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Plenary Article

Neuroglobin overexpression improves sensorimotor outcomes in a mouse model of traumatic brain injury

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HIGHLIGHTS

- Sensorimotor outcomes were improved post-TBI in transgenic neuroglobin mice.
- Neuroglobin gene expression increased 7 days post-TBI in wild-type mice.
- Neuroglobin protein is present throughout the brain of transgenic neuroglobin mice.
- Increasing neuroglobin early after TBI may improve outcomes.

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ABSTRACT

There is a significant need for novel treatments that will improve traumatic brain injury (TBI) outcomes. One potential neuroprotective mechanism is to increase oxygen binding proteins such as neuroglobin. Neuroglobin has a high affinity for oxygen, is an effective free radical scavenger, and is neuroprotective within the brain following hypoxia and ischemia. The purpose of this study was to determine whether neuroglobin overexpression improves sensorimotor outcomes following TBI in transgenic neuroglobin overexpressing (NGB) mice. Additional study aims were to determine if and when an endogenous neuroglobin response occurred following TBI in wild-type (WT) mice, and in what brain regions and cell types the response occurred. Controlled cortical impact (CCI) was performed in adult (5 month) C57/BL6 WT mice, and NGB mice constitutively overexpressing neuroglobin via the chicken beta actin promoter coupled with the cytomegalovirus distal enhancer. The gridwalk task was used for sensorimotor testing of both WT and NGB mice, prior to injury, and at 2, 3, and 7 days post-TBI. NGB mice displayed significant reductions in the average number of foot faults per minute walking at 2, 3, and 7 days post-TBI when compared to WT mice at each time point. Neuroglobin mRNA expression was assessed in the injured cortex of WT mice prior to injury, and at 1, 3, 7, and 14 days post-TBI using quantitative real time polymerase chain reaction (qRT-PCR). Neuroglobin mRNA was significantly increased at 7 days post-TBI. Immunostaining showed neuroglobin primarily localized to neurons and glial cells in the injured cortex and ipsilateral hippocampus of WT mice, while neuroglobin was present in all brain regions of NGB mice at 7 days post-TBI. These results showed that overexpression of neuroglobin reduced sensorimotor deficits following TBI, and that an endogenous increase in neuroglobin expression occurs during the subacute period. Increasing neuroglobin expression through novel therapeutic interventions during the acute period after TBI may improve recovery.

1. Introduction

Traumatic brain injury (TBI) is a major public health concern with severe consequences that include long-term loss of function, profound disability, and death. Accordingly, there is a significant need for novel treatments that will improve TBI outcomes. Improved TBI outcomes may be achieved through treatments that target globin genes (e.g., hemoglobin, myoglobin, cytoglobin, and

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neuroglobin). The globin gene family translates proteins that are vital for binding oxygen within various body tissues. Following upregulation, globin proteins also play a neuroprotective role in the brain [12,26]. The work presented here focuses on neuroglobin, one of the most recently characterized members of the globin gene family. Neuroglobin was first described in 2000 as a protein with a high affinity for binding oxygen that is present in the brain [4]. In addition to its ability to bind oxygen, neuroglobin also functions as an antioxidant and free radical scavenger [18]. Neuroglobin is expressed throughout the central and peripheral nervous systems of vertebrates [4,14,23,28]. The primary sites of neuroglobin expression are the limbic system and cerebral cortex [14]. Neuron survival is enhanced via an increased endogenous production of neuroglobin following hypoxia and ischemia [13]. Therefore, neuroglobin has been suggested to be neuroprotective in experimental models of stroke [17,27] and in TBI [22,28].

Previous research has demonstrated that transgenic neuroglobin overexpressing (NGB) mice display significantly reduced cortical lesion volumes in comparison to wild-type (WT) mice 21 days after TBI [28]. Furthermore, overexpression of neuroglobin in Wistar rats significantly reduced neuron necrosis and apoptosis after TBI in comparison to controls [22]. In humans, genetic polymorphisms in neuroglobin have been shown to positively influence recovery in TBI patients [6]. The present study was designed to determine whether NGB mice displayed improved sensorimotor recovery after TBI in comparison to WT mice. In addition, related study objectives were to determine if and when an endogenous neuroglobin response occurred following TBI, and in what brain regions and cell types the response occurred. It was hypothesized that overexpression of neuroglobin would reduce sensorimotor deficits following TBI in NGB mice when compared to WT mice.

2. Methods

Adult (5 month) male C57/BL6 WT, and transgenic (B6.Cg-Tg(CAG-Ngb,-EGFP)1Dgrn/J; The Jackson Laboratory, ME) NGB mice were used for this study. NGB mice overexpressed neuroglobin via the chicken beta actin promoter coupled with the cytomegalovirus distal enhancer. Animal care and use procedures were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and conducted according to the Institute of Laboratory Animal Research guidelines.

A controlled cortical impact (CCI) was used to induce a moderate TBI as previously described [19]. The CCI injury model targeted the primary and secondary motor and primary somatosensory cortical regions. CCI reproduces many of the features of brain injuries including motor deficits, memory loss, and neuron loss [7]. Responses following controlled cortical impact include glial activation, increased cytokine expression, blood brain barrier disruption, and neuron loss [20,21]. The CCI procedure was performed while mice were anaesthetized with 2.5% isoflurane in 30% O_2 and air. Briefly, a midline incision was made to expose the skull. A Wild operating microscope was used to view the skull at 60× magnification, and a 3.5 mm diameter craniotomy was performed with a dental drill on the right side of the mid-sagittal suture, with the following coordinates: Anterior–Posterior (AP) coordinates centered at bregma, 2.5 mm lateral to the midline. Drilling was performed in a careful manner to leave the dura intact, and avoid blood vessels traveling through the superior sagittal sinus. Once the brain was exposed, the cortex was impacted (3 mm diameter injury tip centered at 2.5 mm lateral to bregma) with a custom made, electronically controlled, CCI injury device (P01-23×80, LinMot Inc., Zurich, Switzerland). CCI impact parameters were as follows: impact velocity (1.5 m/s), impact depth (1.0 mm), and contact time (85 ms). These impact parameters have resulted in a low (5%) mortality rate. Following impact, the skin was closed with a sterile suture. The duration of each CCI procedure was approximately 30 min.

Sensorimotor deficits (i.e., foot faults) were evaluated using the gridwalk task as previously described [19]. 6 WT and 7 NGB mice were tested on the gridwalk at four time points (1 day pre-TBI, 2 days post-TBI, 3 days post-TBI, and 7 days post-TBI). Mice were given one trial per day, at the same time of day, and scored by an observer who was unaware of the groups. Mice were placed on a grid area measuring 32 cm × 20 cm × 50 cm with 11 mm × 11 mm diameter openings. Mice were allowed to walk on the grid for 5 min, during which their total walking time was measured, and the number of foot faults for each foot counted. Any step passing through a grid hole was considered a foot fault. Foot fault data was normalized by dividing the total counted foot faults by the total time spent walking to obtain a measure of foot faults per minute of walking. Data were expressed as the mean number of foot faults per minute ± SEM. Two-way repeated measures ANOVA with a Fisher's LSD post hoc for multiple comparisons was used for analyzing significant differences in mean number of foot faults per minute at each time-point.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was utilized for our gene expression time course study as previously described [1]. 6 WT mice were sacrificed via intraperitoneal injection of beuthanasia prior to TBI, and at 1 day, 3 days, 7 days, and 14 days post-TBI. Animals were decapitated at each time point, and whole brains were removed and stored in RNA later (Ambion, Austin, TX) for 24 h at 4 °C to preserve the RNA. After the 24 h incubation period, whole brains were dissected in order to collect the injured cortex (right hemisphere). Samples were homogenized and total RNA was extracted, purified, and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Complementary DNA (cDNA) was synthesized by using 1 μg total RNA from each sample and random hexamers in a Taqman reverse transcription reaction (Applied Biosystems, Foster City, CA, USA). The cDNA and gene specific primers (see Supplementary Materials for primer sequences) were subjected to PCR amplification using an Applied Biosystems 7300 Real Time PCR System Machine (Applied Biosystems, Foster City, CA). PCR reactions were conducted in duplicate, and GAPDH was used as the housekeeping gene. Sequence Detection Software 2.0 (Applied Biosystems, Foster City, CA) was used to collect and analyze data. Neuroglobin gene expression was calculated relative to GAPDH by the ΔΔCt method, and reported in accordance with The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [5]. A two-way ANOVA was used for analyzing significant differences in mRNA expression between time points. Fisher's LSD was used for post hoc comparisons.

Immunohistochemistry was conducted to assess the location of specific cell types expressing neuroglobin as previously described [20]. 4 WT and 4 NGB mice were sacrificed by transcardial perfusion under anesthesia (isoflurane) at 7 days post-TBI. Brains were fixed by perfusion with 4% buffered formaldehyde, dissected, postfixed overnight, cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.2), and sectioned through the coronal plane at 50 μm on a microtome. Sections were rinsed in PBS with 0.4 mg/ml glycine for 5 min, permeabilized with 0.2% Triton X-100, and quenched for peroxidase with 3% H_2O_2. Sections were incubated overnight at 4 °C with the primary antibody (neuroglobin rabbit anti-mouse 1:200, Sigma AB-N7162). After overnight incubation, all sections were rinsed with PBS and incubated with a biotinylated secondary antibody (1:500 goat anti-rabbit; Vector Labs BA-1000) for 2 h at room temperature. An ABC Elite kit (Vector Labs, Burlingame, CA) was used to produce the immunoperoxidase reaction. Color was visualized using diaminobenzidine (DAB) solution (Vector Labs, Burlingame, CA). A Nikon inverted stage microscope was used to