Neurochemistry International 92 (2016) 43–48

Contents lists available at ScienceDirect

Neurochemistry International

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

Rapid communication

Evidence of extensive RNA oxidation in normal appearing cortex of multiple sclerosis brain

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ARTICLE INFO

Article history:
Received 26 May 2015
Received in revised form
22 November 2015
Accepted 2 December 2015
Available online 17 December 2015

Keywords:
Multiple sclerosis
Oxidative stress
RNA oxidation

ABSTRACT

The role of reactive oxygen species (ROS) in the progression of neurodegenerative and neuroinflammatory disorders such as multiple sclerosis (MS) has been highlighted in recent years. Due to the debilitated cellular antioxidant defense mechanism in the neurons in MS, and their vulnerability to ROS effects, the cellular components in neuronal cells are susceptible to oxidative damage. The damage due to ROS in various biomolecules including proteins and DNA has already been shown in MS lesions. Using an *in situ* approach we have detected hitherto unidentified RNA oxidative damage in the neuronal cells of normal appearing cortex of postmortem MS brains. We analyzed the presence of oxidative damage marker nucleoside 8-hydroxyguanosine (8-OHG) to determine the presence of oxidized RNA in MS brain. Immunohistochemical analyses with anti 8-OHG antibody showed significant oxidation in the cytoplasm and to a conspicuously lesser extent in the nucleus of neuronal cells within the normal appearing cortex of MS brain, whereas similar areas were weakly immunopositive in control brain tissues. Pretreatment with RNase 1 greatly reduced the immune reaction with anti 8-OHG antibody while it was only slightly diminished by DNase I pre-treatment, indicating extensive oxidative damage in the RNA pool of MS brain. The abundance of 8-OHG, hence the high extent of RNA oxidative damage was further confirmed by immunoprecipitation and HPLC analyses of total RNA isolated from MS brain. To our knowledge, this is the first evidence of increased RNA oxidation in normal appearing cortex of MS brain. The current study begins to define the link of RNA oxidation to MS pathophysiology.

1. Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the central nervous system (CNS) (Trapp and Nave, 2008; Nakahara et al., 2012). Multiple sclerosis is widely recognized as an immune mediated inflammatory disease of the CNS which leads to demyelination resulting in axonal degeneration and hence neuronal miscommunication in the CNS. Although the mechanism of axonal injury in MS is believed to be inflammation-led neurodegeneration, accumulating evidence supports the possibility of a reverse mechanism (Bar-Or et al., 2011). Multiple factors are known to play their roles in the process of neurodegeneration, including oxidative stress (OS) and inflammation (Uttara et al., 2009). The cause of high oxidative stress in cells is either the inability of cellular defense mechanism to detoxify the reactive oxygen species (ROS), such as, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or hydroxyl radicals ($^{\bullet}OH$) or its failure to repair the resulting oxidative damage caused by the generation of excess ROS. A disturbance in the physiological balance of the redox state of a cell thus produces toxic effects via the accumulation of the ROS that triggers damage in different biomolecules including proteins, lipids, and nucleic acids (Lee et al., 2012). Brain, being the most vibrant site of oxidative phosphorylation, produces more ROS as a byproduct of energy metabolism (Uttara et al., 2009). Since MS has been primarily considered an immune mediated inflammatory disorder, its neurodegenerative aspects have received less priority in the past. But over the last decade, significant effort has been spent to investigate the contribution of oxidative stress and mitochondrial dysfunction in MS progression (Bolanos et al., 1997; Smith et al., 1999; Dutta et al., 2006). Oxidative damage to intracellular biomolecules was viewed as just an epiphenomenon of neurodegeneration; however, numerous researchers have shown the

Abbreviations: ROS, reactive oxygen species; MS, multiple sclerosis; OS, oxidative stress; CNS, central nervous system; 8-OHG, 8-hydroxyguanosine.

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http://dx.doi.org/10.1016/j.neuint.2015.12.002
0197-0186/

functional importance of oxidative imbalance as a critical event in mediating disease pathogenesis (Zhu et al., 2005; Tanaka et al., 2007). In support of this idea, oxidative modifications in different biomolecules including proteins, lipids, and DNA have been reported in MS brains (Smith et al., 1999; Lu et al., 2000; Bizzozero et al., 2005; Broadwater et al., 2011).

Multiple studies have shown a significant increase in cellular oxidative markers namely 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) levels in the brains of patients with classical neurological disorders, such as, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) and some of the studies showed that most of the oxidized nucleosides are associated with cytoplasmic RNA and are restricted to vulnerable neurons (Nunomura et al., 2009, 2012; Poulsen et al., 2012; Jorgensen, 2013; Nunomura, 2013). Interestingly, there are reports showing the selective susceptibility of specific mRNA species toward oxidative damage; some of which have shown to be involved in the disease pathogenesis (Shan et al., 2003, 2007). Oxidized mRNAs not only produce truncated proteins but also they cannot be translated properly leading to reduced protein expression and the loss of protein function. Although there are some studies that showed DNA oxidation in MS lesions (Vladimirova et al., 1998; Lu et al., 2000; Haider et al., 2011; Fischer et al., 2013), to the best of our knowledge, there is no known study describing RNA oxidative damage in normal appearing areas of MS brain. Since selective RNA oxidation can be a preceding event that contributes to neurodegeneration, we hypothesize that OS induced RNA oxidation is linked to MS pathology.

2. Materials and methods

2.1. Demographic details of the tissue donors

The details of the tissue donors' demographic information is summarized in Table S1 in the Supplementary information. Frozen tissue blocks from postmortem MS and non-MS brains were obtained from the Rocky Mountain MS Center, and the Human Brain and Spinal Fluid Resource Center at UCLA. The average age and average postmortem intervals (PMI) of the MS donors were 62.3 years (standard deviation SD ± 10.5) and 8.68 h (SD ± 6.6) respectively while those of non-MS donors were 66.8 (SD ± 6.1) years and 10.5 h (SD ± 5.8).

2.2. Immunohistochemistry

2.2.1. DAB staining

Frozen tissue blocks from postmortem MS and non-MS brains were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) for 48 h and washed in PBS (2 × 24 h). Then the tissue blocks were sliced 20 μm thick sections perpendicular to the outer edge of the cortex on a PBS media cooled Vibratome®. Tissue sections (TS) were stored in PBS in 24-well plates at 4 °C in a humidified chamber overnight. Following which the sections were washed with PBS (3 × 5 min). Next, the TS were treated with 1% H_2O_2 to quench the endogenous peroxidase activity, and washed with PBS (3 × 5 min). The sections were then incubated in 3% normal donkey serum in PBS for 30 min and washed with PBS (3 × 5 min), which was followed by incubation in a primary antibody to myelin proteolipid protein (PLP) (Chemicon, MAB388) in a 1:200 dilution with 3% donkey serum in PBS with 0.5% Triton X-100 as the diluent. Each section was covered with diluted antibody solution and incubated at 4 °C for 48 h. The sections were rinsed with PBS (3*10 min). A biotinylated anti-mouse secondary antibody was applied at a 1:500 dilution in PBS containing 3% donkey serum and 0.5% Triton X-100 for 1 h at room temperature. The sections were then rinsed with PBS again (3 × 10 min) and then incubated in freshly prepared Vector Elite ABC solution (Vector labs) for 30 min. Then the TS were rinsed in PBS (3 × 10 min) and then peroxidase substrate solution, diaminobenzidine (DAB) was applied until the desired staining level was achieved. The sections were rinsed three times in PBS for 10 min each, blotted and allowed to air dry. Each slide was protected from UV radiation using Permount® under the coverslip and allowed to dry overnight in a hood. Images were acquired using an Olympus BX53 microscope using bright field imaging mode.

2.2.2. Immunofluorescence staining

Following the 1% H_2O_2 treatment, the TS were incubated in diluted primary anti DNA and RNA oxidative damage marker antibody (8-OHG antibody, QED Biosciences) solution overnight at 4 °C, washed three times in PBS (10 min each) and were incubated in donkey anti-mouse Alexa 488 secondary fluor antibody (1:500, Invitrogen) in 3% normal donkey serum in PBS with 0.5% Triton-X 100, for 2 h. For co-immunostaining, the TS were incubated in diluted primary antibody solutions (anti 8-OHG antibody and anti NeuN antibody, abcam or anti ASPA antibody, EMD Millipore) overnight at 4 °C, washed three times in PBS (10 min each) and were incubated in donkey anti-rabbit Alexa fluor 488 secondary fluor antibody (1:500, abcam) and donkey anti-mouse Alexa fluor 555 secondary antibody (1:500, Invitrogen) in 3% normal donkey serum in PBS with 0.5% Triton-X 100, for 2 h. Following three 10 min washes in PBS, the secondary antibody treated sections were incubated in lipofuscin autofluorescence quenching solution composed up of 50 mM ammonium acetate and 10 mM cupric sulfate for 1.5 h. Sections were washed three times in PBS (10 min each), placed below coverslips under Vectashield mounting media, sealed with clear nail polish, and kept refrigerated until microscopically imaged. Images were acquired using an Olympus FV1000 confocal microscope equipped with two lasers (Ar 488 nm and HeNe 555 nm) and analyzed with ImageJ.

2.3. Nucleic acid isolation and immunoprecipitation

Nucleic acids were isolated from postmortem brain tissues using Tripure isolation reagent (Roche Lifesciences) according to the manufacturer's protocol with slight modification and quantified using a NanoDrop®. RNA and DNA were immunoprecipitated separately with anti 8-OHG antibody by using some modifications in the previous protocol (Shan et al., 2007). Briefly, 25 μg of total RNA (each from both MS and control brain tissue) were incubated with 30 μg of 8-OHG antibody at room temperature for 2 h. Then 35 μL of immobilized protein L agarose gel beads (Pierce) were added to the RNA-antibody mixture and incubated overnight at 4 °C. The beads were washed three times (3 × 5 min) with 200 μL of 0.04% (v/v) Nonidet P-40 (Roche Applied Science) solution in sterile PBS. The oxidized RNA: antibody: protein L agarose beads complexes were separated from non-oxidized RNAs (which remained in the supernatant) by centrifugation at 1500 rpm for 5 min at 4 °C. The oxidized RNAs were mixed with following reagents: 3 mL of PBS with 0.04% Nonidet P-40, 300 μL of 10% (w/v) sodium dodecyl sulfate (SDS), and 3 mL of PCI (phenol: chloroform: isoamyl alcohol; 25:24:1), and the mixture was incubated at 37 °C for 30 min (with occasional vortexing) and separated to an aqueous and an organic phase by spinning at 13,200 rpm for 15 min at 4 °C. The aqueous layer containing oxidized RNA was separated and mixed with 40 μL of 3 M sodium acetate buffer (pH 5.3), 2 μL of 10 μg/μL glycogen and 1 mL of absolute ethanol. The sample was then