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OXIDATIVE STRESS AND REDUCED GLUTAMINE SYNTHETASE ACTIVITY IN THE ABSENCE OF INFLAMMATION IN THE CORTEX OF MICE WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Abstract—Pathological changes occur in areas of CNS tissue remote from inflammatory lesions in multiple sclerosis (MS) and its animal model experimental allergic encephalomyelitis (EAE). To determine if oxidative stress is a significant contributor to this non-inflammatory pathology, cortex tissues from mice with clinical signs of EAE were examined for evidence of inflammation and oxidative stress. Histology and gene expression analysis showed little evidence of immune/inflammatory cell invasion but reductions in natural antioxidant levels and increased protein oxidation that paralleled disease severity. Two-dimensional oxyblots and mass-spectrometry-based protein fingerprinting identified glutamine synthetase (GS) as a particular target of oxidation. Oxidation of GS was associated with reductions in enzyme activity and increased glutamate/glutamine levels. The possibility that this may cause neurodegeneration through glutamate excitotoxicity is supported by evidence of increasing cortical Ca^{2+} levels in cortex extracts from animals with greater disease severity. These findings indicate that oxidative stress occurs in brain areas that are not actively undergoing inflammation in EAE and that this can lead to a neurodegenerative process due to the susceptibility of GS to oxidative inactivation. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: oxidative stress, experimental allergic encephalomyelitis, multiple sclerosis, glutamine synthetase.

Multiple sclerosis (MS) is a chronic inflammatory disorder of the CNS which generally manifests in an initial relapsing-remitting clinical course that culminates in permanent neurological damage. The main pathological features of the disease include focal CNS inflammation with axonal demyelination and cell death. Transection of axons by inflammatory lesions is considered the principal cause of neurodegeneration, although the mechanism of neurodegeneration is still under debate (Qi et al., 2006). Certain of the pathological features of the onset of MS are modeled by experimental autoimmune encephalomyelitis (EAE), an inflammatory disorder induced by immunization of susceptible rodent species with myelin antigens. EAE is characterized by blood-brain barrier (BBB) breakdown, perivascular infiltration of immune cells, microglia activation, and demyelination (Raine, 1984). The demyelinating lesions are mainly localized in the spinal cord and cerebellum, with minimal involvement of the cerebral cortex (Brown et al., 1982; Tran et al., 1997). However, studies to elucidate the mechanisms of neurodegeneration that underlie the clinical development of EAE have shown that suppression of the inflammatory response does not necessarily prevent the axonal and neuronal damage responsible for the loss of function (Jeffery, 2007; Metz et al., 2007; Lu et al., 2009). Moreover, in both MS and EAE enhanced expression of genes involved in anti- and pro-inflammatory mechanisms has been identified in CNS tissues, such as cerebral cortex, that are distant from lesion activity (Zeis et al., 2008a, b). These findings indicate that pathological changes occur in the CNS tissues either independent of, or as a prelude to lesion formation.

There is considerable evidence that oxidative damage plays a role in the pathogenesis of MS and EAE (LeVine, 1992; Gilgun-Sherki et al., 2004; Ferretti et al., 2006). Reactive oxygen species (ROS), produced at high levels during a CNS inflammatory response, overcome endogenous antioxidant defenses and contribute to the pathogenesis of CNS inflammation through the oxidation of a variety of targets including biomolecules in the myelin sheath (Smith et al., 1999). However, protein oxidation has also been detected in the CNS tissues before the infiltration of inflammatory cells begins in animals immunized to develop EAE (Qi et al., 2006). This suggests that oxidative stress plays an early role in neurodegeneration that may precede overt inflammation. In the present study we have investigated this possibility using a proteomic approach coupled to quantification of key brain metabolites in the cerebral cortex of PLSJL mice immunized with myelin basic protein (MBP). Clinical severity of EAE is variable in this model with 60–80% of the animals normally developing signs of disease which correlate with pathological changes in the spinal cord but not cerebellum or cerebral cortex (Fabis et al., 2008). We therefore examined brain tissues from mice

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Abbreviations: BBB, blood-brain barrier; EAE, experimental allergic encephalomyelitis; Gln, glutamine; Glu, glutamate; GS, glutamine synthetase; GSH, glutathione; GSSG, glutathione disulfide; MBP, myelin basic protein; MS, multiple sclerosis; ROS, reactive oxygen species.

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that failed to develop EAE as well as animals with increasing clinical severity. Our data show that in the cortex of mice with clinical signs of EAE, despite the absence of local immune cell infiltration, there is selective oxidation of glutamine synthetase (GS), a protein that is particularly important for neural homeostasis. The resulting reduction in GS function, which is concomitant with reduced antioxidant capacity, is likely associated with glutamate excitotoxicity.

EXPERIMENTAL PROCEDURE

Immunization protocol and clinical score classification

As detailed previously (Fabis et al., 2007), female 10–11-week-old PLSJL mice (Jackson Laboratories, Bar Harbor, ME, USA) were either left untreated (controls) or immunized s.c. at three sites with 200 μl of an emulsion containing 100 μg of guinea pig MBP in Complete Freund's Adjuvant supplemented with 4 mg/ml *Mycobacterium tuberculosis* $H_{37}RA$ (BD, Franklin Lakes, NJ, USA). Immunized mice also received 400 ng of pertussis toxin (List Biological Laboratories, Campbell, CA, USA) i.p. on days 0 and 2. The animals were scored daily for clinical signs of EAE. Animals assessed in this study had scores assigned as follows: 0, immunized but appearing normal; 3, tail paralysis and hindlimb weakness or ataxia; 5, complete hindlimb paralysis but without forelimb involvement, alert and capable of eating and drinking. Tissue samples were obtained from non-immunized animals (NI), MBP-immunized mice that had shown clinical signs for at least 3 days with a clinical score of either 3 or 5 as well as MBP-immunized mice that did not develop signs of disease (clinical score 0) over the same period of time after immunization. All procedures were conducted in accordance with federal guidelines under animal protocols approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Histological and quantitative RT-PCR assessment of inflammation

For histochemical analysis, cerebellum and cortex from eight control and MBP-immunized mice without disease (score 0) or clinical signs of EAE of 3 and 5 were removed and snap frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Torrance, CA, USA). The cerebellum and cortex sections (10 μm) were stained with Mayer's Hematoxylin (0.1%) and eosin Y (1%) (Sigma-Aldrich, St. Louis, MO, USA) and photographed using a Leitz microscope (Wild Leitz, Wetzlar, Germany) and digital camera at initial magnifications of 4 and 40×. Isolation of mRNA and quantification of specific mRNA levels were performed on tissue samples from nine mice per group by real-time quantitative RT-PCR using previously described primers, probes, and methodologies (Scott et al., 2002) and a Bio-Rad iCycler iQ real-time detection system (Bio-Rad, Hercules, CA, USA). Data were calculated based on the threshold cycle (Ct), the PCR cycle at which the fluorescent signal becomes higher than that of the background (cycles 2–10) plus 10 times the SD of the background. Synthetic cDNA standards were used to determine copy numbers. Data are expressed as the fold increase in mRNA copy numbers in test tissues over levels in control tissue samples from naive mice, with all values normalized to the mRNA content of the ribosomal housekeeping protein L13 in each sample.

Brain tissue sampling

Mouse brain samples were minced in liquid nitrogen atmosphere. Brain powders were suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, and proteinase inhibitors, and sonicated for 30 s. Homogenates were centrifuged at 14,000×g for 10 min to remove debris. Care was taken to avoid protein oxidation or degradation during sample preparation. Protein concentration in the supernatant was determined by the Bradford method (Bio-Rad).

2D gel electrophoresis and Western blotting

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was performed in a Bio-Rad system using 110 mm immobilized pH gradient (IPG) strips and Criterion 10% gels (Bio-Rad). Sample preparation was carried out as described (Castegna et al., 2002a,b). Briefly, for each sample two preparations were made, one for Oxyblot analysis and one for mass spectrometry analysis. Protein homogenates corresponding to 200 μl of each sample were incubated with 800 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH)/2M HCl (for Oxyblot analysis) or 2 M HCl (for mass spectrometry analysis) for 20 min at room temperature. Then trichloroacetic acid was added at the final concentration of 0.15%, with 10 min incubation on ice. Samples were centrifuged at 14,000×g for 10 min, the pellet washed three times with an ethanol-ethyl acetate solution (1:1) and then resuspended in 200 μl 2D PAGE sample buffer (8 M urea, 2 M thiourea, 20 mM dithiothreitol, 0.2% (v/v) Biolytes 3–10, 2% CHAPS and Bromophenol Blue).

For the first dimension, 200 μg protein was applied to a rehydrated IPG strip, and isoelectric focusing was carried out at 20 °C. Before the second dimension separation, the gel strips were equilibrated for 10 min in 37.5 mM Tris–HCl (pH 8.8) containing 6 M urea, 2% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 10 min in the same buffer except that dithiothreitol was replaced with 4.5% iodoacetamide. The IPG strips were rehydrated as described above and placed on Criterion gels (Bio-Rad). After unstained molecular weight protein standards had been applied, electrophoresis was started. Isoelectric focusing was performed as follows: 300 V for 1 h, then linear gradient to 8000 V for 5 h and finally 20,000 V/h. Second-dimension gels were run at 200 V for 60 min. Gel staining, performed with Sypro Ruby (Bio-Rad), and immunoblotting analysis was performed as described in (Castegna et al., 2002a).

The specificity of protein-DNP hydrazone antibody was assessed as described (Sultana et al., 2006) and the spots corresponding to GS identified as described (Castegna et al., 2002a). Briefly, each gel spot was excised from the underivatized gel, subjected to in-gel-digestion, and the peptides were extracted. The mixture was subjected to mass spectrometry and sequences matched to GS using a database as described (Castegna et al., 2002a).

Analysis of gel images

The analysis of gels and membranes to compare protein and carbonyl content between control and EAE samples was performed using PDQuest software (Bio-Rad). Images from SYPRO Ruby stained gels for proteomic analyses were obtained with a ChemiDoc XRS system (Bio-Rad) using a fluorescence reference plate to avoid saturation as recommended by the manufacturer. Oxyblot images were obtained with a GS-800 Densitometer (Bio-Rad).

Sample preparation for liquid chromatography tandem mass spectrometry analysis (LC-MS/MS)

For mass spectrometry analysis of nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced $NADP^+$ (NADPH), nicotinamide adenine dinucleotide (NAD^+), reduced NAD^+ (NADH) and reduced glutathione (GSH) and oxidized glutathione disulfide

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