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Arginase overexpression in neurons and its effect on traumatic brain injury

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ABSTRACT

Arginine is a semi-essential amino acid which serves as a substrate for nitric oxide (NO) production by nitric oxide synthase (NOS) and a precursor for various metabolites including ornithine, creatine, polyamines, and agmatine. Arginase competes with nitric oxide synthase for substrate arginine to produce orthinine and urea. There is contradictory evidence in the literature on the role of nitric oxide in the pathophysiology of traumatic brain injury (TBI). These contradictory perspectives are likely due to different NOS isoforms – endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) which are expressed in the central nervous system. Of these, the role of nNOS in acute injury remains less clear. This study aimed to employ a genetic approach by overexpressing arginase isoforms specifically in neurons using a Thy-1 promoter to manipulate cell autonomous NO production in the context of TBI. The hypothesis was that increased arginase would divert arginine from pathological NO production. We generated 2 mouse lines that overexpress arginase I (a cytoplasmic enzyme) or arginase II (a mitochondrial enzyme) in neurons of FVB mice. We found that two-weeks after induction of controlled cortical injury, overexpressing arginase I but not arginase II in neurons significantly reduced contusion size and contusion index compared to wild-type (WT) mice. This study establishes enhanced neuronal arginase levels as a strategy to affect the course of TBI and provides support for the potential role of neuronal NO production in this condition.

1. Introduction

Arginine is a semi-essential amino acid that serves as a precursor for several metabolites including ornithine, agmatine, creatine, polyamines and nitric oxide (NO). These metabolites have a wide range of physiological functions including modulation of cerebral blood flow, energy production, regeneration and extracellular matrix remodeling. These processes influence outcomes following traumatic brain injury by protecting or repairing the brain. Interestingly, severe traumatic brain injury patients have reduced levels of plasma arginine and alterations in several of its downstream metabolites [1]. Administration of L-arginine post induction of controlled cortical injury in rats has been shown to increase cerebral blood flow and reduce contusion volume [2]. Studies have shown that this effect of L-arginine administration is due to increased NO production and is absent in mice deficient in endothelial nitric oxide synthase (eNOS), an enzyme that utilizes arginine to produce NO. [3, 4]

In addition to endothelial NO, several reports in the literature have implicated neuronal or glial NO in traumatic brain injury (TBI). However, the literature reveals conflicting data on whether NO has neurotoxic or neuroprotective effect post-TBI. For instance, administration of L-NAME, an inhibitor of all forms of NOS, is associated with both positive and adverse outcomes in TBI [5, 6]. Inhalation of nitric oxide post-TBI has been shown to increase cerebral blood flow (CBF) and prevent ischemia in C57/BL6 mice [7]. The same study showed that after prolonged exposure to inhaled NO, the mice had reduced lesion volume and improved neurological function. On the contrary, another study has shown that higher levels of NO metabolites in extracellular fluid adjacent to neurons, correlates with poorer survival in TBI patients [8]. Moreover, a placebo controlled randomized phase II trial in humans for nitric oxide synthase inhibitor, VAS203, resulted in better Glasgow Scale Outcomes for patients 6 months after

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administration [9]. These contradictory findings may be due in part to the spatial and temporal differences in NO production [10]. NO can be generated from arginine by three isoforms of the enzyme nitric oxide synthase (NOS), which has cell-specific expression. Endothelial NOS (eNOS) is expressed in vascular endothelium and the choroid plexus, neuronal NOS (nNOS) is expressed in neurons while inducible NOS (iNOS) is expressed in macrophages and glial cells. While multiple studies have been performed to understand the role of eNOS on CBF, the role of NO derived from nNOS is underreported and still widely debated. The aim of this study was to understand the role of arginine, and consequently NO, in neurons after TBI.

In addition to lower plasma levels of L-arginine post-TBI, multiple studies have also reported an increase in plasma arginase levels and activity [11]. Increased arginase levels and activity has also been noted in different tissues such as mesenteric arteries and mononuclear cells [12, 13]. Arginase is an enzyme that utilizes arginine as a substrate to generate ornithine and urea. It competes with NOS for utilization of arginine. Genetic modulation of arginase serves as a useful tool to manipulate arginine levels in a cell-specific manner. Therefore, we generated genetic mouse models that overexpress arginase only in neuronal cells. The rationale is that overexpression of arginase in neurons would channel arginine to ornithine and urea production, away from nitric oxide synthesis, thereby lending insight into the role neuronal nitric oxide and its effect on TBI.

2. Materials and methods

All animal experiments performed in this study followed the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

2.1. Animal models

In order to generate the animal models needed in this study, we used two different constructs. For the neuronal overexpression lines, we generated the constructs by cloning either Arginase I or Arginase II cDNA under the promoter of the neuronal Thy-1 gene. Both constructs also contain the tyrosinase gene under the control of the K14 (keratinocyte specific promoter), to detect insertion of the construct using eye color change, as well as insulators. These constructs were then injected into embryos of FVB/N mice. Founders were bred with WT FVB females to establish and maintain the lines as heterozygous transgenic mice. To confirm presence of the transgene, animals were genotyped using DNA isolated from 1 to 2 mm tail clippings. Polymerase chain reaction was performed for 30 cycles (1 min denaturing, 1 min annealing at 58° Celcius and 1 min elongation at 72° Celcius) annealing temperature for 1 min, followed by running the product on a gel. For all experimental procedures described, both male and female mice were used.

2.2. Western blotting

Expression of Arginase I or II protein was confirmed by Western blotting. Whole brain tissues from neuronal arginase overexpressing mice were used to prepare protein lysates. Brain lysates from WT mice were used for comparison. Protein lysates from liver and kidney tissues of wild-type (WT) mice were used as positive controls for Arginase I and Arginase II expression respectively. Lysates from WT brain tissues were used as negative control. Antibodies for arginase I and II were purchased from Santa Cruz Biotechnology (catalog numbers sc-18,354 and sc-20,151 respectively).

2.3. Enzyme activity assay

Arginase enzyme activity assay was performed using the technique described by Zawada et al. [14] Mouse brains were homogenized for 10 s at speed 6 in Ultra-turrax homogenizer in a buffer composed of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X and phenylmethylsufonyl fluoride protease inhibitor. Proteins were quantified using the microBCA Protein Assay Kit from Thermo Scientific Pierce (catalog number 23235). The following components were mixed for the reaction: 200 µg extracted brain protein, 50 mM arginine, in 10 mM potassium phosphate buffer (pH 8.5) for a total of 100ul. The reaction was performed at 37° Celsius for 30 min and stopped by boiling. Precipitated proteins were then spun down and the supernatant was collected. Urea was quantified by adding 150ul of primaquine/o-phtalaldehyde solution and reading at 450 nm after 30 min, in parallel with a standard curve for urea.

2.4. Amino acid measurements

Brain arginine and ornithine levels were measured in transgenic and WT mice using a Biochrom 30 HPLC amino acid analyzer. In brief 30-50ul of tissue homogenate was mixed with equal volumes of Seraprep SP100 (Pickering Laboratories) and lithium dilution buffer. Protein was precipitated by centrifugation and 10ul supernatant was injected to the analyzer. Physiological amino acid standards (Sigma-Aldridge) were used to calibrate and determine analyte concentration. Results were analyzed using EZchrom Elite software.

2.5. Surgical preparation

The mice were anesthetized with 5% isoflurane in 100% oxygen in a vented anesthesia chamber and maintained on 2% isoflurane for the duration of the experiment using a volume-controlled ventilator (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten – Germany) adjusted to obtain and end-tidal CO2 level [EtCO2] between 35 and 40 mmHg monitored with microcapnography (Columbus Instruments, Columbus, OH, USA). Rectal temperature was maintained between 36 and 37 °C using a heat pad and a rectal thermistor. Blood pressure was monitored via a femoral artery cannula.

2.6. Controlled cortical impact

Controlled cortical impact (CCI) injury was produced as previously described [15]. Briefly, after craniectomy and dural exposure, the 3 mm diameter impactor tip was positioned perpendicular to the exposed surface of the brain at an angle of approximately 45° to the vertical. CCI (3 m/s, 1.5 mm deformation, 100 ms of duration) was performed using a voltage driven impactor (Benchmark Stereotaxic Impactor, myNeuroLab, St Louis, MO, USA).

2.7. Histology

Intact mouse brains were collected two weeks post-injury. First, the mice were anesthetized and then perfused with phosphate-buffered saline followed by 10% formalin. Brains were harvested from the mice cut in half and fixed in 10% formalin. The paraffin blocks were sectioned in slices of 6 µm and mounted on slides.

For H&E staining for contusion volume measurements, samples were de-paraffinized and rehydrated with reducing concentration of ethanol, rinsed with distilled water and stained with Mayer hematoxylin solution. Next, samples with treated with 1% sodium bicarbonate for 1 min and then rinsed with distilled water followed by 80% ethanol before counterstaining with eosin-y solution for 1 min. Last, samples were dehydrated with increasing concentrations of ethanol, and treated with xylene twice for 2 min each and mounted with a xylene based medium. For neuron morphology, Nissl staining on cortical sections and cerebellar sections was performed after deparaffinization and rehydration. A 0.1% cressyl violet solution was used for the Nissl staining.