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Accumulation of protein carbonyls within cerebellar astrocytes in murine experimental autoimmune encephalomyelitis

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Abstract

Recent work from our laboratory has implicated protein carbonylation in the pathophysiology of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). The present study was designed to determine the changes in protein carbonylation during the disease progression, and to identify the target cells and modified proteins in the cerebellum of EAE animals, prepared by active immunization of C57/BL6 mice with MOG₃₅₋₅₅ peptide. In this model, protein carbonylation was maximal at the peak of the disease (acute phase) to decrease thereafter (chronic phase). Double immunofluorescence microscopy of affected cerebella showed that carbonyls accumulate in white matter astrocytes, and to a lesser extent in microglia/macrophages, both in the acute and chronic phase. Surprisingly, T cells, oligodendrocytes and neurons were barely stained. By 2D-oxyblot and mass spectrometry, β -actin, β -tubulin, GFAP and HSC-71 were identified as the major targets of carbonylation throughout disease. Using a pull-down/western blot method we found a significant increase in the proportion of carbonylated β -actin, β -tubulin and GFAP in the chronic phase but not in the acute phase. These results suggest that as disease progresses from the inflammatory to the neurodegenerative phase there may be an inappropriate removal of oxidized cytoskeletal proteins. Additionally, the extensive accumulation of carbonylated GFAP in the chronic phase of EAE may be responsible for the abnormal shape of astrocytes observed at this stage.

Keywords

astrocyte; cytoskeleton; experimental autoimmune encephalomyelitis; GFAP; multiple sclerosis; oxidative stress; protein carbonylation

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human CNS and a major cause of neurological disability among young adults in North America and Europe (Trapp and Syts, 2009). The pathological changes that contribute to neurological disability in MS include inflammation, demyelination, oligodendrocyte death and axonal degeneration (Kornek and Lassmann, 1999). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for CNS autoimmune disorder, recapitulating a number of clinical and pathological features of MS (Gold *et al.*, 2000). Several EAE models have been developed throughout the years that reflect the different clinical courses of MS. MOG₃₅₋₅₅ peptide-induced EAE in the C57BL/6 mouse, the animal model used in this study, is

characterized by the presence of inflammatory (non-demyelinated) lesions throughout the CNS at the peak of disease and extensive demyelination with minor inflammation during the chronic phase (Kuersten *et al.*, 2007). These features make this an ideal model to study the pathophysiological mechanisms underlying disease progression.

There is substantial amount of data indicating that oxidative stress plays a major role in the pathogenesis of both MS and EAE. Excessive production of reactive oxygen species (ROS), primarily by activated microglia/macrophages and astrocytes, leads to severe oxidative stress, which contributes significantly to tissue damage (Gilgun-Sherki *et al.*, 2004). The principal outcome of oxidative stress is the chemical transformation of lipids, proteins, and nucleic acids by ROS. Of these, proteins are the major target for oxidants as the result of their abundance and their high reaction rate constants (Davies, 2005). While the polypeptide backbone and the side chains of most amino acids are susceptible to oxidation, the non-enzymatic introduction of aldehyde or ketone functional groups to specific amino acid residues (i.e. carbonylation) constitutes the most common oxidative alteration of proteins (Bizzozero, 2009). Protein carbonyls can be introduced in proteins directly via metal ion-catalyzed oxidation of certain amino acid residues (Requena *et al.*, 2001) or indirectly by the attachment of bi-functional reactive carbonyl species (e.g. 4-hydroxynonenal, acrolein, malondialdehyde, glyoxal) (Esterbauer *et al.*, 1991). In either case, carbonylation often leads to loss of protein function and the formation of toxic cross-linked protein aggregates (Bizzozero, 2009).

Accumulation of protein carbonyls has been implicated in the etiology and/or progression of several neurodegenerative disorders including Alzheimer's disease (Aksenov *et al.*, 2001), Parkinson's disease (Floor and Wetzel, 1998), and amyotrophic lateral sclerosis (Ferrante *et al.*, 1997). Our recent discovery that protein carbonyls accumulate in the brain of MS patients (Bizzozero *et al.*, 2005; Hilgart and Bizzozero, 2008) and in the spinal cord of rats with acute EAE (Smerjac and Bizzozero, 2008), suggests that this type of chemical modification may also play a critical pathophysiological role in inflammatory demyelinating diseases. The present study was designed to assess the levels of protein carbonylation and to identify the target cells and the modified proteins in the cerebellum of EAE mice during the course of the disease. The results show that most of the carbonyls accumulate in white matter astrocytes, and to a lesser extent in microglia/macrophages, present at the site of inflammatory lesions both at the peak of disease and during the chronic phase. Surprisingly, T cells, oligodendrocytes and neurons were barely stained. At all disease stages, the major carbonylated proteins were identified as β -actin, β -tubulin, GFAP and heat shock cognate-71 (HSC-71). While both oxidative stress and total protein carbonylation decrease later in the disease, we observed that the proportion of the carbonylated forms of the cytoskeletal proteins was notably higher in the chronic animals. This suggests impairment in the removal of oxidized proteins as disease progresses. A preliminary account of this work has been presented in abstract form (Zheng and Bizzozero, 2009).

Materials and Methods

Induction of Experimental Autoimmune Encephalomyelitis (EAE)

Housing and handling of the animals as well as the euthanasia procedure were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. Eight-week-old female C57BL/6 mice were purchased from Harlan Bioproducts (Indianapolis, IN) and housed in the UNM-animal resource facility. To induce EAE, animals received a subcutaneous injection into the lower back area of 200 μ l of MOG₃₅₋₅₅ peptide (200 μ g) (AnaSpec, San Jose, CA) in saline mixed with complete Freund's adjuvant (CFA) supplemented with 4 mg/ml of heat killed *Mycobacterium tuberculosis* H37Ra (Chondrex Inc; Redmond, WA).

Control animals were given CFA without MOG peptide. Two-hours and 48h after EAE induction, all animals received an i.p. injection of 0.3 µg of pertussis toxin (List Biological Laboratories; Campbell, CA) in 100 µl of saline. Seven days after disease induction mice received a second immunization with the MOG peptide in CFA. Animals were weighed and examined daily for the presence of neurological signs. At prescribed days post-immunization (DPI), EAE mice and CFA-injected controls were euthanized by decapitation. The cerebellum was removed and either fixed with methacarn (methanol : chloroform : acetic acid, 60 : 30 : 10 by vol) or homogenized in PEN buffer (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 0.1 mM neocuproine) containing 2 mM 4,5 dihydroxy-1,3 benzene disulfonic acid and 1 mM dithiothreitol (DTT). Protein homogenates were stored at -20°C until use. Protein concentration was assessed with the Bio-Rad DC™ protein assay (Bio-Rad Laboratories; Hercules, CA) using bovine serum albumin as standard.

Biochemical determination of oxidative markers

The amount of non-protein thiols, of which > 90% is reduced glutathione (GSH) was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Bizzozero *et al.*, 2006). Lipid peroxidation was assessed by measuring the amount of thiobarbituric acid reactive substances (TBARS) in the tissue homogenates (Ohkawa *et al.*, 1979). The relative amount of protein carbonyls was measured with the OxyBlot™ protein oxidation detection kit (Intergen Co., Purchase, NY) as described elsewhere (Bizzozero *et al.*, 2006).

Two-dimensional oxyblots of cerebellar proteins

Cerebellar proteins (5 µg) were first incubated with 2,4-dinitrophenyl-hydrazine (DNPH) to convert the carbonyl groups into 2,4-dinitrophenyl (DNP) hydrazone derivatives, and were then analyzed by 2D-gel electrophoresis (Smerjac and Bizzozero, 2008). After electrophoresis, proteins were blotted to polyvinylidene difluoride (PVDF) membranes. DNP-containing proteins were detected using rabbit anti-DNP antiserum (1:5000) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000). Blots were developed by enhanced chemiluminescence (ECL) using the Western Lightning ECL™ kit from Perkin-Elmer (Boston, MA). Blots were stripped and re-probed with antibodies against specific cytoskeletal proteins including β-tubulin (1:1000, mouse monoclonal; Sigma, St Louis, MO), GFAP (1:1000, mouse monoclonal; Sigma) and β-actin (1:1000, mouse monoclonal; GeneTex, Irvine, CA). As before, blots were developed by ECL.

Identification of major carbonylated proteins by mass-spectrometry

Spot matching between the 2D-oxyblots and the coomassie blue stained 2D-gels was performed using the Discovery Series PDQuest 2-D Analysis Software Version 7.0.1 (Bio-Rad). Protein spots were excised from the gel and subjected to *in situ* digestion with trypsin (Bizzozero *et al.*, 2002). Before mass-spectrometry, peptides were cleaned-up and concentrated using C-18 Zip-tips (Millipore Corp., Billerica, MA) and mixed with α-cyano-4-hydroxycinnamic acid. Mass spectra were acquired on a Biosystems 4700 Proteomics Analyzer (TOF/TOF) (Applied Biosystems/MDX Sciex, Foster City, CA) in positive ion reflection mode and using a S/N threshold of 30. Monoisotopic peak lists were generated employing a GPS Explorer™ software (v3.5, Applied Biosystems) and were submitted to the MASCOT search tool for protein identities. For all searches, precursor ion mass tolerance was 100 ppm. Protein identification was considered significant with a Mascot score corresponding to $p < 0.05$.

Quantification of carbonylation levels in specific proteins

The extent of protein carbonylation was determined using a pull-down/western blot method (Bizzozero *et al.*, 2006). Briefly, protein carbonyls were biotinylated by reaction with biotin

hydrazide in the presence of cyanoborohydride. A small aliquot of these protein homogenates was saved for western blotting and the rest was processed to isolate the biotinylated proteins using streptavidin-agarose. Proteins were eluted from the beads with SDS-sample buffer and analyzed by western blotting on 10% polyacrylamide gels. Blots were probed with antibodies against individual protein species and developed by ECL as described above. Films were scanned in a Hewlett Packard Scanjet 4890 and the images were quantified using the NIH Image 1.63 imaging analysis program. Band intensities from the total and streptavidin-eluted fractions were used to calculate the percent of protein modified by carbonylation.

Immunohistochemical detection of protein carbonyls

Tissue specimens were fixed overnight in methacarn and then mounted in paraffin. Tissue was sectioned in the sagittal plane (6- μ m thick) and mounted on VectabondTM-treated slides (Vector Laboratories, Burlingame, CA). Sections were deparafinized with xylenes and a graded alcohol series, and then rinsed with phosphate-buffered saline (PBS) solution for 10 min. Lesions were detected by staining with hematoxylin and eosin (H&E). Adjacent sections were incubated for 30 min with 1 mg/ml DNPH prepared in 2 N HCl to convert carbonyl groups into DNP-hydrazones. Sections were rinsed three times with PBS, blocked with 10% (v/v) normal goat serum and incubated overnight with rabbit anti-DNP antibody (1:1000) (Sigma). After removing the primary antibody with PBS containing 0.1% Triton X-100, sections were incubated for 3 h with Alexa Fluor[®] 647 goat anti-rabbit antibody (1:100, Molecular Probes, Eugene, OR). Sections were rinsed twice with PBS containing 0.1% Triton X-100, once with PBS, and then mounted in a buffered glycerol solution containing p-phenylenediamine as anti-fade reagent. Images were captured with a Zeiss 200m microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY) equipped with a Hamamatsu C4742-95 digital camera (Hamamatsu Corp., Bridgewater, NJ).

For double immunofluorescence, DNPH-treated sections were incubated with the mixture of two primary antibodies overnight at 4°C, followed by incubation with the corresponding fluorescent secondary antibodies (Alexa Fluor[®] 488 and Alexa Fluor[®] 647, 1:100, Molecular Probes). After washing, the sections were cover slipped with anti-fade fluorescent mounting medium. The various cell types were detected by using antibodies against GFAP (1:500, mouse monoclonal; Sigma), Iba1 (1:250, mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA), CD3 (1:100, mouse monoclonal, Santa Cruz), adenomatous polyposis coli protein C-terminal (1:125, mouse monoclonal, Chemicon, Temecula, CA) and NeuN (1:250, mouse monoclonal, Chemicon). To quantify the percentage of each cell type that shows positive carbonyl deposits, slide-mounted sections were scanned at a 60X magnification and were digitalized with a MagnaFire Camera (Optronics, Galeta, CA). Images were imported into Image J software to obtain merged pictures. Three fields (100 μ m $\times$ 75 μ m) per slide and three slides per animal were chosen for quantification.

Statistical Analysis

Results were analyzed for statistical significance with ANOVA utilizing GraphPad Prism[®] program (GraphPad Software Inc., San Diego, CA).

Results

Characteristics of EAE mice

EAE in female C57BL/6 mice was induced by active immunization with MOG₃₅₋₅₅ peptide as described under Materials and Methods. Symptoms were graded according to the following scale: 0, no symptoms; 1, tail weakness; 1.5, clumsy gait; 2, hind limb paresis;

2.5, partial hind limb dragging; 3, hind limb paralysis; 3.5, hind limb paralysis with fore limb paresis; 4, complete paralysis; and 5, moribund. In this EAE model, neurological symptoms begin at 14 DPI (7 days after the boost with MOG peptide) reaching a peak at 30 DPI, and most animals remain ill (score 3.0-3.5) throughout the entire experimental period (60 DPI) (Fig. 1A). Acute disease was defined as having maximal neurological symptoms of EAE without any improvement for at least three consecutive days. At this stage the cerebellar pathology is characterized perivenular infiltration of inflammatory cells mostly within the white matter (Fig. 1C). Chronic EAE was defined arbitrarily as animals that remain in the stationary phase of the disease for 30 days (60 DPI). At this stage there is reduced perivascular and parenchymal inflammation, and lessened transmigration of inflammatory cells into the cerebellum (Fig. 1D). CFA-injected animals, which were sacrificed at 30DPI (control young) and 60DPI (control old), did not exhibit any neurological sign or cerebellar pathology (Fig. 1B). Western blot analysis of cerebellar proteins using antibodies against several myelin proteins, neurofilament proteins and GFAP revealed that cerebella from acute EAE animals have increased gliosis without apparent demyelination or axonal injury. In contrast, chronic EAE cerebellar tissue has reduced gliosis and augmented demyelination and axonal damage (data not shown). These results are in agreement with the current notion that acute EAE is mainly an inflammatory disorder while chronic EAE is mostly a demyelinating/neurodegenerative disorder (Kuersten *et al.*, 2007).

Increase oxidative stress in acute and chronic EAE

As shown in Fig. 2A, GSH levels in EAE cerebella was 73% and 85% of control values at the peak of disease and in the chronic phase, respectively. This indicates that the CNS of the affected animals is indeed subjected to considerable oxidative stress. Interestingly, the amount of TBARS, a marker of lipid peroxidation, in EAE animals was similar to that in controls both at peak of the disease and in the chronic phase (Fig. 2B). Quantitative analysis of the oxyblots revealed a significant enhancement in protein carbonyl levels in the acute phase with little or no changes in the chronic phase of EAE (Fig. 2C).

Protein carbonyls accumulate within astrocytes present at the lesion's sites

Immunohistochemical localization of carbonyls groups was carried out after derivatizing these moieties with DNPH (Fig. 3). Validation of this technique was performed by omitting the DNPH-treatment, the anti-DNP antibody or the secondary anti-rabbit IgG antibody (not shown). We also carried out a positive control in which carbonyls were generated by incubating cerebellar sections with $\text{FeSO}_4/\text{H}_2\text{O}_2$ (panel E) and a specificity control in which endogenous carbonyls were removed by incubation with NaBH_4 (panel F). Using this technique we found that carbonyl staining in the cerebellum of both acute (panel B) and chronic EAE mice (panel D) is highly intense in the white matter, where the majority of inflammatory lesions are present. As expected from the biochemical data shown in Fig 3C, there is higher density of carbonyl staining in the acute phase than in chronic phase. In addition, the morphology of most carbonyl-positive cells appears to be that of astrocytes. This was confirmed by immunostaining carbonyls and GFAP simultaneously (Fig. 4). It is worth noting that, in the acute tissue, astrocytes have normal morphology and show colocalization with GFAP and carbonyl in their distal processes, while in the chronic tissue, astrocytes exhibit an abnormal morphology. Indeed, some astrocyte processes are completely retracted.

Double immunofluorescence with antibodies against DNP and different cell-specific markers was used to identify other major cell targets of carbonylation. As depicted in Fig. 5, the majority of astrocytes (~90%) present at the site of inflammatory lesions in chronic EAE showed positive carbonyls staining, and similar results were found in acute EAE (data not

shown). A significant proportion of microglial cells/macrophages (40%) also showed carbonyl staining, while <10% of T cells (CD3⁺), <5% of oligodendrocytes (APC⁺) and <10% of neurons (NeuN⁺) were stained with the anti-DNP antibody.

Identification of the major protein targets of carbonylation in EAE

Two-dimensional-oxyblot of cerebellar proteins from acute EAE mice shows the presence of 4 major carbonylated polypeptides and several minor species that become visible at much longer exposure times (Fig. 6). These major oxidized species were also present in oxyblots of cerebellar proteins from chronic EAE animals (not shown). This particular 2D-pattern of carbonyls was somewhat reminiscent to that of found in the spinal cord of EAE rats, where cytoskeletal proteins are the major targets of carbonylation (Smerjac and Bizzozero, 2008). Thus, identification of carbonyl-containing proteins was initially carried out by spot matching DNP-labeled proteins on a 2D-oxyblot with those of specific cytoskeletal elements. Using this approach we identified three of the major carbonylated spots as β -actin, β -tubulin and GFAP. The other major spot in the 2D-oxyblot did not correspond to any of the other cytoskeletal species and was identified by mass-spectrometry as HSC-71 (*P63017*; mascot score= 208).

Experiments were also conducted to ascertain the chemical nature of the carbonyls. Western blot analysis using antibodies against acrolein, 4-hydroxynonenal and malondialdehyde failed to detect the presence of any modified protein in either control or EAE mice (data not shown), suggesting that reactive carbonyl species-protein adducts are not formed in this disease. Thus, most carbonyl groups present in the oxidized proteins are likely to be α -amino adipic semialdehyde and glutamic semialdehyde, which are formed by direct, metal-ion catalyzed, oxidative deamination of the amino acid side chain of proline, arginine and lysine (Requena *et al.*, 2001).

Accumulation of carbonylated cytoskeletal proteins in chronic EAE

Quantification of the extent of carbonylation of individual proteins was performed using a pull-down/western blot procedure. To this end, protein carbonyl moieties from control and EAE cerebellar homogenates were first converted into biotinylated residues by reaction with biotinhydrazide. Biotin-containing proteins were then isolated with streptavidin-agarose and analyzed by western blotting employing antibodies against the β -tubulin, β -actin and GFAP. HSC-71 was not studied due to the lack of an appropriate antibody. A number of preliminary studies were carried out to ascertain (1) the concentration of biotin hydrazide and time necessary for complete blockage of carbonyl groups, (2) the amount of streptavidin-agarose necessary for complete binding of biotinylated proteins, and (3) the composition and number of rinses that ensure the proper removal of non-biotinylated proteins from the agarose beads before elution. Also, no material was recovered from the streptavidin agarose when either the biotin hydrazide was omitted from the derivatization step or when the carbonyl groups were eliminated by pre-incubation with sodium borohydride, indicating that the procedure for isolating carbonylated proteins is indeed specific. Fig. 7 shows the percentage of individual proteins that is modified by carbonylation in control and EAE animals, which was calculated from the amount of each protein in the bound and total fractions. Surprisingly, the proportion of oxidized β -actin, β -tubulin and GFAP did not increase in acute EAE as compared to young control mice. The reason for the apparent discrepancy between these findings and those obtained with the 2D-oxyblots (Fig. 6) is due to the fact that there is more β -actin and GFAP in the cerebellum of acute EAE than in control animals. In contrast, there is a five-fold increase in the proportion of carbonylated GFAP and a two-fold increase in the proportion of carbonylated β -actin and β -tubulin in chronic EAE mice relative to its control. These results suggest that as disease

progresses from the inflammatory to the neurodegenerative phase there may be an inappropriate removal of the carbonylated forms of these cytoskeletal proteins.

Discussion

This is the first study on the accumulation of oxidized CNS proteins during the course of chronic EAE. We focused our research on the cerebellum since this CNS region is commonly affected in MS (Ramasamy *et al.*, 2009) and in MOG peptide-induced EAE (MacKenzie-Graham *et al.*, 2009). Furthermore, the pathophysiological changes in the cerebellum remain largely unexplored when compared to the spinal cord, the CNS area most studied in EAE. Using classical immunocytochemical techniques, we found that the majority of the carbonyl groups are localized within astrocytes located in the vicinity of inflammatory lesions both at the peak of the disease and during the chronic phase. A number of microglial cells/macrophages was also found to contain detectable levels of carbonyls in EAE, while T cells, oligodendrocytes and neurons were mostly unstained. This result is not totally unexpected since upon inflammatory activation both microglia and astrocytes produce large amounts of ROS (Keller *et al.*, 1999) that could generate significant amounts of carbonyls within these cells. Furthermore, microglial cells contain higher levels of glutathione and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) than astrocytes (Dringen, 2005), which may protect the former from a more severe oxidative damage. Nonetheless, it was somewhat surprising to find that neurons and oligodendrocytes do not have extensive carbonyl staining, particularly when these two cell types are considered by many investigators to be highly susceptible to oxidative stress (Halliwell, 2006; Benarroch, 2009). Interestingly, the notion that astrocytes are less sensitive to oxidative damage than other CNS cells has been recently challenged. It has been found that cerebellar astrocytes in the unperturbed mouse contain significantly lower levels of reduced glutathione than neurons and oligodendrocytes (Miller *et al.*, 2009). Another possibility to explain the selective oxidation of cells in our model is that the ROS generated by activated microglia/macrophages and astrocytes are short lived and do not reach other cell targets. Finally, oligodendrocytes and neurons may have a more efficient proteolytic machinery to remove oxidized proteins thus reducing the build up of carbonylated proteins in these cells. This may be also the case for T cells, whose proteasomal activity is enhanced during inflammation (Mattingly *et al.*, 2007).

Another significant finding in this study was the identification of β -actin, β -tubulin, GFAP and HSC-71 as major targets of protein carbonylation in the cerebellum of both acute and chronic EAE. It should be noted that the detection and identification of oxidized species using a 2D-oxyblot is biased toward abundant cell proteins and that longer exposure times reveals the presence of other modified species. However, many proteins are as abundant as β -actin, β -tubulin and GFAP and yet they have minimal carbonylation (e.g. spectrin) or their oxidation does not change in EAE (e.g. vimentin), which suggests specificity. Indeed, metal ion-catalyzed oxidation of *Escherichia coli* proteins seems to be highly selective with most carbonylation sites present in RKPT-enriched regions that are exposed to the solvent (Maisonneuve *et al.*, 2009). It has been known for some time that in neurodegenerative disorders cytoskeletal proteins are particularly susceptible to carbonylation (Aksenov *et al.*, 2001; Muntané *et al.*, 2006). Furthermore, previous work from our lab has also identified β -actin, β -tubulin and GFAP as significant targets of carbonylation in the brain of MS patients (Hilgart and Bizzozero, 2008) and in the spinal cord of rats with acute EAE (Smerjac and Bizzozero, 2008).

Carbonylation of cytoskeletal proteins has been reported to cause loss of function. For instance, actin filaments and microtubules both destabilize and disassemble upon oxidation of their protein components (Dalle-Donne *et al.*, 2001; Neely *et al.*, 2005). Oxidative

damage of GFAP has been described in Alzheimer's disease (Korolainen *et al.*, 2005; Pamplona *et al.*, 2005), aceruloplasminaemia (Kaneko *et al.*, 2002), Pick's disease (Muntané *et al.*, 2006) and MS (Hilgart and Bizzozero, 2008). Whether the accumulation of oxidized GFAP is associated with loss of function, as demonstrated for the other cytoskeletal proteins, is not known. Because GFAP is a main intermediate filament of cytoskeleton that modulates astrocyte stability and shape, it is possible that the accumulation of oxidized GFAP may have an effect on astrocyte morphology. Interestingly, carbonyl-positive astrocytes in chronic EAE, where the proportion of oxidized GFAP is relatively high, have very short processes and there is redistribution of this protein from the processes to the soma. In contrast, normal morphology of activated astrocytes with long processes was observed at the peak of the disease where, despite the large excess of GFAP, the proportion of oxidized protein is just above control values. Carbonylation may also affect other GFAP properties such as the anchoring of the glutamate transporter GLAST to the plasma membrane of astrocytes, which seems to play an important role in protecting the brain against glutamate-mediated excitotoxicity (Sullivan *et al.*, 2007). Significant carbonylation of HSC-71 was detected in both EAE and control cerebella, suggesting that this chaperone is highly susceptible to oxidation. HSC-71 is a constitutively expressed and multifunctional chaperone protein present in both neurons and activated astrocytes (Kanninen *et al.*, 2004). Based on its involvement in the structural maintenance of the proteasome and conformational recognition of misfolded proteins by proteases, HSC-71 expression has been proposed as a defensive mechanism of response to unfavorable conditions. While our study is the first to describe carbonylation of HSC-71, other chaperones are known to exhibit an age-associated increase in carbonylation including BiP/Grp78, protein disulfide isomerase, and calreticulin (Rabek *et al.*, 2003).

In present study, the total amount of carbonylated protein at the peak of disease is significantly increased compared to that of control animals, while little change is found during the chronic phase of EAE. This is likely due to the lower number/activity of inflammatory lesions, the site where protein carbonyls build-up, in the chronic phase of EAE. Indeed, immunohistochemical studies revealed that carbonyls accumulate in the diseased white matter in both phases of EAE (Fig. 3). Moreover, the percentage of oxidized GFAP, β -tubulin and β -actin in the chronic phase are considerably higher than those in the acute phase. While the proportion of carbonylated cytoskeletal proteins measured in the cerebellum homogenate of chronic animals seems low (~2% for GFAP), one has to consider that these oxidized molecules may be distributed heterogeneously. Thus, the proportion of modified cytoskeletal proteins in cells near inflammatory foci may be much higher than that determined in the pull-down assays from the entire tissue. The amount carbonylated protein is determined by the rate of generation and degradation of carbonyls. Proteolysis is considered the only physiological mechanism for elimination of carbonylated proteins since there is no evidence for enzymatic reduction of protein-bound carbonyl groups to alcohols (Bizzozero, 2009). Therefore, since protein carbonyls cannot be repaired and since there is less oxidative stress in chronic than in acute EAE, it is fair to conclude that the accumulation of carbonylated cytoskeletal proteins in the cerebellum of chronic EAE mice may be due to impaired degradation. This, in turn, could be caused by reduced activity of the degradation system and/or by decreased susceptibility of the oxidized proteins to proteolysis. Degradation of carbonylated proteins is thought to be carried out in an ATP- and ubiquitin-independent manner by the 20S proteasome, which selectively recognizes and digests partially unfolded oxidized proteins (Rivett, 1985; Grune *et al.*, 1995). Interestingly, preliminary studies in our laboratory discovered a significant decrease in proteasome activity in chronic EAE, which might explain the accumulation of oxidized proteins as disease progresses (Zheng and Bizzozero, 2010). At this time, however, we cannot exclude that oxidized cytoskeletal proteins are also less susceptible to digestion by the proteasome

and other cellular proteases as previously proposed (Friguet *et al.*, 1994). Studies in our laboratory are underway to examine this possibility.

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Fig. 1. Clinical course and cerebellar pathology of EAE in C57BL/6 female mice. EAE was induced by active immunization with MOG₃₅₋₅₅ peptide as described under Materials and Methods. Animals were monitored daily for signs of clinical disease and scored as indicated in the text. Clinical scores represent the mean of 6 control and 10 EAE mice (panel A). Panels B-D depict representative H&E-stained cerebellar sections from control, acute EAE and chronic EAE mice, respectively. GL, granule cells layer; ML, molecular layer; WM, white matter. In panel C, arrows point to perivenular white matter lesions with abundant lymphocyte infiltration in the acute EAE. In panel D, arrows point to perivenular white matter lesions in chronic EAE that show reduced inflammatory activity. Bar= 300μm.

Fig. 2.

Levels of oxidative stress markers in the cerebellum of acute and chronic EAE mice. Aliquots of cerebellar homogenates from control and EAE mice were used to determine the levels of reduced glutathione (panel A), TBARS (panel B) and protein carbonyls (panel C) as described in Materials and Methods. Values represent the mean $\pm$ SEM of 5 animals in each experimental group. Asterisks denote values that are statistically different ($p < 0.05$) from their respective controls.

Fig. 3. High levels of carbonyls in cerebellar white matter of acute and chronic EAE mice. Cerebellar sections (6 μ m-thick) from control, acute EAE and chronic EAE mice were incubated with DNPH to convert carbonyls into DNP-derivatives, which were detected by sequential incubation with rabbit anti-DNP and *Alexa Fluor*[®] 647 (red) goat anti-rabbit antibodies as described under Materials and Methods (panels A-D). Panels E and F show cerebellar sections from control animals that were pretreated with Fe/H₂O₂ to generate carbonyls (positive control) and with NaBH₄ to remove endogenous carbonyls (negative control), respectively. Bar= 300 μ m. Other abbreviations are as in Fig 1.

Fig. 4. Colocalization of carbonyls and GFAP in the cerebellum of acute and chronic EAE mice. Double immunofluorescence analysis was performed as described in Methods and Materials. Green channel is for GFAP-positive astrocytes while red channel is for carbonyls. Immunofluorescent images show that the majority of white matter astrocytes in the cerebellum of acute and chronic EAE mice contain significant amounts of carbonyls. In acute EAE, cerebellar astrocytes have normal morphology and show colocalization of GFAP and carbonyl staining in their distal processes. In chronic EAE, cerebellar astrocytes exhibit abnormal morphology including the retraction of some processes. Bar= 50 μ m.

Fig. 5.

Carbonyl staining in the cerebellum of chronic EAE mice colocalizes mostly with astrocytes and some microglial cells. Double immunofluorescence analysis was performed as described in Methods and Materials. Green channel is for the various cell markers while red channel is for carbonyls. Immunofluorescent images show that the majority of cerebellar astrocytes (GFAP⁺) (panel A) and a significant proportion of microglia/macrophages (Ib1a⁺) in chronic EAE mice (panel B) are positive for carbonyls. In contrast, only a few CD3⁺-Tcells (panel C), NeuN-expressing neurons (panel D) and APC⁺-oligodendrocytes (panel E) show positive carbonyl staining. Bar= 50μm.

Fig. 6.

β -Actin, β -tubulin, GFAP and HSC-71 are the major carbonylated proteins in EAE cerebellum. Cerebellar proteins from control and EAE mice were derivatized with 2,4-dinitrophenylhydrazine. Proteins were separated by 2-D-gel electrophoresis and blotted to PVDF membranes. Membranes were immunostained with anti-DNP, then stripped and reprobed with antibodies against the major cytoskeletal proteins. β -Actin, β -tubulin and GFAP on the oxyblots were identified by spot matching. HSC-71 was identified by mass spectrometry as described in Materials and Methods. Only the pH 3-6 region of the oxyblots is shown since the pH 6-10 region was devoid of visible carbonyl-positive spots.

Fig. 7.

GFAP, β -actin and β -tubulin are more carbonylated in chronic than in acute EAE. Carbonylated proteins were converted into biotinylated proteins and were isolated using streptavidin-agarose as described under Materials and Methods. Aliquots of the starting material (total) and the streptavidin-purified fraction (bound) were separated on SDS-gels and transferred to PVDF membrane. Blots were probed with antibodies against various proteins and were developed by ECL (panel A). Densitometric scans were obtained to calculate the proportion of each protein that is carbonylated (panel B). Values represent the mean $\pm$ SEM of five animals. * $p < 0.05$