysis of variance test and least significant difference t-test. HPF: High power field; h: hour; d: day; min: minute.

Figure 3   Traumatic brain injury (TBI)-induced changes in Bax protein expression (immunocytochemical staining).

Strong immunocytochemical staining for Bax was observed in the cytoplasm and synapses of neurons in the cerebral cortex (A, Scale bar: 25 µm; TBI 30 minutes). Compared with the sham surgery group (Sham) (B; scale bar: 50 µm), the number of Bax-positive neurons was significantly increased in rat cerebral cortex 30 minutes to 5 days after TBI, with a peak at 2 hours (C, Scale bar: 50 µm; TBI 2 hours) post TBI.

(D)  aP < 0.001, vs. sham. Data were expressed as mean ± SD, n = 6. Statistical analyses were performed using one-way analysis of variance test and least significant difference t-test. HPF: High power field; h: hour; d: day; min: minute.

Figure 4   Traumatic brain injury (TBI)-induced changes in Bcl-2 protein expression (immunocytochemical staining).

Strong immunocytochemical staining for Bcl-2 was observed in the cytoplasm and synapses of neurons in the cerebral cortex (A, Scale bar: 25 µm; TBI 30 minutes). Compared with the sham surgery group (Sham) (B; scale bar: 50 µm), the number of Bcl-2-positive neurons was significantly increased in rat cerebral cortex 30 minutes to 5 days after TBI (C, Scale bar: 50 µm; TBI 2 hours).

(D)  aP < 0.001, bP < 0.05, vs. sham. Data were expressed as mean ± SD, n = 6. Statistical analyses were performed using one-way analysis of variance test and least significant difference t-test. HPF: High power field; h: hour; d: day; min: minute.
DISCUSSION

Neuroglobin has become a hot focus of neuroscience research. Khan et al[6] reported that neuroglobin-overexpressing transgenic mice were resistant to cerebral and myocardial ischemia. Compared with wild-type littermates, the volumes of cerebral and myocardial infarcts were reduced by 30% and 25%, respectively, in the transgenic mice. These results suggest that neuroglobin is a potential target for the diagnosis and treatment of neurodegenerative diseases. However, the role of neuroglobin in TBI and in neuronal cell death post TBI is unclear. Major questions remained. Does neuroglobin take part in the protective response to TBI? Can neuroglobin regulate neuronal cell death post injury? To tackle these questions, we set out to explore the relationship between neuroglobin, TBI and neuronal apoptosis.

Neuroglobin and TBI

In the present study, we found that neuroglobin gene and protein expression were activated and exhibited a dual peak pattern post TBI. The first peak occurred at the ultra-early phase post-injury (30 minutes post injury), which might be a protective response to traumatic stress. Mechanical injury might directly impair the function of mitochondria, inactivate the Na⁺/K⁺-ATPase, and cause oxidative stress to neurons, leading to disruption of mitochondrial energy metabolism and compromise membrane integrity[7]. All these might induce compensatory up-regulation of neuroglobin expression to supply oxygen. A previous study[8] showed that at 1 hour post injury, the apparent diffusion coefficient of brain tissue increased strikingly, corresponding to post-injury vasogenic brain edema and increased extracellular fluid volume due to post-injury transient blood brain barrier opening.

With the development of vasogenic brain edema, cerebral blood flow decreases and leads to cerebral hypoperfusion. The resulting ischemia and hypoxia might contribute to the up-regulation of neuroglobin in the brain post-injury. With its high oxygen affinity, neuroglobin might supply more oxygen to brain tissue subjected to hypoxia and ischemia. Unfortunately, the compensatory ability of neuroglobin is limited. Kimelberg[9] revealed that the ratio of glial cells to neurons was approximately 20:1 in humans and 10:1 in rats. Although there is high expression of neuroglobin post injury, it does not persist. Consequently, it may not satisfy the oxygen consumption requirement of the large number of glial cells. Injury to glial cells is a pivotal factor in cytotoxic brain edema.

Interestingly, 12 hours post injury, neuroglobin mRNA transcription increased, with an additional peak at 48 hours post injury. Correspondingly, neuroglobin protein expression also increased 48 to 72 hours after TBI. Obviously, these changes were not the result of primary mechanically traumatic injury, but might be related to edema and ischemic injury to neurons post TBI. Studies revealed that cytotoxic brain edema is initiated 45 minutes post TBI, subsequent to vasogenic edema, and is the major cause of secondary diffuse brain swelling[8, 10]. A pathophysiological study using Marmarou’s model indicated that secondary brain swelling is initiated at 6 hours, peaks at 24 hours and then subsides gradually.
over several days post injury[11]. Secondary diffuse brain swelling inevitably results in reduced cerebral perfusion. Sun et al[10] have suggested that neuroglobin is a hypoxia-inducible neuroprotective factor in hypoxic-ischemic injury. They found that factors that enhance the expression of hypoxia-inducible genes, such as CoCl$_2$ and deferoxamine, increased neuroglobin expression. Gene analysis showed that the 5'-untranslated region of neuroglobin contains several copies of the hypoxia-inducible factor-1 binding sequence, 5'-RCGTT-3', located upstream of the transcription initiation site[13]. This suggests the specific involvement of the hypoxia signaling pathway in neuroglobin induction.

The mechanisms underlying the neuroprotection afforded by neuroglobin may include protection against oxidative stress after TBI[12-14], protection against neuronal death via G protein signaling[14], parenchymatous and vasomotor protective effects resulting from enhancement of endothelial nitric oxide synthase expression in vascular endothelial cells[6], and improvement of mitochondrial function during hypoxia[15].

Based on the evidence above, we consider that the up-regulation of neuroglobin expression might be a protective response to secondary ischemic-hypoxic insult and oxidative stress.

**Neuroglobin and neuronal apoptosis post TBI**

Neuronal death, including necrosis and apoptosis, after TBI is a major cause of neurological deficits and mortality[16-17]. The Bcl-2 family is one of the two main conserved protein families involved in cellular apoptosis. Bax and Bcl-2 are pro- and anti-apoptotic members of the Bcl-2 family, respectively. In homeostatic conditions, the anti-apoptotic members (Bcl-2) maintain mitochondrial integrity by preventing the pro-apoptotic members (Bax and Bak) from causing mitochondrial outer membrane permeability changes. In this regard, modulation of the Bax/Bcl-2 ratio might affect mitochondrial outer membrane permeability. Pavlov et al[18] reported that cells overexpressing Bcl-2 were less vulnerable to the creation of the large pore at the outer membrane, presumably because of reduced presence of Bax at this location.

The present study found that Bcl-2 expression in the cerebral cortex increased and remained high up to 5 days post injury, which was similar to that of neuroglobin, suggesting that both neuroglobin and Bcl-2 might serve as anti-apoptotic factors that are sensitive to traumatic stress and secondary hypoxic-ischemic insults. Interestingly, although the Bax/Bcl-2 ratio increased sharply after injury, the rate of increase of this ratio decreased while neuroglobin expression increased sharply between 30 minutes and 1 hour post TBI. Coincidentally, 48 to 72 hours post injury, a similar phenomenon occurred. These observations suggest that neuroglobin might play an important anti-apoptotic role by regulating the Bax/Bcl-2 ratio post TBI. Accumulating evidence indicates that the mitochondrial has a pivotal role in post traumatic neuronal death by integrating numerous noxious signals responsible for structure and functional damage. Most of these events disrupt permeability of the mitochondrial membrane, which therefore may represent a potential target for new therapeutic strategies[7].

In combination with previous work, our present results help clarify the role of neuroglobin in TBI and neuronal apoptosis post brain trauma. In the ultra-early and acute phases post injury, neuroglobin expression increases in response to acute traumatic stress and secondary ischemic-hypoxic insults, respectively, which suggests that neuroglobin might take part in the protective response to TBI.

In the various stages of injury, neuroglobin protects neurons in different ways. In the ultra-early stage of injury, neuroglobin expression increases to enhance oxygen supply and maintain mitochondrial membrane stability. In contrast, in the acute phase of injury, neuroglobin acts as an oxidative stress sensor. Furthermore, neuroglobin might play an anti-apoptotic role by regulating the Bax/Bcl-2 ratio post TBI. Thus, neuroglobin is a crucial neuroprotectant following brain injury.

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**MATERIALS AND METHODS**

**Design**
This is a randomized, controlled animal experiment.

**Time and setting**
All experiments were performed in the Neurosurgical Laboratory and Gerontological Research Institute of General Hospital of Chinese PLA from August 2006 to March 2007.

**Materials**
Adult male Sprague-Dawley rats, weighing 350–400 g, were bought from the Laboratory Animal Center of the PLA Military Medical Science Academy (license No. SCXK-2002-001). All experiments were performed in accordance with the **Guidance Suggestions for the Care and Use of Laboratory Animals**, formulated by the Ministry of Science and Technology of China[19].
Methods

Surgical procedure

Rats were used to study the expression of neuroglobin after severe closed TBI using Marmarou’s weight-drop model[20]. The rats were subjected to TBI under transient 3% isoflurane anesthesia (2 minutes). After a midline skull incision, a metallic disk, 10-mm in diameter and 3-mm thick, designed to protect against skull fracture, was placed directly onto the clear part of the skull. A cylindrical metallic 450 g weight was dropped from a height of 1.7 m through a metal tube onto the disk. After impact, the skin incision was sutured. The rats were allowed to return to their plexiglass cages to recover from anesthesia. The animals were individually housed and allowed free access to food and water. Rats in the sham surgery group received the same anesthesia and midline skull incision as the experimental ones except for injury, and their brains were treated as above.

Immunocytochemistry

At 30 minutes, and 1, 2, 6, 12, 24, 48, 72 hours and 5 days post injury, rats were re-anesthetized. The brains were removed quickly and separated into two parts from the coronal suture. A standard block, 1.0 to 2.0 mm posterior to the bregma, was obtained and embedded in paraffin. A series of consecutive sections (5 μm in thickness) were prepared from the paraffin-embedded tissues for immunocytochemical detection. After dewaxing and rehydration, sections were washed three times with 0.01 M PBS. Endogenous peroxidase activity was inactivated by incubation in 3% H,O, for 10 minutes. After washing in PBS, the sections were incubated in PBS containing 5% bovine serum for 1 hour at room temperature to prevent nonspecific immunoreactions. The sections were then incubated with a rabbit anti-neuroglobin polyclonal antibody (working dilution 1:200), a rabbit anti-Bax polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a rabbit anti-Bcl-2 polyclonal antibody (1:200; Santa Cruz Biotechnology) at 4°C overnight[21]. Sections were then washed in PBS and stained using ChemMate™ EnVision™ Detection Kit, Peroxidase/3,3′-diaminobenzidine (DAKO, Carpinteria, CA, USA), according to the manufacturer’s instructions. Immunoreactivity was observed under a light microscope (Olympus, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was isolated from rat brain cortex tissue using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. cDNA synthesis was performed using ReverTra Ace-α-TM first strand kit (Toyobo, Osaka, Japan), according to the manufacturer’s instructions. Quantitative reverse transcription-PCRs were performed using 100 ng cDNA (RNA equivalent) as a template in a 20 μL reaction. The primers and probes for rat neuroglobin and GAPDH are as follows.

| Primer   | Neuroglobin | GAPDH  |
|----------|-------------|--------|
| Forward: | 5’-CCAAAGC  | Forward: 5’-CAAGT |
| TAG AGG ACC TGT | CAA CGG CAC AGT |
| Reverse: | 5’-GGTCGGG  | Reverse: 5’-TGGTGA |
| ACC CAG GCA CTT-3’ | AGA GGC CAG TAG |
| Probe    | FAM-CACCTA CTC TCG | FAM-TCT TCC AGG |
| AGA AGG AGG TGA | AGC GAG ATC CCG |
| GCC T-TAMRA | CTA AC-TAMRA |

GAPDH was selected as internal control. The TaqMan Universal PCR Master Mix was from Applied Biosystems and amplifications were performed using an ABI Prism 7300 instrument (Applied Biosystems, Foster, CA, USA).

Data analysis

Images of the stained sections were captured with an Olympus BX-60 microscope (Olympus, Tokyo, Japan) connected to a CCD camera system (Olympus DP10). Cerebral cortex of each section was analyzed. Numbers of neuroglobin-positive, Bax-positive and Bcl-2-positive neurons per visual field (2.5 mm × 1.8 mm) were calculated using Image J software (NIH, Bethesda, MD, USA). The ratio of Bax/Bcl-2 was calculated with the mean Bax- and Bcl-2-positive neuron numbers in each group. On quantitative reverse transcription-PCR, copies of neuroglobin-cDNA and GAPDH-cDNA were calculated with ABI 7300 System SDS software (Applied Biosystems). The quantity of neuroglobin cDNA was calibrated using the internal control (GAPDH) as follows: DNA_{neuroglobin}/DNA_{GAPDH}. Numbers of neuroglobin-positive, Bax-positive, Bcl-2-positive neurons and the quantity of neuroglobin mRNA were expressed as mean ± SD. Statistical analyses were performed using one-way analysis of variance and least significant difference t-test. Statistical analyses were performed with SPSS 11.0 software (SPSS, Chicago, IL, USA).

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Conflicts of interest: None declared.

Ethical approval: This study was approved by the Medical Ethics Committee, General Hospital of Chinese PLA.

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