Free Radical Biology and Medicine 78 (2015) 89–100

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Protein carbonylation after traumatic brain injury: cell specificity, regional susceptibility, and gender differences

Rachel C. Lazarus [a], John E. Buonora [a,1], David M. Jacobowitz [a,b], Gregory P. Mueller [a,b,c,*]

[a] Program in Neuroscience, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
[b] Department of Anatomy, Physiology, and Genetics, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA
[c] Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA

ARTICLE INFO

Article history:
Received 21 July 2014
Received in revised form
7 October 2014
Accepted 10 October 2014
Available online 23 October 2014

Keywords:
Carbonylation
Reactive oxygen species
Reactive carbonyl species
Traumatic brain injury
Median eminence
Dorsal third ventricle
Astrocytes
Ependymal cells
Free radicals

ABSTRACT

Protein carbonylation is a well-documented and quantifiable consequence of oxidative stress in several neuropathologies, including multiple sclerosis, Alzheimer's disease, and Parkinson's disease. Although oxidative stress is a hallmark of traumatic brain injury (TBI), little work has explored the specific neural regions and cell types in which protein carbonylation occurs. Furthermore, the effect of gender on protein carbonylation after TBI has not been studied. The present investigation was designed to determine the regional and cell specificity of TBI-induced protein carbonylation and how this response to injury is affected by gender. Immunohistochemistry was used to visualize protein carbonylation in the brains of adult male and female Sprague–Dawley rats subjected to controlled cortical impact (CCI) as an injury model of TBI. Cell-specific markers were used to colocalize the presence of carbonylated proteins in specific cell types, including astrocytes, neurons, microglia, and oligodendrocytes. Results also indicated that the injury lesion site, ventral portion of the dorsal third ventricle, and ventricular lining above the median eminence showed dramatic increases in protein carbonylation after injury. Specifically, astrocytes and limited regions of ependymal cells adjacent to the dorsal third ventricle and the median eminence were most susceptible to postinjury protein carbonylation. However, these patterns of differential susceptibility to protein carbonylation were gender dependent, with males showing significantly greater protein carbonylation at sites distant from the lesion. Proteomic analyses were also conducted and determined that the proteins most affected by carbonylation in response to TBI include glial fibrillary acidic protein, dihydropyrimidase-related protein 2, fructose-bisphosphate aldolase C, and fructose-bisphosphate aldolase A. Many other proteins, however, were not carbonylated by CCI. These findings indicate that there is both regional and protein specificity in protein carbonylation after TBI. The marked increase in carbonylation seen in ependymal layers distant from the lesion suggests a mechanism involving the transmission of a cerebral spinal fluid-borne factor to these sites. Furthermore, this process is affected by gender, suggesting that hormonal mechanisms may serve a protective role against oxidative stress.

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Introduction

Traumatic brain injury (TBI) is a major public health issue affecting over 1.7 million Americans annually, with falls, collision incidents, and motor vehicle accidents being the leading causes of injury [1]. The consequences of TBI can be complex and long lasting, resulting in serious disorders that involve progressive cognitive deficits, epilepsy, and profound behavioral alterations. Although the molecular and cellular disturbances involved in these longer-term responses are not well understood, one common element in the pathology seems to be the establishment of a chronic inflammatory state that can persist in brain for weeks, months, and perhaps even years after TBI [2,3].

Abbreviations: CCI, controlled cortical impact; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; DAPI, 4′,6-diamidino-2-phenylindole; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IACUC, Institutional Animal Case and Use Committee; PBS, phosphate-buffered saline; RCS, reactive carbonyl species; ROS, reactive oxygen species; TBI, traumatic brain injury; TBS-T, Tris-buffered saline–Tween 20; TFA, trifluoroacetic acid; USUHS, Uniformed Services University of the Health Sciences

* Corresponding author at: Department of Anatomy, Physiology and Genetics; Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA. Fax: +(301) 295 3566.
E-mail address: Gregory.mueller@usuhs.edu (G.P. Mueller).
[1] *Present address:* U.S. Army Graduate Program in Anesthesia Nursing, Fort Sam Houston, TX 78234–7585, USA.

http://dx.doi.org/10.1016/j.freeradbiomed.2014.10.507
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A hallmark of inflammation is oxidative stress, which can be caused by metabolic dysfunction with numerous potential causes [4,5]. At the tissue and cellular levels, the pro-oxidative forces of inflammation following an injury can outweigh the capacity of antioxidative, protective mechanisms such as superoxide dismutase and glutathione peroxidase. The resulting state drives the formation of reactive oxygen species (ROS) and reactive carbonyl species (RCS), products of lipid peroxidation by ROS. In TBI, this condition results from injury-induced ischemia/reperfusion, hypoxia, elevated intracranial pressure, glutamate excitotoxicity, and intracellular calcium overload [6,7], all of which contribute to the breakdown of mitochondrial bioenergetics due, in part, to abnormally aggregated proteins [3].

An important modification of oxidative stress is protein carbonylation, which involves the introduction of carbonyl groups into protein-bound amino acids. Protein carbonylation may occur either through direct oxidation of amino acid targets by ROS or via interaction with RCS, which are themselves the product of lipid peroxidation [8]. By either mechanism, the resulting carbonyl modifications can disrupt protein function and thereby contribute to injury pathology [9]. Carbonylation has long been utilized as a metric of oxidative stress levels in various neurodegenerative pathologies, including multiple sclerosis [10] and Alzheimer's disease [11]. Although several studies in TBI have explored protein carbonylation, this work has been largely limited to the use of this modification as a measure of total oxidative stress [12]. Here, we identify four proteins that are preferentially carbonylated in response to TBI, investigate the susceptibility of various brain regions to carbonylation, identify cell types within these regions that are most affected by this modification, and, furthermore, differentiate gender-based differences in carbonylation after TBI. The results of this work shed light onto into the differential susceptibility of specific brain regions and cell types to TBI-induced oxidative stress, providing insights into the mechanisms of TBI pathology.

Materials and methods

Controlled cortical impact (CCI)

Adult male and adult female Sprague–Dawley rats (8–9 weeks of age) were purchased from Charles River Laboratories (Morrisville, NC, USA). Rats were housed in a barrier facility for animals accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Before experimental procedures, rats were anesthetized with isoflurane (Baxter Healthcare Corp., Deerfield, IL, USA) vaporized in medical-grade oxygen (100%, Roberts Oxygen Co. Inc., Rockville, MD, USA), placed in a digital cranial stereotactic device (Leica Microsystems, Buffalo Grove, IL, USA), and subjected to unilateral CCI injury over the left hemisphere. Briefly, the rats underwent a free-hand craniotomy (−3.8 mm from bregma in males, −3.0 mm in females). CCI was administered through the ImpactOne stereotaxic impactor (Leica Microsystems), which delivered a 3-mm flat-tipped impactor at 20° to a depth of 2 mm at 5 m/s with a 500-ms dwell time. After CCI, the incision was closed and the rats received buprenorphine 0.05 mg/kg for postoperative analgesia. Throughout all procedures, rat body core temperature was maintained at 37 ± 0.3 °C. Sham-operated rats received no anesthesia, incision, or craniotomy. Animals were euthanized 5 days after injury under anesthesia (10% chloral hydrate solution, 0.4 ml/100 g). All animal handling procedures were performed in compliance with guidelines from the National Research Council for the ethical handling of laboratory animals and were approved by the Institutional Animal Care and Use Committee of USUHS (IACUC Protocol APG 12-827, Bethesda, MD, USA).

Immunoblotting: protein carbonylation detection and identification in brain tissue

Tissue collection and preparation

Brains were removed immediately after euthanization, frozen on powdered dry ice, and stored at −80 °C until used. Brains were hand-dissected to produce blocks of penumbral structures enriched in carbonylated proteins as identified immunohistochemically (see below). The contralateral brain region and equivalent region from the naïve animals were similarly collected.

One-dimensional gel electrophoresis

Protein fractions were prepared by homogenization in 5 volumes/tissue weight extraction solution (8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Chaps), and 50 mM dithiothreitol (DTT; Sigma–Aldrich, St. Louis, MO, USA) containing 0.8% ampholytes (pH 3–10; Invitrogen Life Technologies, Carlsbad, CA, USA) and 1 × Complete protease inhibitor mix (Roche, Indianapolis, IN, USA)). After homogenization and subsequent centrifugation (20,000*g*, 10 min, 4 °C), the resulting supernatant was stored at −80 °C until used. The efficiency of tissue solubilization was > 95%, as judged by protein assay and Coomassie staining of one-dimensional gels.

Protein samples were prepared for gel electrophoresis by combining with an equal volume of 2 × reducing loading buffer (Novex NuPAGE LDS sample buffer; Invitrogen; containing 50 mM DTT) and heating at 70 °C for 10 min. Samples were then fractionated (25 µl/4.16 µg per lane) using NuPAGE 10% Bis–Tris gels (Novex–Invitrogen) and transferred to nitrocellulose blots using an iBlot transfer apparatus (Invitrogen).

Fluid-phase isoelectric focusing (IEF)

To reduce the complexity of tissue homogenates for subsequent proteomic analyses, samples were prefractionated by fluid-phase IEF before one-dimensional gel electrophoresis. Samples of injured and contralateral hemisphere, and the corresponding regions from control naïve animals ($n = 8$ for each region), were homogenized in 5 volumes (wt/vol) IEF denaturant consisting of 7.7 M urea, 2.2 M thiourea, and 4.4% Chaps containing 1 × Complete protease inhibitor mix (Roche) and clarified by centrifugation (20,000*g*, 10 min, 4 °C). Tissue region pools were prepared and 200-µl aliquots of each pool supernatant were further diluted to 2.865 ml having a final composition of IEF denaturant plus ampholytes (150 µl, pH 3–10; Invitrogen), DTT (50 µl, 2 M stock), and bromophenol blue (10 µl, 10 mg/ml stock). The resulting sample was loaded into the IEF fractionator and focused using the following conditions: 100 V, 1.2 mA, 0 W (15 min); 200 V, 2.0 mA, 0 W (1 h); 400 V, 2.0 mA, 1 W (1 h); 600 V, 1.5 mA, 1 W (1 h). This resulted in fractions of proteins within the following p*I* ranges: 3.0–4.6, 4.6–5.4, 5.4–6.2, 6.2–7, and 7–9.1. IEF-fractionated proteins were further fractionated by size by one-dimensional gel electrophoresis (see *One-dimensional gel electrophoresis*).

Postelectrophoretic detection of carbonylated proteins

Postelectrophoretic detection of carbonylated proteins was performed as described by Conrad et al. [13], with minor modifications. Briefly, nitrocellulose membranes were washed in 20% methanol/80% Tris-buffered saline/Tween 20 (TBS-T), equilibrated in 2 N hydrochloric acid (HCl), and then incubated with 0.5 mM 2,4-dinitrophenylhydrazine (DNPH; Sigma–Aldrich) in 2 N HCl (10 min, in the dark). The derivatized membranes were then washed three times with 2 N HCl (10 min per wash) followed by 50% methanol (five times, 10 min per wash). Membranes were then equilibrated in TBS-T, blocked with 5% fetal bovine serum/TBS-T, and probed overnight at 4 °C with rabbit anti-DNP

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