Redox Biology 8 (2016) 59–67

Contents lists available at ScienceDirect

Redox Biology

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

Research paper

Cellular and subcellular oxidative stress parameters following severe spinal cord injury

Nishant P. Visavadiya [a,1], Samir P. Patel [a,1,*], Jenna L. VanRooyen [a], Patrick G. Sullivan [b], Alexander G. Rabchevsky [a]

[a] Spinal Cord and Brain Injury Research Center, Department of Physiology, University of Kentucky, Lexington, KY 40536-0509, USA
[b] Spinal Cord and Brain Injury Research Center, Department of Anatomy & Neurobiology, University of Kentucky, Lexington, KY 40536-0509, USA

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
24 December 2015
Accepted 29 December 2015
Available online 30 December 2015

Keywords:
4-Hydroxynonenal
3-Nitrotyrosine
Protein carbonyl
ROS
RNS
Mitochondria

ABSTRACT

The present study undertook a comprehensive assessment of the acute biochemical oxidative stress parameters in both cellular and, notably, mitochondrial isolates following severe upper lumbar contusion spinal cord injury (SCI) in adult female Sprague Dawley rats. At 24 h post-injury, spinal cord tissue homogenate and mitochondrial fractions were isolated concurrently and assessed for glutathione (GSH) content and production of nitric oxide ($NO^{\bullet}$), in addition to the presence of oxidative stress markers 3-nitrotyrosine (3-NT), protein carbonyl (PC), 4-hydroxynonenal (4-HNE) and lipid peroxidation (LPO). Moreover, we assessed production of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in mitochondrial fractions. Quantitative biochemical analyses showed that compared to sham, SCI significantly lowered GSH content accompanied by increased $NO^{\bullet}$ production in both cellular and mitochondrial fractions. SCI also resulted in increased $O_2^{\bullet-}$ and H_2O_2 levels in mitochondrial fractions. Western blot analysis further showed that reactive oxygen/nitrogen species (ROS/RNS) mediated PC and 3-NT production were significantly higher in both fractions after SCI. Conversely, neither 4-HNE levels nor LPO formation were increased at 24 h after injury in either tissue homogenate or mitochondrial fractions. These results indicate that by 24 h post-injury ROS-induced protein oxidation is more prominent compared to lipid oxidation, indicating a critical temporal distinction in secondary pathophysiology that is critical in designing therapeutic approaches to mitigate consequences of oxidative stress.

1. Introduction

Traumatic spinal cord injury (SCI) includes primary mechanical and secondary pathophysiological mechanisms of injury which ultimately cause motor, sensory and/or autonomic dysfunction. The initial insult primarily elicits tissue pathology at the injury epicenter. A number of secondary injury events follow which cause the damage to spread, including ischemia/reperfusion injury, inflammatory processes, edema, reactive oxygen/nitrogen species (ROS/RNS) generation, glutamate-mediated excitotoxicity, intracellular calcium accumulation, activation of proteases and caspases, as well as cellular necrosis and apoptosis around the injury epicenter [1–6]. SCI triggers a rapid increase in extracellular glutamate concentrations which precipitates calcium influx into cells via voltage-gated ion channels [7]. Elevated intracellular calcium is consequently taken up into mitochondrial compartments, leading to a failure of aerobic energy metabolism, inhibition of ATP synthesis, decrease in mitochondrial membrane potential, increased generation of ROS/RNS, and onset of mitochondrial permeability transition; all of which constitute mitochondrial dysfunction [8–10].

Previous studies have documented that by 24 h following contusion SCI, oxidative stress markers specific to lipid and protein oxidation, namely 4-hydroxynonenal (4-HNE), 3-nitrotyrosine (3-NT) and protein carbonyl (PC) formation, all increase in injured tissue homogenates [11–13] and in isolated mitochondria [9,14]. However, there has never been a comparative assessment of oxidative stress parameters in cellular versus subcellular fractions following contusion SCI, concurrently. Accordingly, the present study was designed to provide a comprehensive assessment of free radical production and free radical-mediated adduct formation (i.e., PC, 3-NT and 4-HNE) in tissue homogenate and mitochondria following acute severe contusion SCI in rats. In summary, compared to lipid oxidation, acute ROS-induced protein oxidation appears to be a key target to mitigate consequences of injury-induced oxidative stress.

* Correspondence to: Spinal Cord and Brain Injury Research Center (SCoBIRC) B461, Biomedical & Biological Sciences Research Building, 741 South Limestone Street, Lexington, KY 40536-0509, United States.
E-mail address: skpate2@uky.edu (S.P. Patel).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.redox.2015.12.011

2. Materials and methods

2.1. Spinal cord injury

Spinal cord injury was carried out on adult female Sprague-Dawley rats (Harlan Labs, IN) weighing 225–250 g. Animals were housed in a core facility at the University of Kentucky and allowed access to water and food *ad libitum*. All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and according to NIH guidelines. Prior to injury, rats were anesthetized with Ketamine (80 mg/kg i.p., Fort Dodge Animal Health, Fort Dodge, IA) and Xylazine (10 mg/kg i.p., Lloyd Laboratories, Shenandoah, IA). A dorsal laminectomy was performed at the 12th thoracic vertebra to expose the first and second lumbar (L1/L2) spinal cord levels using published methods [15,16]. Spinal cord contusions ($n=6$) were performed using the well-characterized Infinite Horizon impactor device (PSI, Lexington, KY) at 250 kDyn force [17]. Control sham rats ($n=6$) received laminectomy only at the 12th thoracic vertebra, but no injury was performed. After injury, the wound was irrigated with saline, the muscles sutured together in layers with 3–0 Vicryl (Ethicon, Inc., Somerville, NJ), and the skin layers were closed with wound clips (Stoelting Co., Wood Dale, IL). Hydrogen peroxide and betadine were used to clean the wound area and animals injected (s.c.) with pre-warmed lactated Ringer's solution (10 ml split into 2 sites bilaterally) and Cefazolin (33.3 mg/kg) before returned to their cages with food and water *ad libitum*. Upon regaining consciousness, both sham and injured rats received Buprenorphine-HCl (0.03 mg/kg; Reckitt Benckiser Pharmaceuticals Inc. Richmond, VA) (s.c.) every 8 h.

2.2. Preparation of tissue homogenate and mitochondrial fractions

At 24 h post-injury, all sham and injured animals were deeply anesthetized with CO_2, decapitated, and a 1.5 cm segment of spinal cord at the L1/L2 spinal level centered on the injury site was rapidly dissected and placed in an ice cold dissecting plate containing isolation buffer consisting of 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, and pH adjusted to 7.2 with KOH) [14,16]. The spinal cord tissue homogenate was prepared using a Potter-Elvehjem homogenizer containing 2 mL of ice-cold isolation buffer, and then 400 μl of tissue homogenate was frozen immediately in liquid nitrogen for biochemical analyses. To isolate the mitochondria, the remaining tissue homogenate was centrifuged twice at $1400 \times g$ for 3 min at 4 °C to obtain a pellet containing the nuclear fraction (NU). The supernatant (cytosolic fraction: CY) was re-centrifuged at $13{,}000 \times g$ at 4 °C for 10 min and the pellet was subsequently re-suspended and placed into a nitrogen cell disruption chamber (1200 psi, 10 min, 4 °C) to release synaptosomal mitochondria, producing the mitochondrial fraction. The mitochondrial fraction was then centrifuged at $13{,}000 \times g$ for 10 min and resultant mitochondrial pellet was washed in isolation buffer without EGTA and centrifuged for 10 min at $10{,}000 \times g$ at 4 °C. The final purified mitochondrial pellet was resuspended in 50 μl isolation buffer without EGTA. The protein concentration of total homogenate and mitochondrial fraction was measured using the BCA protein assay kit.

For oxidative stress profiles, $O_2^{\bullet -}$ and H_2O_2 production assays were carried out in the mitochondrial fraction while other assays were carried out in both tissue homogenate and mitochondrial fraction. This is due to the fact that while ROS are generated in multiple compartments, the vast majority of cellular ROS (estimated at approximately 90%) can be traced back to the mitochondria in which ROS are generated as by-products of cellular metabolism. Further, the non-mitochondrial ROS released or formed in the cytosol are buffered generally under strong reducing conditions by intracellular thiols, particularly glutathione (GSH) and thioredoxin ($TRXSH_2$) by the activities of their reductases. Hence, the mitochondrial electron transport chain contains several redox centers that leak electrons to oxygen, constituting the primary/major source of ROS in tissue [18–21].

2.3. GSH measurements

Reduced form of glutathione (GSH) content was measured using the fluorescent probe monochlorobimane (MCB) as described [22]. Tissue homogenate or mitochondria (2 μg/μl) were incubated in 100 μM MCB and 1 U/ml GST reaction mixture for 30 min at 37 °C in the dark. The reaction samples were then centrifuged at $8000 \times g$ for 8 min. Finally, the fluorescence of tagged GSH in each supernatant was measured using a fluorescence reader (excitation=380 nm; emission=470 nm). Briefly, the MCB Glutathione Detection Kit utilizes MCB dye that has a high affinity for reduced GSH. The unreacted dye is virtually non-fluorescent. After glutathione-S-transferase (GST)-catalyzed reaction of the dye with GSH, the resulting blue fluorescence intensity reflects the amount of GSH present in the samples.

2.4. Lipid peroxidation measurements

Tissue homogenate or mitochondria (100 μg/100 μl assay system) were incubated in eppendorf tubes containing 10% trichloroacetic acid (TCA). Following subsequent addition of 0.67% thiobarbituric acid (TBA), tubes were placed in a boiling water-bath for 20 min and then centrifuged at $3000 \times g$ for 10 min. The amount of malondialdehyde (MDA), present as thiobarbituric acid reactive substances (TBARS) formed in the supernatant, was measured at 532 nm using the molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [23].

2.5. Nitric oxide measurements

Tissue homogenate or mitochondria (100 μg/100 μl assay system) were incubated with a reaction mixture containing Griess reagent (1% sulfanilamide, 2% HCl and 0.1% naphthyl ethelene diamine dihydrochloride) and vanadium (III) chloride–based reduction. Vanadium (III) in dilute acid solution causes reduction of nitrate to nitrite. The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide, and subsequent coupling with naphthylethelene diamine, was read at 540 nm as described [24].

2.6. Immunoblots of tissue homogenates and mitochondria

Proteins (20 μg) from the various spinal cord fractions were suspended in Laemmli buffer under reducing conditions (100 mM DTT) and then separated by SDS–PAGE using Criterion 4–20% Tris–HCl (10–250 kD) gel (Bio-Rad, Hercules, CA). Proteins captured by western blot gels were trans-blotted onto polyvinylidene difluoride (PVDF), blocked with 5% nonfat dry milk for an hour at room temperature, and then incubated at 4 °C overnight in a primary antibody solution. Alternatively, detection of PC formation was performed according to published methods [25] using an OxyBlot kit (cat #S7150; Millipore Inc). The antibodies used for western blots were 3-nitrotyrosine rabbit polyclonal (3-NT – 200 nl/ml; cat #9691; Cell Signaling technology Inc.), 4-hydroxynonenal (mouse monoclonal HNE – 150 ng/ml; cat # HNEJ-2; JaICA Co., Ltd) and rabbit polyclonal (HNE – 100 nl/ml; cat #HNE11-S; alpha-diagnostic international), β-actin mouse monoclonal (25 ng/ml, cat #A2228; Sigma-Aldrich Inc.) and voltage-dependent anion channel rabbit polyclonal (VDAC; 150 ng/ml; cat