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Approved by the Dissertation Committee:

, Chairperson

CARBONYLATION OF NEURONAL CYTOSKELETAL PROTEINS AND THEIR PROTEOLYTIC DEGRADATION IN ACUTE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

BY

SUZANNE SMERJAC

B.S., Chemistry, Colorado School of Mines, 2001

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

May 2011

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Abstract

Oxidative stress and its damage resulting in carbonylation of proteins is known to take place in the CNS tissue of multiple sclerosis patients and of mice with experimental autoimmune encephalomyelitis (EAE), and has been implicated in the pathophysiology of these diseases. In order to investigate the possible consequences of this protein damage during acute myelin basic protein (MBP) -directed autoimmune neuroinflammation the first goal of this thesis was to characterize oxidative stress and protein carbonylation, including identification of the major oxidized protein species, in the Lewis rat model of EAE. I discovered that there is significant oxidative stress in the spinal cord of EAE rats characterized by reduced levels of glutathione, increased lipid peroxidation products, and accumulation of protein carbonyls during the inflammatory stages of the disease. Using two-dimensional oxyblot I was able to identify various CNS cytoskeletal proteins, including the neurofilaments (NFH, NFM, and NFL) and β-tubulin, as major targets of this oxidative damage. There are many possible fates for carbonylated proteins including

accumulation, formation of high molecular weight aggregates and proteolysis. The specific fate likely depends not only on the particular species being oxidized but also on the level of damage it sustains. Western blotting for the neurofilaments and β-tubulin revealed that there is significant degradation of these cytoskeletal proteins during the disease and that the level of specific oxidative damage to NFL changes during the disease course. The second major objective of this thesis was to investigate the role of calpain and the proteasome, which have been proposed to selectively degrade carbonylated proteins, in the removal of these cytoskeletal proteins during neuroinflammation and to examine whether oxidative modification of the proteins made them more susceptible to degradation. This idea was tested by inhibiting calpain or the proteasome in EAE Lewis rats by intrathecal injection of calpeptin or epoxomicin during the peak of clinical disease. After inhibiting calpain, I found significant increases in total protein carbonylation and in the amount of neurofilament proteins and β-tubulin that are spared from degradation, but no changes are seen in the specific oxidation of any of these protein species. Inhibition of the proteasome did not affect total protein carbonylation or degradation, but did change the specific oxidation of NFM. These results suggest that (1) calpain is the primary clearance mechanism of neurofilament and β-tubulin in acute EAE, but that carbonylation of these proteins does not enhance their susceptibility to calpain-mediated proteolysis and (2) the proteasome may contribute to removal of carbonylated cytoskeleton, though it does not play a significant role in the axonal damage observed in acute EAE. This is the first study to investigate the relationship between protein carbonylation and targeted degradation *in vivo*, and provides insight into the relationship between oxidative stress and axonal damage during neuroinflammation.

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1. Introduction and Background

1.1 Multiple Sclerosis and its animal model Experimental Autoimmune Encephalomyelitis (EAE)

Multiple Sclerosis (MS) is an inflammatory disorder of the CNS and is a leading cause of neurologic disability in young adults. Believed to be autoimmune in nature, MS is characterized by perivenular infiltration of lymphocytes and macrophages in the CNS white matter with the ensuing development of well-defined areas of demyelination and astrocytic proliferation and varying degrees of oligodendrocyte death and axonal damage (Keegan & Noseworthy 2002). Neurologic disability in MS is attributed to pathological changes (Kornek & Lassmann 1999). Most patients with MS (85%) have a relapsing-remitting form (RR-MS), with periods of neurologic disability, termed "attacks," followed by complete recovery. RR-MS can transform into secondary progressive MS (SP-MS), characterized by increasing irreversible neurologic decline with attacks superimposed (Keegan & Noseworthy 2002). Axonal degeneration in MS is thought to be a major component of the irreversible neurologic disability that patients experience during the progressive stages of the disease. Although the cause of axonal transection is not known, pathological studies in MS brains implicate inflammatory mediators as a primary source. The number of transected axons, determined by identification of axonal ovoids, is increased 13-fold in active compared to chronic lesions (Trapp *et al.* 1998). This initial damage during inflammation has long lasting effects. Axonal density in normal appearing white matter of the spinal cord is reduced up to 57% in SP-MS patients (Lovas *et al.* 2000), and 50-80% in the spinal cord of paralyzed patients (Trapp *et al.* 1998).

Experimental autoimmune encephalomyelitis (EAE) shares a number of clinical and pathological features with MS, and is routinely employed to study the mechanistic bases of disease and to test therapeutic approaches (Gold *et al.* 2000). Animal models show similar association of inflammation as the primary cause of axonal damage. In Lewis rat EAE, acute axonal pathology, measured by axonal staining for β-amyloid precursor protein in active lesions, is present and correlates with the extent of macrophage infiltration (Kornek *et al.* 2000). In a chronic EAE model, with repeated inflammatory events in the spinal cord, no permanent neurologic disability was seen after the first attack despite a 22% axonal loss (Wujek *et al.* 2002). After multiple attacks mild permanent disability was seen with 28-48% axonal loss, and non-remitting paralysis was associated with 43-59% axonal loss. Thus, the number of attacks correlated strongly with both percent axonal loss and sustained disability. These studies support the notion that compensatory mechanisms will allow for neurologic recovery despite damage to axons during inflammatory events (in the relapsing-remitting stage MS) until a threshold level of axonal damage is sustained, after which more permanent disabilities (seen in the secondary-progressive MS) manifest themselves.

EAE, a widely-used animal model of MS, can be induced in a variety of mammalian species by active immunization with myelin-specific antigens (e.g., myelin basic protein (MBP), myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG)) or by adoptive transfer of T-cells from immunized animals into naïve recipients (Day 2005). MBP-induced EAE in the Lewis rat, the animal model used in this study, is an acute monophasic clinical disease that resolves spontaneously within 5 days of onset. At the peak of disease there are perivenular infiltrates of inflammatory

cells within the spinal cord. There is, however, no demyelination of CNS axons, making this an ideal model to study the neuroinflammatory aspects of MS. Inflammatory lesions are the primary location of damaged axons in MS and EAE (Kornek *et al*. 2000).

1.2 Oxidative Stress and its role in MS and EAE

1.2.1 Oxidative stress and its resulting damage to cellular components

Oxidative stress occurs when reactive oxygen species are increased beyond the levels at which cellular antioxidant defense systems can mitigate their damage to cellular components (Mancuso *et al.* 2009). Oxidative stress and its resulting damage to cellular structures are thought to be an important pathophysiological feature of MS and EAE (reviewed in Gilgun-Sherki *et al.* 2004, LeVine 1992). A number of reactive oxygen species (ROS) are generated both intracellularly and extracellularly in MS and EAE (LeVine 1992, Smith *et al.* 1999, Bizzozero 2009). The major ROS generated extracellularly by infiltrating phagocytes and by activated microglia are hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and peroxynitrite (ONOO-) (Smith *et al*. 1999). H_2O_2 is formed by non-enzymatic dismutation of superoxide, which is the product of one-electron reduction of O_2 via NADPH oxidase. H_2O_2 is a highly diffusible and relatively unreactive molecule, and therefore it can rapidly enter cells. In the presence of transition metals (Fe^{2+}, Cu^{+}), H_2O_2 readily decomposes into the highly reactive hydroxyl radical ($OH^{\cdot}$) which can oxidize a large variety of macromolecules. Hypochlorite is a stable and potent oxidant produced by reaction of H_2O_2 with chloride ions via myeloperoxidase, an enzyme present in neutrophils and some types of monocytes. The intracellular ROS (mostly $O_2^{\cdot-}$ and H_2O_2) are largely made by dysfunctional mitochondria (Smith *et al*. 1999), though the contribution from other sources like xanthine oxidase,

microsomal cytochrome P-450, and cyclo/lipooxygenases may be also significant. Several processes that occur in MS and EAE, such as glutamate excitotoxicity, axonal depolarization, and glutathione depletion, lead to mitochondrial damage and ROS production (Smith *et al*. 1999). Of these processes, glutamate excitotoxicity is the most important. Activated immune cells (macrophages and microglia) release large amounts of glutamate, and excitotoxic damage has been shown to take place in both EAE (Pitt *et al.* 2000, Smith *et al.* 2000) and MS (Stover *et al.* 1997, Werner *et al.* 2001). The pathogenic role of glutamate excitotoxicity in inflammatory demyelination is supported by three major findings. First, the type of demyelinating lesions and axonal damage caused by excitotoxins are histologically similar to those observed in MS (Matute 1998). Second, the treatment of EAE mice with AMPA/kainate antagonists ameliorates the clinical symptoms, increases oligodendrocyte survival and reduces dephosphorylation of NFH, an indicator of axonal damage (Pitt *et al*. 2000, Smith *et al*. 2000). In addition, administration of the glutamate antagonist riluzole has been shown to lessen the clinical severity, inflammation, demyelination and axonal damage of EAE animals (Gilgun-Sherki *et al.* 2003). Third, CSF levels of glutamate in MS show a positive correlation with the severity and course of the disease (Barkhatova *et al.* 1998, Stover *et al*. 1997). It is noteworthy that high quantities of glutamate may also be released in MS as consequence of sustained, spontaneous, repetitive electrical discharges in demyelinated (Kapoor *et al.* 1997) and injured axons (Stys 2005). However, regardless of the cellular origin of this neurotransmitter, the prolonged activation of brain cells by glutamate triggers membrane depolarization and influx of calcium, which in turn results in mitochondrial production of ROS. The above studies indicate that ROS can be generated

not only by inflammatory cells but also by dysfunctional mitochondria as the result of glutamate excitotoxicity.

The occurrence of oxidative stress in tissues is concurrent with the reduced levels of glutathione, a major cellular antioxidant, and the increase in lipid peroxidation products, including 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and acrolein. Lipid peroxidation products have been found in both CSF and plasma of MS patients (Hunter *et al.* 1985, Naidoo & Knapp 1992). Increased free radical activity and a decrease in both antioxidants and major antioxidant enzymes have also been reported (Langemann *et al.* 1992, LeVine 1992). While oxidation of lipids and nucleic acids in these disorders has been studied extensively, until recently protein oxidation has received little attention. Protein carbonylation is an oxidative damage to proteins that occurs in many neurodegenerative disorders such as Alzheimer's disease (Aksenov *et al.* 2001), Parkinson's disease (Floor & Wetzel 1998) and amyotrophic lateral sclerosis (Ferrante *et al.* 1997). Carbonylation of proteins can have deleterious effects on their biological activity. For example, carbonylation of both actin and tubulin protein molecules can lead to instability and depolymerization of the filaments (Yan & Sohal 1998). Our laboratory has shown that protein carbonyls also build up in white matter and gray matter of MS patients (Bizzozero *et al.* 2005b) and in murine EAE (Zheng & Bizzozero 2010a). In the present study I aimed to characterize protein oxidation in MBP-induced EAE in the Lewis rat, an animal model that mimics neuroinflammatory features of MS, and investigate the mechanisms and consequences of this oxidative damage during neuroinflammation.

1.2.2 Mechanisms and implications of protein carbonylation

Carbonylation of proteins is an irreversible form of damage since there are no known enzymatic mechanisms that can repair proteins once they become carbonylated (Bizzozero 2009). Protein carbonyls are relatively difficult to induce compared to other oxidative modifications and are considered to occur under conditions of severe or prolonged oxidative stress (Dalle-Donne *et al.* 2003). Protein carbonyls are a useful maker of oxidative stress because they are a permanent modification for the life of the protein (Bizzozero 2009). Although the protein backbone and the side chains of most amino acids are susceptible to oxidation, the non-enzymatic addition of aldehydes or ketones to specific amino acid residues (i.e., carbonylation) constitutes the major and most common oxidative alteration (Berlett & Stadtman 1997, Dalle-Donne *et al*. 2003, Nystrom 2005).

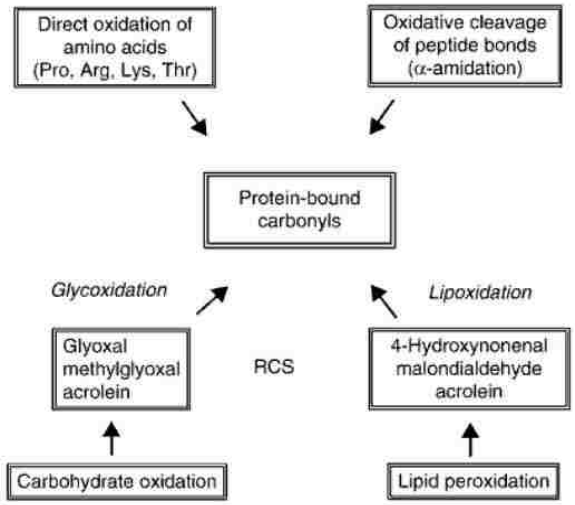

Figure 1.1 Mechanisms of formation of protein-bound carbonyls.
(From Bizzozero O.A., Protein carbonylation in neurodegenerative and demyelinating CNS disease. Handbook of Neurochemistry and Molecular Neurobiology. 2009. pp, 543-562)

Carbonyl groups can be introduced into proteins either by direct (oxidative) reaction of ROS (e.g., hydrogen peroxide, lipid hydroperoxides) with protein side chains or the

protein backbone, or via indirect (non-oxidative) addition of reactive carbonyl species (RCS) to Cys, His and Lys residues (Figure 1.1). RCS are carbonyl-containing molecules resulting from the oxidation of lipids (e. g. 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), acrolein) or carbohydrates (e.g., glyoxal, methylglyoxal). When this oxidative attack affects the protein backbone, either through alpha-amidation pathway (Figure 1.2) or through oxidation of glutamyl side chains, the resulting peptide has an alpha-ketoacyl

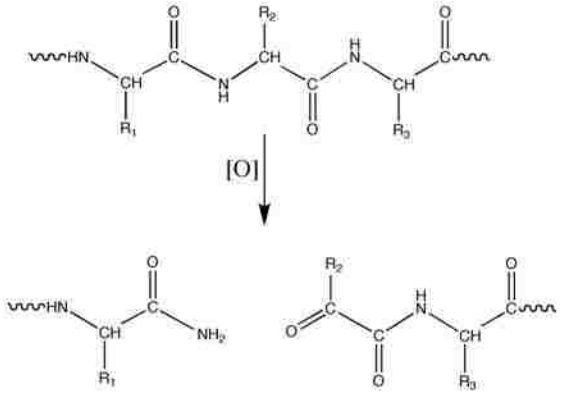

Figure 1.2. Cleavage of the protein backbone via the α-amidation pathway.

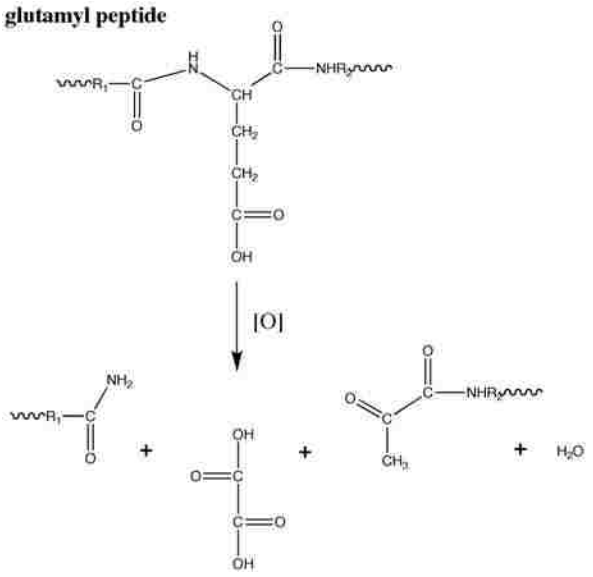

Figure 1.3. Oxidation of the protein backbone through glutamyl side chain.

derivative blocking its N-terminal amino acid (Berlett and Stadtman, 1997) (Figure 1.3). The metal-catalyzed oxidation of the amino acid side chains of arginine, lysine, proline, and threonine also result in protein carbonyls. Oxidative deamination reactions transform prolyl and arginyl residues into glutamic semialdehyde (Figure 1.4) and lysyl residues into aminoadipic semialdehyde (Figure 1.5). Oxidation of the secondary alcohol group of threonine residues results in its conversion to α-amino-β-ketobutyryl (Figure 1.6).

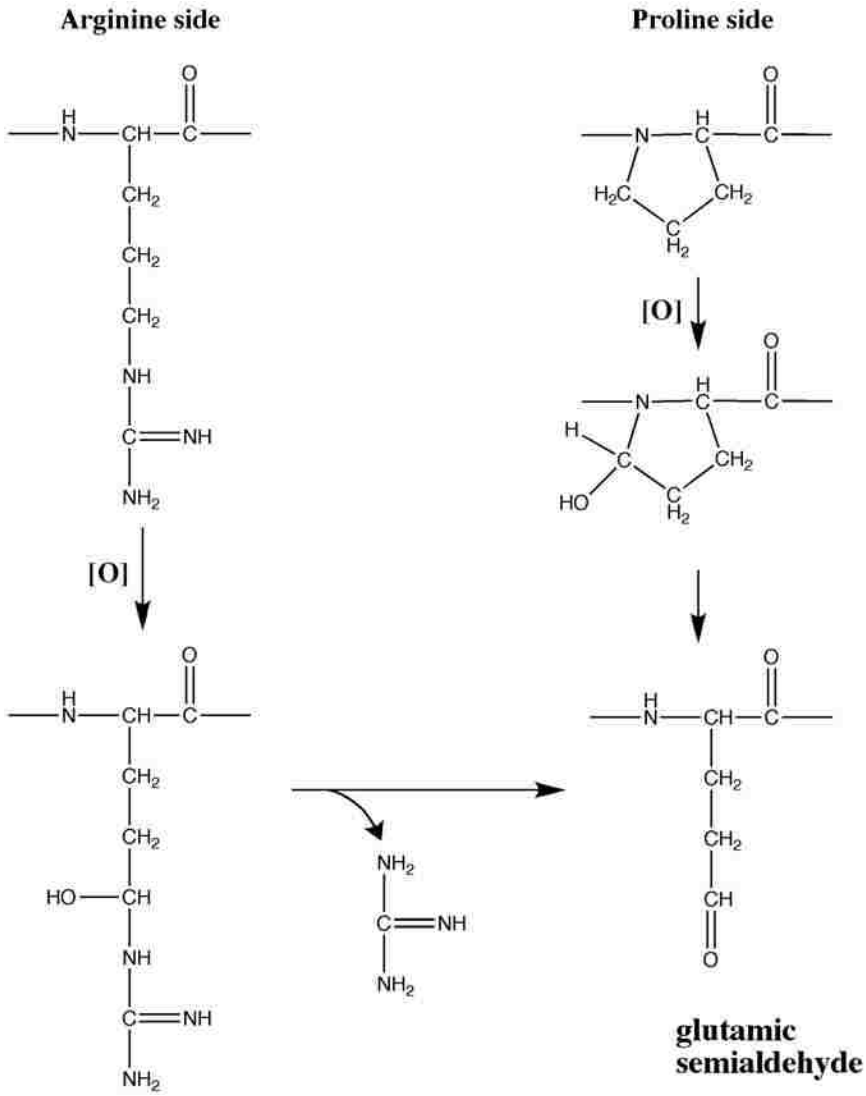

Figure 1.4. Oxidative deamination reaction mechanism of arginine and proline side chains resulting in transformation to glutamic semialdehyde.

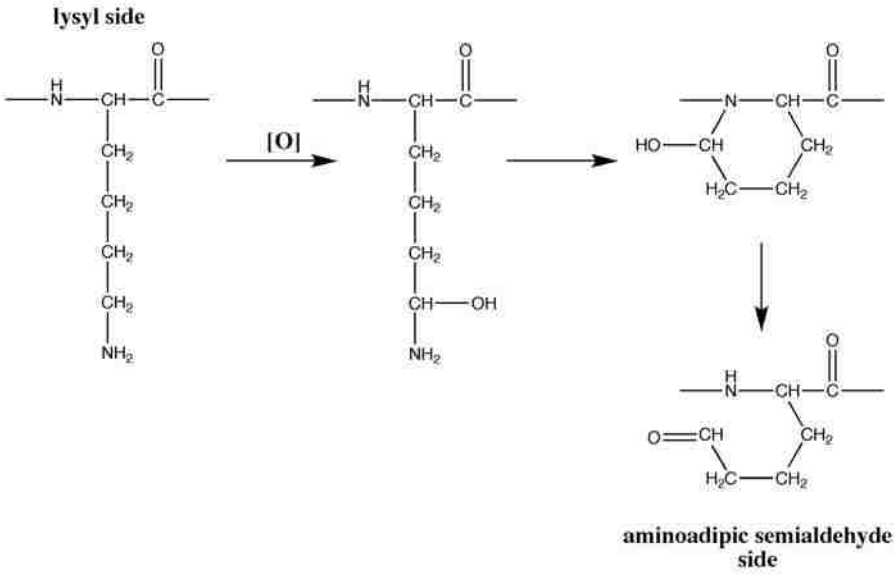

Figure 1.5. Oxidative deamination reaction mechanism of lysine side chains.

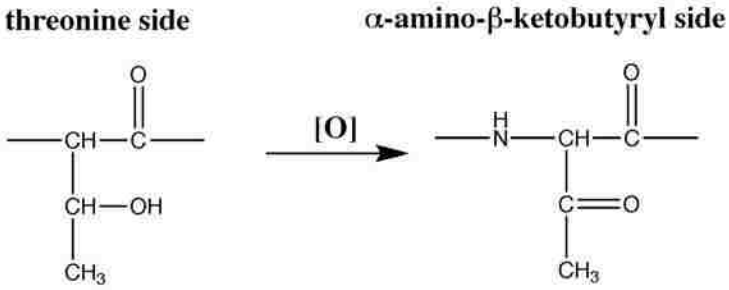

Figure 1.6. Direct oxidative attach of the threonine side chain.

As indicated above, protein carbonyls can also arise from indirect mechanisms. These reactions are not the result of direct attack by reactive oxygen species. Instead, as lipid and carbohydrates are oxidized the carbonyl containing byproducts of these reactions can attach to the protein. As previously discussed, peroxidation of

polyunsaturated fatty acids (PUFA) forms the aldehyde lipid peroxidation products 4-HNE and MDA. 4-HNE is capable of reacting with lysine, histidine, and cysteine, residues. Malondialdehyde reacts with protein amino groups. Other reactive carbonyl species, including reducing sugars and their oxidation products (e. g., glyoxal, methylglyoxal), can participate in non-oxidative reactions to introduce carbonyls into proteins.

While both direct and indirect oxidative mechanisms can introduce carbonyls into proteins, the relative contribution of the different mechanisms is still controversial. While inhibition of lipid peroxidation will reduce the amount of protein carbonyls in diseased tissues, but modified amino acids resulting from direct pathways have been found to be the major modification in human brain tissue. Increased mitochondrial production of hydrogen peroxide and superoxide using glutathione depletion in rat brain slices increases protein carbonyls and is not reversed using RCS scavengers (Zheng & Bizzozero 2010c). Furthermore, cerebellar proteins from EAE mice at the peak of neurological disease do not contain acrolein, 4-HNE or MDA adducts, as determined by western blot with specific antibodies against these moieties (Zheng & Bizzozero 2010a). These findings suggest that direct oxidation of amino acids is the chief mechanism resulting in protein carbonyls.

Cytoskeletal proteins are established targets of protein carbonylation in many diseases including CNS disorders (Aksenov *et al*. 2001, Muntane *et al.* 2006). As described in Chapter 2, I observed that β-actin, β-tubulin, and the three neurofilament chains are the major targets of carbonylation in acute EAE. More importantly, carbonylation of cytoskeletal proteins is known to have deleterious effects on the

macromolecule structures. For instance, actin filaments and microtubules both destabilize or disassemble upon oxidation of their protein components (Banan *et al.* 2001, Banan *et al.* 2004, Dalle-Donne *et al.* 2001, Neely *et al.* 2005, Ozeki *et al.* 2005). The secondary structure of neurofilaments is also altered when the individual proteins become carbonylated (Gelinas *et al.* 2000) often leading to the formation of dense aggregates (Smith *et al.* 1995). Oxidized neurofilament proteins also have increased propensity toward proteolysis (Troncoso *et al.* 1995). One of the aims of this thesis is to investigate potential consequences of protein carbonylation on the metabolic stability of cytoskeletal proteins in EAE.

1.2.3 Accumulation of protein carbonyls in the CNS of MS patients and EAE animals

Protein carbonylation is an irreversible form of damage resulting from severe or prolonged oxidative stress conditions (Dalle-Donne *et al*. 2003), which occurs in many neurodegenerative disorders including Alzheimer's disease (Aksenov *et al*. 2001), Parkinson's disease (Floor & Wetzel 1998) and amyotrophic lateral sclerosis (Ferrante *et al*. 1997). Our discovery that protein carbonyls are augmented in MS (Bizzozero *et al.* 2005b) and in mice with EAE (Zheng & Bizzozero 2010a), suggests that this type of chemical modification may also play a critical pathophysiological role in inflammatory demyelinating diseases. A major goal of this study was to investigate whether protein carbonyls accumulate during the inflammatory period in acute EAE and whether those carbonylated proteins persist in CNS tissue upon resolution of the single neuroinflammatory event that characterizes MBP-induced EAE in the Lewis rat. As described in Chapter 2, I observed that protein carbonylation increases coincident with the peak neurologic disability in the spinal cord tissue of EAE rats, but the animals are

able to clear the carbonylated proteins from their CNS tissue when neurologic symptoms disappear. Because carbonylation is irreversible, the reduction in protein carbonyls indicates that proteolytic mechanisms are working to remove these damaged proteins from the spinal cord.

1.3 Proteolytic mechanisms involved in axonal damage and protein carbonyl removal

Magnetic resonance spectroscopic imaging studies closely link the amount of axonal damage to the neurologic disability seen in individual MS patients (De Stefano *et al.* 1998, Matthews *et al.* 1998). Significant loss of axons, up to 60%, in early and chronic MS lesions has been reported (Lovas *et al*. 2000, Mews *et al.* 1998). Axons are particularly susceptible to injury during neuroinflammation, although neuronal cell bodies are also damaged (Coleman & Perry 2002). Complete axonal transection leading to the build up of neurofilament proteins in amyloid precursor protein (APP)-positive ovoids has also been observed in MS lesions (Ferguson *et al.* 1997, Trapp *et al*. 1998). Although axonal damage can be found in normal appearing white matter, much of the damage to axons is localized in areas of high microglia, macrophage and cytotoxic T-lymphocyte activity. Upon activation these cells all produce ROS causing oxidative damage to axons in the inflammatory lesions. Additionally, the high metabolic requirements of neurons increase the propensity of oxidative damage to axonal proteins by mitochondrial sources of ROS (Neumann 2003). In this study I investigate damage and proteolysis of major axonal proteins, focusing on cytoskeletal proteins. As described in chapter 2, I show that there is significant degradation of major axonal cytoskeleton proteins and that these proteins are also carbonylated.

There are multiple possible fates for carbonylated proteins, and the outcome likely depends on the specific protein being carbonylated, as well as the level of oxidative damage it sustains. The concentration of carbonylated proteins within cells is normally extremely low; although even moderate increases in carbonylation can have tremendous effects on protein function, aggregate formation, or metabolic stability. Maintenance of low carbonylation level is achieved through the action of several proteolytic systems that preferentially digest oxidized proteins. It has been proposed that targeted digestion is a consequence of unfolding as a result of the oxidative modification (Grune *et al.* 2004). The core 20S proteasome is known to catalyze the degradation of oxidized proteins in an ATP- and ubiquitin-independent manner, and is thought to be the primary clearance mechanism for carbonylated proteins in cells (Grune *et al.* 1997). For example, during myocardial ischemia/reprofusion oxidized actin is removed by the 20S proteasome (Divald & Powell 2006). Oxidized protein aggregates accumulate during aging, and it has been proposed that this may be a direct consequence of reduced proteosomal activity with aging (Agarwal & Sohal 1994, Bota & Davies 2002). Proteasomal activity is also reduced in chronic murine EAE and may contribute to accumulation of protein carbonyls in that model (Zheng & Bizzozero 2010b). However, other proteases have been implicated in the hydrolysis of oxidized proteins as well. For example, the calcium-dependent cysteine protease calpain has been shown to preferentially degrade oxidized neurofilament over non-oxidized protein *in vitro* (Troncoso *et al*. 1995). In culture, calcium import into cells both activates calpain and stimulates carbonylation of mitochondrial apoptosis inducible factor protein (AIF); in this system calpain-mediated proteolysis of AIF occurs at a rate five times faster than degradation of non-oxidized AIF (Norberg *et al.* 2010). Calcium

homeostasis is altered during EAE and it is known that calpain is activated in neurons (Guyton *et al.* 2005), glia and inflammatory cells (Shields & Banik 1998) coincident with inflammation in the Lewis rat model.

While many proteins become more susceptible to degradation by cellular proteases upon carbonylation of one or more residues (Stadtman 1990), other oxidized proteins become more resistant to proteolysis. For example, mildly carbonylated aconitase is proteolytically degraded but when carbonyl modifications affect more amino acid residues high-molecular-weight aggregates form, and unlike the mildly oxidized species these aggregates are not susceptible to break down by the mitochondrial Lon protease (Bota & Davies 2002). Additionally, carbonylated proteins can inhibit the proteases that degrade them, and carbonylation of the proteases themselves impairs their function (Friguet *et al.* 1994, Grune *et al*. 2004, Shacter 2000). Ultimately, it is this phenomenon that likely causes the long-term accumulation of carbonylated species in diseased tissues. As their concentration within cells builds up, carbonylated proteins tend to cross-link forming high-molecular-weight aggregates, which are both cytotoxic and no longer susceptible to proteolysis (Nie *et al.* 2007, Smith *et al*. 1995).

While there is significant evidence for preferential proteolysis of carbonylated proteins *in vitro*, the function and relative contributions of these proteolytic mechanisms has not been adequately demonstrated *in vivo*. For this reason I tested two major proteolytic systems, calpain and the 20S proteasome, known to preferentially digest oxidatively modified proteins. By inhibiting these systems in the CNS tissue of EAE animals I sought to investigate which, if either, of these pathways was responsible for clearance of carbonylated proteins during neuroinflammation. As described in chapter 3,

modest inhibition of calpain led to accumulation of protein carbonyls in the CNS tissue, but small reduction in proteasome activity did not significantly affect carbonyl levels. My results suggest that the accumulation of protein carbonyls after calpain inhibition appears to be a consequence of overall sparing of cytoskeletal proteins, which are major targets of carbonylation, rather than the blocking of a pathway that specifically targets oxidized proteins for removal.

1.4 Hypothesis

I proposed to test the hypothesis that oxidative stress conditions occur in acute experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, and lead to increased oxidation (carbonylation) of cytoskeletal proteins. Oxidation of these proteins augments their susceptibility to proteolytic degradation and thereby contributes to the axonal damage that is characteristic of inflammatory demyelinating disorders.

1.5 Rationale for a study of protein oxidation and its consequences in EAE

This study built upon findings in our laboratory that protein carbonyls accumulate in the CNS tissue of people with multiple sclerosis. The goal of this thesis work was to characterize oxidative stress and its proteolytic consequences in the Lewis rat EAE model for MS. This animal model mimics the myelin-directed neuroinflammatory events in MS, not the chronic demyelination seen in later disease stages.

The study in Chapter 2 was designed to characterize markers oxidative stress in EAE spinal cord tissue throughout the disease course, and to determine if EAE displays oxidative damage to proteins similar to that seen in MS. I sought to determine whether

changes in protein carbonylation levels occur during the course of EAE and to identify specific proteins that are carbonylated in this disorder. The focus was on cytoskeletal proteins because (1) their abundance in cells makes them common targets of various ROS and RCS (Dalle-Donne *et al.* 2006), and (2) they are major targets of oxidation in MS (Bizzozero 2007). The results clearly show that the oxidative stress conditions generated during the course of MBP-induced EAE in the Lewis rat are sufficient to cause protein carbonylation. I found that cytoskeletal proteins, including neurofilaments, β-tubulin, β-actin, and GFAP were major targets of protein carbonylation, and neurofilaments and β-tubulin were degraded during the disease course. This chapter also shows that the percentage of neurofilament light chain protein (NFL) that is carbonylated changes during the disease course. A decrease in the percentage of oxidized NFL suggests that carbonylation of this protein may trigger proteolytic degradation of these molecules in EAE.

The goal of the studies described in Chapter 3 was to explore the possibility of a link between the carbonylation and degradation of cytoskeletal proteins during EAE. The claim that oxidative protein damage targets proteins for degradation has been asserted in the literature and is generally accepted as true. However, the studies that address the topic use either cell-free or cell culture systems. I used the EAE model to investigate the relationship between cytoskeletal oxidation and degradation *in vivo*, and to identify the proteolytic machinery responsible for cytoskeletal degradation. To this end, EAE animals were treated with inhibitors for calpain or the proteasome for 48 hours during the peak disease activity. By inhibiting the cellular mechanisms of proteolytic degradation I was able to investigate the contribution of the different degradation pathways to

cytoskeletal loss and to accumulation of carbonylated protein species. In order to address whether carbonylated proteins are preferentially degraded, I determined the specific proportion of carbonylated cytoskeletal proteins in inhibitor-treated and vehicle-treated EAE animals. This approach assesses the relative degradation of oxidized and normal cytoskeletal proteins. If indeed my hypothesis were correct, then inhibiting a pathway that preferentially degrades oxidized proteins would lead to accumulation of carbonylated proteins and higher specific oxidation of cytoskeletal species. Treating EAE animals via intrathecal (i.t.) injection of the calpain inhibitor calpeptin prevented significant degradation of neurofilaments and β-tubulin proteins and led to an overall increase in the accumulation of protein carbonyls in the spinal cord tissue. However, I was unable to detect changes in the specific oxidation of any of these proteins in the calpeptin-treated animals compared to vehicle-injected EAE rats. Treatment with the proteasome inhibitor epoxomicin did not significantly alter cytoskeletal levels or specific oxidation. These findings suggest that calpain plays a critical role in removal of cytoskeletal proteins in EAE, but does not preferentially digest carbonylated proteins.

Chapter 4 recaps the data presented in chapters 2 and 3. In this chapter I work to bring the findings of this thesis together and put the work into context, assessing the overall contribution of my thesis to understanding the mechanisms that lead to cytoskeletal protein carbonylation and digestion during inflammation in EAE. Taken together the findings of this thesis provide valuable insights into the relationship beteween neuroinflammation and oxidation of neuronal cytoskeleton and into the mechanisms involved in removal of damaged cytoskeletal proteins. These studies, to the best of my knowledge, are also the first to explore the role of different proteolytic

mechanisms in the removal of carbonylated proteins in an *in vivo* system and to demonstrate that oxidative damage to proteins is only a minor player in their degradation during acute inflammation. This work also shows that neither calpain nor the proteasome appear to preferentially target carbonylated cytoskeletal proteins for removal.

2. Cytoskeletal protein carbonylation and degradation in experimental autoimmune encephalomyelitis

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2.1 Abstract

Protein carbonylation, the non-enzymatic addition of aldehydes or ketones to specific amino acid residues, has been implicated in the pathophysiology of multiple sclerosis (MS). In this study we investigated whether protein carbonyls (PCOs) also accumulate in the spinal cord of Lewis rats with acute experimental autoimmune encephalomyelitis (EAE). Western blots analysis after derivatization with dinitrophenyl hydrazine (oxyblot) showed elevated protein carbonylation at the time of maximal clinical disability. During the same period glutathione levels were substantially reduced, suggesting a causal relationship between these two markers. In contrast, lipid peroxidation products accumulated in EAE spinal cord well before the appearance of neurological symptoms. Carbonyl staining was not restricted to inflammatory lesions but present throughout the spinal cord particularly in neuronal cell bodies and axons. By 2-dimensional-oxyblot we identified several cytoskeletal proteins, including β-actin, GFAP and the neurofilament proteins as the major targets of carbonylation. These findings were confirmed by pull-down experiments, which also showed an increase in the number of carbonylated β-actin molecules and a decrease in that of oxidized neurofilament proteins in EAE. These data suggest the possibility that oxidation targets neurofilament proteins for degradation, which may contribute to axonal pathology observed in MS and EAE.

2.2 Introduction

Experimental autoimmune encephalomyelitis (EAE) shares a number of clinical and pathological features with MS, and is routinely employed to study the mechanistic bases of disease and to test therapeutic approaches (Gold *et al*. 2000). This animal model of MS can be induced in a variety of mammalian species by active immunization with myelin-

specific antigens (e.g., MBP, PLP, MOG) or by adoptive transfer of T-cells from immunized animals into naïve recipients (Day 2005). MBP-induced EAE in the Lewis rat, the animal model used in this study, is an acute monophasic clinical disease that resolves spontaneously within 5 days of onset. At the peak of disease there are perivenular infiltrates of inflammatory cells within the spinal cord. There is, however, no demyelination of CNS axons, making this an ideal model to study the neuroinflammatory aspects of MS.

In recent years a significant body of experimental evidence has accumulated demonstrating that oxidative stress is a major player in the pathogenesis of both MS and EAE (for reviews, see Gilgun-Sherki *et al*. 2004, LeVine 1992). While oxidation of lipids and nucleic acids in these disorders has been studied extensively, protein oxidation has received little attention. The non-enzymatic addition of aldehydes or ketones to specific amino acid residues (i.e. carbonylation) constitutes the major and most common oxidative alteration (Berlett & Stadtman 1997, Dalle-Donne *et al*. 2003, Nystrom 2005). Carbonyl groups can be introduced into proteins either by direct (oxidative) reaction of ROS (e.g., hydrogen peroxide, lipid hydroperoxides) with protein side chains or indirect (non-oxidative) addition of reactive carbonyl species (RCS) to Cys, His and Lys residues. RCS are carbonyl-containing molecules resulting from the oxidation of lipids (e.g., 4-hydroxynonenal, malondialdehyde (MDA), acrolein) or carbohydrates (e.g., glyoxal, methylglyoxal). Protein carbonylation is an irreversible form of damage that results from severe or prolonged oxidative stress conditions (Dalle-Donne *et al*. 2003), which occurs in many neurodegenerative disorders including Alzheimer's disease (Aksenov *et al*. 2001), Parkinson's disease (Floor & Wetzel 1998) and amyotrophic lateral sclerosis

(Ferrante *et al.* 1997). Our discovery that protein carbonyls are augmented in MS (Bizzozero *et al.* 2005a) suggests that this type of chemical modification may also play a critical pathophysiological role in inflammatory demyelinating diseases.

There are multiple possible fates for carbonylated proteins; the outcome likely depends on the specific protein being carbonylated as well as the level of oxidative damage it sustains. The concentration of carbonylated proteins within cells is normally extremely low; although even moderate increases in the carbonylation can have tremendous effects on protein function, aggregate formation, or metabolic stability. Maintenance of low carbonylation level is achieved through the action of several proteolytic systems that preferentially digest oxidized proteins. The core 20s proteasome is known to catalyze the degradation of oxidized proteins in an ATP- and ubiquitin-independent pathway, and is thought to be the primary clearance mechanism for carbonylated proteins in cells (Grune *et al*. 1997). However, other proteases have been implicated in the hydrolysis of oxidized proteins as well. For example, the calcium-dependent cysteine protease calpain has been shown to preferentially degrade oxidized neurofilament over non-oxidized protein (Troncoso *et al*. 1995). While many proteins become more susceptible to degradation by cellular proteases upon carbonylation of one or more residues (Stadtman 1990), other oxidized proteins become more resistant to proteolysis. Additionally, carbonylated proteins can inhibit the proteases that degrade them, and carbonylation of the proteases themselves impairs their function (Friguet *et al*. 1994, Shacter 2000). Ultimately it is this phenomenon what likely causes the accumulation of carbonylated species in diseased tissues. As their concentration within cells build up, carbonylated proteins tend to crosslink forming high-molecular-weight

aggregates, which are both cytotoxic and no longer susceptible to proteolysis (Nie *et al*. 2007, Smith *et al*. 1995).

The present study was designed to determine if EAE displays oxidative damage to proteins similar to that seen in MS. We sought to determine whether changes in protein carbonylation levels occur during the course of EAE and to identify specific proteins that are carbonylated in this disorder. Our focus was on cytoskeletal proteins because (1) their abundance in cells makes them common targets of various ROS and RCS (Dalle-Donne *et al*. 2006), and (2) they are major targets of oxidation in MS (Bizzozero *et al.* 2007). The results clearly show that the oxidative stress conditions generated during the course of MBP-induced EAE in the Lewis rat are sufficient to cause protein carbonylation and that this process may trigger proteolytic degradation of oxidized cytoskeletal proteins. To the best of our knowledge this is the first study demonstrating protein carbonylation in EAE. A preliminary account of these findings has been presented in abstract form (Smerjac & Bizzozero 2006, Smerjac & Bizzozero 2007).

2.3 Materials and Methods

2.3.1 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. Seven-week-old male Lewis rats were purchased from Harlan Bioproducts (Indianapolis, IN) and housed in the UNM-animal resource facility. EAE was induced in these animals as described by Schaecher *et al.* 2002) with slight modifications. Briefly, rats received two subcutaneous

injections into the lower back area of 100µl each containing 200 mg/ml of guinea pig spinal cord homogenate plus 280 µg/ml of guinea pig myelin basic protein (Sigma, St. Louis, MO) in saline mixed with complete Freund's adjuvant (CFA) supplemented with 1 mg/ml of heat killed Mycobacterium tuberculosis H37Ra (Chondrex Inc; Redmond, WA). Control animals were given CFA without myelin antigens. Two hours after EAE induction, all animals received an i.p. injection of 2 µg of pertussis toxin (List Biological Laboratories; Campbell, CA) in 100 µl of saline. Animals were weighed and examined daily for the presence of neurological signs. At prescribed days post-injection (DPI) animals were euthanized by decapitation. Spinal cords were dissected and either fixed with methacarn 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 -80˚C until use. Protein concentration was assessed with the Bio-Rad DCT protein assay (Bio-Rad Laboratories; Hercules, CA) using bovine serum albumin as standard.

2.3.2 Determination of glutathione (GSH)

The amount of non-protein thiols, of which > 90% is GSH (Vitvitsky *et al.* 2006), was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma). Briefly, an aliquot of the spinal cord homogenate prepared in PEN buffer without DTT was mixed with an equal volume of 2% sulfosalicylic acid to precipitate the proteins. After centrifugation at 10,000g for 15 min, the supernatants were mixed with 0.2M sodium phosphate buffer pH 7.5 containing 0.3 mM DTNB, 10 mM EDTA and 1% SDS and incubated for 15 min at room temperature. Absorbance was measured at 412 nm using a Hewlett-Packard 8452-A Diode Array Spectrophotometer. The amount of thiol groups was calculated using a

molar extinction coefficient of 13,600 cm-1 for the thionitrobenzoate anion (Riddles *et al.* 1979).

2.3.3 Measurement of lipid peroxidation

Lipid peroxidation was assessed by measuring the amount of thiobarbituric acid reactive substances (TBARS), which are byproducts of lipid peroxidation, in the tissue homogenates (Ohkawa *et al.* 1979). One-hundred-µl aliquots (~500µg of protein) were mixed with 25µl of 2% (w/v) butylated hydroxytoluene and 875µl of 1% (w/v) thiobarbituric acid (Aldrich; Milwaukee, WI) prepared in 10% (w/v) trichloroacetic acid. Samples were incubated for 20 min at 90˚C. Aggregated material was removed by centrifugation at 10,000g for 15 min and the absorbance of the supernatant was measured at 532 nm. The amount of TBARS was calculated using a standard curve prepared with 1,1,3,3-tetraethoxypropane (Aldrich).

2.3.4 Assessment of protein carbonylation by western blotting

Protein carbonyl groups were measured with the OxyBlot™ protein oxidation detection kit (Intergen Co., Purchase, NY), following the protocol provided by the manufacturer. In brief, proteins (5µg) were incubated with 2,4-dinitrophenyl-hydrazine to form the 2,4-dinitrophenyl (DNP) hydrazone derivatives. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride (PVDF) membranes. DNP-containing proteins were then immunostained using rabbit anti-DNP antiserum (1:500) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:2000). Blots were developed by enhanced chemioluminescence (ECL) using the Western Lightning ECL™ kit from Perkin-Elmer (Boston, MA). The

developed films were scanned in a Hewlett Packard Scanjet 4890 and the images quantified using the Scion Image for Windows, version Alpha 4.0.3.2 (Scion Corporation, Frederick, MD).

2.3.5 Two-dimensional gel electrophoresis of carbonylated spinal cord proteins

DNP-labeled proteins were also dissolved in isoelectric focusing (IEF) buffer (8M urea, 2% CHAPS, 50mM DTT and 0.2% (w/v) Bio-Lyte® 3/10 ampholytes). Proteins were absorbed on 11cm-long ReadyStrip IPG® strips (pH range 3-10 non-linear; BioRad) and IEF was carried out at 30,000 V-hr. IEF strips were equilibrated for 10 min in 375mM Tris-HCl buffer pH 8.8 containing 6M urea, 2% SDS, 20% glycerol with 130mM DTT, followed by 10 min the same buffer containing 135mM iodoacetamide instead of DTT. Proteins were separated on 10% Criterion™ pre-cast gels (BioRad) followed by western blotting carried out as described above. Blots were probed with anti-DNP antibodies, then stripped and probed with antibodies against specific cytoskeletal proteins. Spot matching between blots was performed using The Discovery Series PDQuest 2-D Analysis Software Version 7.0.1 (BioRad).

2.3.6 Identification of carbonylated proteins

Tissue proteins (0.5mg), dissolved in 200 µl 2% SDS, were incubated at room temperature with 5 mM biotin-hydrazide (Sigma). After 60 min, proteins were precipitated with 1 ml of acetone at -20˚C and collected by centrifugation at 10,000g for 10 min. Pellets were washed three times with 1 ml of acetone-water (3:1, v/v) and dissolved in 100 µl of 100 mM Tris-HCl buffer pH 7 containing 1% SDS and 100 mM NaCl. The solutions were diluted 20-fold with 100 mM NaCl and centrifuged at 10,000g

for 10 min to remove any aggregated material. The supernatants were then incubated for 1 h at 20˚C with 50µl of streptavidin-agarose (Sigma) previously equilibrated in 100 mM Tris-HCl pH 7.6 containing 0.05% SDS and 100mM NaCl (buffer A). The resin was washed 5-times with 500 µl of buffer A, 4-times with buffer A containing 1M NaCl, and once with buffer A without NaCl. Bound-proteins were eluted from the resin with 100 µl of SDS-sample buffer containing 1% 2-mercaptoethanol. Aliquots from the total and bound fractions were separated by SDS-PAGE on 10% polyacrylamide gels and blotted against PVDF membranes. Blots were probed with antibodies against NFH (1:1000, rabbit polyclonal; Chemicon, Temecula, CA), NFM (1:4000, rabbit polyclonal, Chemicon), NFL (1:1000, mouse monoclonal, Chemicon), β-tubulin (1:1000, mouse monoclonal; Sigma), GFAP (1:5000, rabbit polyclonal; Dako, Carpinteria, CA) and β-actin (1:1000, mouse monoclonal; Abcam Inc., Cambridge, MA), followed by incubation with the appropriate HRP-conjugated secondary antibody. Blots were developed by ECL as described above.

2.3.7 Statistical Analysis

Results were analyzed for statistical significance with Student's unpaired t test or ANOVA utilizing the GraphPad Prism® (version 4) program (GraphPad Software Inc., San Diego, CA).

2.4 Results

2.4.1 Characteristics of Lewis rat EAE animals

EAE in male Lewis rats was induced 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; 5, moribund. Following immunization with MBP, Lewis rats experienced an acute and monophasic inflammation of the spinal cord resulting in clinical disability. Neurological symptoms begin at 9-11 DPI, peaking at 12-13 DPI, with full recovery by 16 DPI (Table 2.1). CFA-injected control and EAE rats were sacrificed on days 7, 9, 10, 11, 13, and 15 after injection. Animals injected with antigen that did not show symptoms after 12 DPI were excluded from our analysis. Spinal cord sections from vertebrae T6-L2 were removed; portions from each animal were homogenized for biochemical analysis or fixed in methacarn for IHC. Since none of the biochemical parameters corresponding to CFA injected control animals measured in this study changed during the course of the experiment, they were combined to obtain the average control values.

2.4.2 Oxidative stress occurs in the spinal cord of EAE rats: oxidation of glutathione and lipids

To establish the occurrence of oxidative damage in EAE, we measured the levels of GSH in the spinal cords of the affected animals throughout the disease course. As shown in Figure 2.1A, GSH levels in EAE spinal cords was 58% and 72% of control values at 10 and 11DPI, respectively, indicating that the CNS of the affected animals is subjected to considerable oxidative stress. Normal GSH levels were observed prior to 10 DPI and after 11 DPI.

Lipid oxidation, another commonly used marker of oxidative stress conditions, was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) in spinal cords. As depicted in Figure 2.1B, the level of TBARS augmented in

EAE at all time points examined (i.e. 7–15 DPI), demonstrating carbonyl stress conditions in the EAE spinal cord before and after the period of clinical disability.

2.4.3 Oxidative stress damage increases protein carbonylation in EAE

We recently reported that protein carbonylation in brain slices can be stimulated by depleting GSH and/or inducing lipid peroxidation (Bizzozero *et al.* 2006). Because both of these changes take place during the course of EAE, we thought that protein carbonyls might also accumulate in this disorder. The presence of protein carbonyls in the spinal cord tissue of CFA-injected control animals and EAE rats throughout the disease course was investigated using the oxyblot technique. A representative western blot detecting protein carbonyls (i.e. oxyblot) of one control and 5 EAE spinal cords from different DPI is shown in Figure 2.2A. At 11 and 13 DPI, there is a clear increase in the carbonyl content of several protein bands from EAE rat spinal cords relative to control. Quantitative analysis of the oxyblots revealed that at 11 and 13 DPI, total protein carbonyl levels in EAE spinal cords increase by 70% relative to control spinal cords (Figure 2.2B) returning to control values at 15 DPI. Since carbonylation is an irreversible protein modification, the above results indicate that a clearance mechanism to eliminate these damaged proteins must operate in the disease tissue.

The accumulation of carbonyls in EAE spinal cords was also assessed using the DNPH-based IHC procedure that we have recently developed to determine the anatomical distribution of PCOs in brain sections (Bizzozero *et al*. 2006). In this case, however, detection of PCOs was performed by immunofluorescence utilizing a TRITC-labeled secondary antibody. Figure 2.3 shows the IHC distribution of carbonyls in the

spinal cord of a control and an EAE animal at 11 DPI. The amount of carbonyls in both the gray matter (GM) and white matter (WM) from the CFA-injected control is relatively low. There is an overall increase in protein carbonyls in both WM and GM from EAE spinal cords, as opposed to localization around inflammatory lesions. Motor neurons and axons (arrows) were the most intensely stained structures in the diseased tissue, and this distribution is reminiscent to that of cytoskeletal elements. Validation of this IHC technique was performed by omitting the DNPH-treatment, the anti-DNP antibody or the secondary anti-rabbit IgG antibody (not shown). In addition, we carried out a positive control in which carbonyls are generated by incubating spinal cords sections with FeSO4/H2O2 and a specificity control in which endogenous carbonyls are removed by incubation with NaBH4.

2.4.4 Oxidation levels of β-actin and NFL change during disease

The particular staining pattern for carbonyls prompted us to investigate whether the major cytoskeletal proteins are subjected to carbonylation. Identification of carbonyl-containing proteins was initially carried out by spot matching DNP-labeled proteins on a 2-D gel with those of specific cytoskeletal elements. As shown in Figure 2.4, β-actin, GFAP and the neurofilament proteins are among the major carbonylated targets in EAE. Quantification of the extent of oxidation of these species was performed by the pull-down/western blot procedure recently used in our laboratory to identify carbonylated cytoskeletal proteins during GSH depletion (Bizzozero 2007, Bizzozero *et al*. 2006). To this end, protein carbonyls from control and 13 DPI EAE spinal cord homogenates were first converted into biotinylated residues by reaction with biotin-hydrazide. Biotin-containing proteins were then isolated with streptavidin-agarose and analyzed by western

blotting employing antibodies against the three neurofilament chains, β-tubulin, β-actin, and GFAP. The results show that all six of these proteins are targets of carbonylation, with significant changes in the amount of oxidized NFL and β-actin (Table 2.2). It is important to note that, while total protein oxidation increases in EAE (Figure 2.2), the number of molecules that are carbonylated for specific proteins may increase or decrease depending on whether or not the modified species is selectively degraded during the course of the disease.

2.4.5 Neurofilaments and β-tubulin are degraded in EAE spinal cord

The integrity of CNS cytoskeletal network was investigated by western blotting of control and EAE spinal cord proteins. Figure 2.5A shows representative western blots of the three neurofilament chains, β-tubulin, β-actin and GFAP from control and EAE spinal cord (9 and 13 DPI). Figure 2.5B depicts the relative level of these proteins (normalized for coomassie blue staining) compared to average control values throughout the disease course. There are significantly lower levels of NFH, NFM, NFL, and β-tubulin in EAE compared to CFA-treated control. Since cytoskeletal proteins are very stable (Li & Black 1996, Nixon & Logvinenko 1986), this finding clearly indicates that their disappearance from the EAE spinal cord is due to increased degradation rather than diminished synthesis. A decrease in NFL protein at 10-12 DPI has been reported previously (Shields & Banik 1998) and is believed to be indicative of increased calpain activity in EAE spinal cord. GFAP and β-actin are not degraded, and the increase in GFAP protein at 15 DPI corroborates previous reports of gliosis beginning at 14 DPI in this model (Aquino *et al.* 1988). The specific oxidation (percentage of total protein modified by carbonylation) of b-actin (a protein that is not degraded) is higher in EAE, whereas that of NFL

decreases in EAE. This finding suggests that, while b-actin is resistant to proteolysis, the oxidized neurofilament may be preferentially degraded in this disorder.

2.5 Discussion

In this study we have characterized oxidative stress conditions that occur in MBP-induced EAE in the Lewis rat by measuring established markers for oxidative damage throughout the disease course. We discovered that the concentration of free GSH in the spinal cord of EAE rats is greatly decreased at 10-11 DPI, returning to normal values at 13 DPI. There are several, and not mutually exclusive, reasons underlying the reduction of free GSH in the diseased spinal cord. These include (1) oxidation of GSH to GSSG with hydrogen peroxide or lipid hydroperoxides via a reaction catalyzed by glutathione peroxidase; (2) conjugation of GSH with reactive α,β-unsaturated aldehydes derived form lipid peroxidation (e.g., acrolein, 4-hydroxynonenal) by a reaction catalyzed by gluthathione S-transferase; (3) reduced cellular uptake of cysteine, required for GSH synthesis, due to high levels of glutamate (Sagara & Schubert 1998); (4) decreased activity of enzymes involved in the GSH synthesis (e.g., glutathione synthetase) and/or recycling of GSSG (e.g., glutathione reductase); and (5) diminished amount of NADPH, which is needed for GSSG reduction. Preliminary results in our laboratory have shown that the amount of total glutathione (i.e. GSH + GSSG) in EAE spinal cord is also reduced, suggesting that the disappearance of GSH cannot be simply attributed to its oxidation. However, regardless of the mechanism responsible for the decrease in low-molecular-weight thiols, GSH levels return to normal values as inflammation subsides and animals recover.

TBARS, the most commonly used measurement for lipid peroxidation, detects dialdehydes (mostly MDA) resulting from breakdown of lipid hydroperoxides and endoperoxides (Esterbauer *et al.* 1991). In contrast to the reduction of GSH levels, which parallels disease activity, the concentration of TBARS in the EAE spinal cords is elevated at every time point measured (i.e. 7-15 DPI). This result indicates that oxidative stress takes place prior to the appearance of inflammatory lesions in the spinal cord of EAE animals, a pathological process that correlates temporally with neurological symptoms (Schaecher *et al*. 2002). The presence of TBARS in 15 DPI animals, which are recovering neurologically, may be due to continued ROS generation or more likely to the persistence of dialdehydes in the tissue (MDA $t_{½}$ = 2 days) (Aldini *et al.* 2007).

The most important finding in this study is that several carbonylated protein species accumulate in the spinal cord of EAE rats at 11–13 DPI. The degree and distribution of protein carbonylation present during autoimmune neuroinflammation was unexpected and indicates that the oxidative stress conditions in this model are very severe. Nonetheless, in this acute animal model of MS there appears to be a mechanism that eliminates these damaged proteins and bring carbonylation to control levels as the inflammatory process subsides. Indeed, it is believed that the accumulation of carbonylated proteins is a multifaceted process that takes into account both the rate of oxidation (dependent on both ROS formation and antioxidant levels) and the rate of proteolysis of the damaged proteins (Sayre *et al.* 2005). It will be important to know if repeated tissue injury would result in the long-term accumulation of oxidized proteins as seen in multiple sclerosis (Bizzozero *et al*. 2005a), and ongoing studies are addressing this issue in chronic animal models of MS.

Since ROS have variable but still relatively short half-lives (10-9 s for hydroxyl radicals, 10-6 s for superoxide, 10-3 s for peroxynitrite, 100 s peroxyl radicals and 102 s for hydrogen peroxide) (Reth 2002), they normally damage targets that are in close proximity to their source. Thus, if ROS in EAE spinal cords were generated mostly by inflammatory cells, one would expect to find accumulation of protein carbonyls near the inflammatory foci. Instead, we discovered that protein carbonyls are not circumscribed to inflammatory lesions but accumulate throughout the spinal cord. This more generalized carbonyl increase, staining multiple cell types including neurons, suggests that the origin of ROS is likely metabolic. We have recently shown that total or even partial depletion of GSH with diethyl maleate or 1,2-bis(2-chloroethyl)-1-nitrosourea results in abundant protein carbonylation by a process involving mitochondrial production of ROS (Bizzozero *et al*. 2006). While other cellular/subcellular sources of ROS cannot be excluded, it is tempting to speculate that a similar mechanism takes place in EAE. Supporting this idea are the facts that (1) there is mitochondrial damage and augmented superoxide production in EAE and MS (Kalman *et al.* 2007) and (2) protein carbonyls do not increase until after this antioxidant is depleted. The lack of protein carbonylation in the presence of sustained RCS production is noteworthy and suggests that carbonylation of proteins in EAE takes place via a direct mechanism and not indirectly by reaction with dialdehydes (MDA, glyoxal, methyl glyoxal). In this regard, we have recently found that, while dependent on lipid peroxidation, the carbonylation of cytoskeletal proteins during depletion of glutathione probably occurs via lipid hydroperoxides rather than RCS (Bizzozero *et al*. 2007).

Another major finding in this study is the identification of several prominent cytoskeletal proteins as targets of carbonylation in EAE. Cytoskeletal proteins are established targets of protein carbonylation in many diseases including several CNS disorders (Aksenov *et al*. 2001, Muntane *et al*. 2006). More importantly, carbonylation of cytoskeletal proteins is known to have deleterious effects on the macromolecule structures. For instance, actin filaments and microtubules both destabilize and disassemble upon oxidation of their protein components (Banan *et al*. 2001, Banan *et al*. 2004, Dalle-Donne *et al*. 2001, Neely *et al*. 2005, Ozeki *et al*. 2005). The secondary structure of neurofilaments is also altered when the individual proteins become carbonylated (Gelinas *et al*. 2000), often leading to the formation of dense aggregates (Smith *et al*. 1995).

In the present study, we have demonstrated that neurofilament proteins and β-tubulin are extensively degraded in the EAE spinal cord, which likely causes alterations in the cytoskeletal network. We have also observed that the specific oxidation of NFL protein decreases in EAE, suggesting the possibility that oxidized neurofilament may be preferentially degraded in this disease. Although the heavy and medium chain neurofilament proteins do not show changes in the specific oxidation at the 13 DPI time point analyzed, variations in the susceptibility of these oxidized high molecular weight neurofilament chains to degradation or aggregate formation may reconcile these differences. Additionally, the more rapidly formation of newly synthesized, and therefore unoxidized, light chain neurofilament could contribute to the lower specific oxidation at this time point. It is well known that proteolysis of carbonylated proteins in cells under conditions of oxidative stress aids in preventing formation of large aggregates

that could be cytotoxic (Davies & Shringarpure 2006, Shringarpure *et al.* 2001). The 20s proteasome has been shown to selectively recognize and degrade oxidized proteins (Grune *et al.* 1997, Pacifici *et al.* 1993, Rivett 1985), and upon its inhibition carbonylated proteins do indeed accumulate (Lee *et al.* 2001). Calpain is also believed to be responsible for neurofilament degradation in EAE (Shields & Banik 1998), but it is unclear if this calcium-activated protease has preference for oxidized over native polypeptides. Studies using proteasome and calpain inhibitors in EAE have primarily focused on abatement of neurological symptoms without looking at specific proteins that are protected from proteolysis (Guyton MK 2005, Hassen *et al.* 2006, Hosseini *et al.* 2001, Vanderlugt *et al.* 2000). Whether calpain, the proteasome, or a combination of these proteolysis pathways is responsible for the degradation of carbonylated neurofilament proteins in EAE is currently under investigation through use of specific protease inhibitors in vivo. Recognizing the triggers for neurofilament degradation may help us to understand the mechanisms of axonal degeneration during neuroinflammation.

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2.8 Tables and Figures

Table 2.1 Characteristics of disease in EAE animals

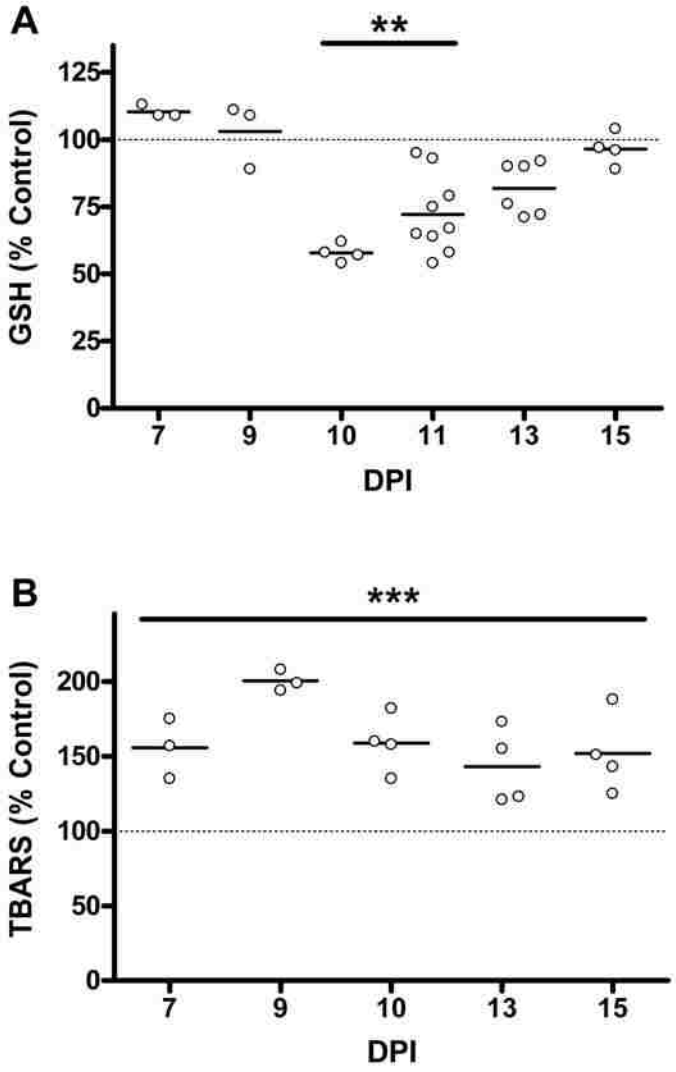
Number of days post-induction (DPI) of EAE	*Number of CFA controls*	*Number of animals that developed EAE*	*Clinical Score at sacrifice*
7	1	0/3	3 = Grade 0
9	1	1/3	2 = Grade 0 1 = Grade 1.5
10	1	4/4	1 = Grade 3 1 = Grade 3.5 2 = Grade 4
11	5	9/9	3 = Grade 3 5 = Grade 3.5 1 = Grade 4
13	4	6/6	1 = Grade 2 4 = Grade 3 1 = Grade 3.5
15	3	4/4	1 = Grade 0 1 = Grade 1 1 = Grade 1.5 1 = Grade 3

Table 2.2 Specific oxidation of cytoskeletal proteins in control and EAE spinal cord

	Control (% oxidized)	*EAE (13 DPI) (% oxidized)*
NFH	0.87 ± 0.09	1.71 ± 0.80
NFM	0.24 ± 0.04	0.32 ± 0.04
NFL	0.19 ± 0.04	0.08 ± 0.04 *
β-Tubulin	0.08 ± 0.04	0.17 ± 0.06
β-Actin	0.39 ± 0.09	0.76 ± 0.06 *
GFAP	1.87 ± 0.49	0.63 ± 0.38

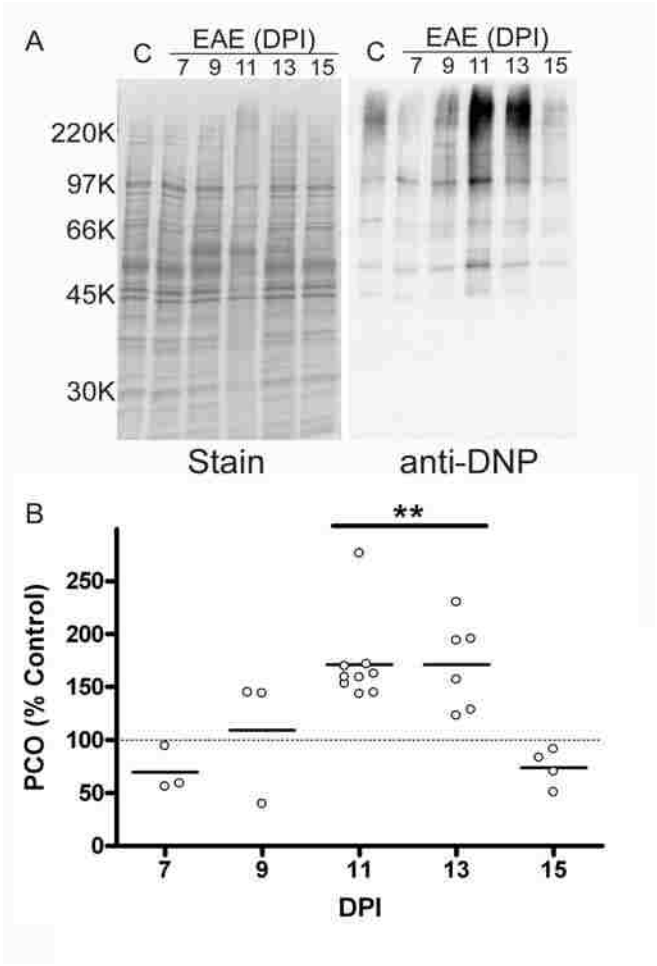
Spinal cord homogenates from control and EAE (13 DPI) animals were derivatized with biotin hydrazide and carbonylated proteins were isolated with streptavidin-agarose as described in Materials and Methods. Total and bound fractions were analyzed by SDS-PAGE followed by western blotting for six cytoskeletal proteins (3 neurofilament chains, β-tubulin, β-actin, and GFAP). Specific oxidation (percentage of total protein modified by carbonylation) is reported. Data was analyzed for statistical significance using t-test, (*) $p<0.05$. β-actin showed increased carbonylation, whereas NFL had less oxidation indicating the oxidized NFL may be degraded.

Figure 2.1 Temporal profile of glutathione oxidation and lipid peroxidation in EAE spinal cord



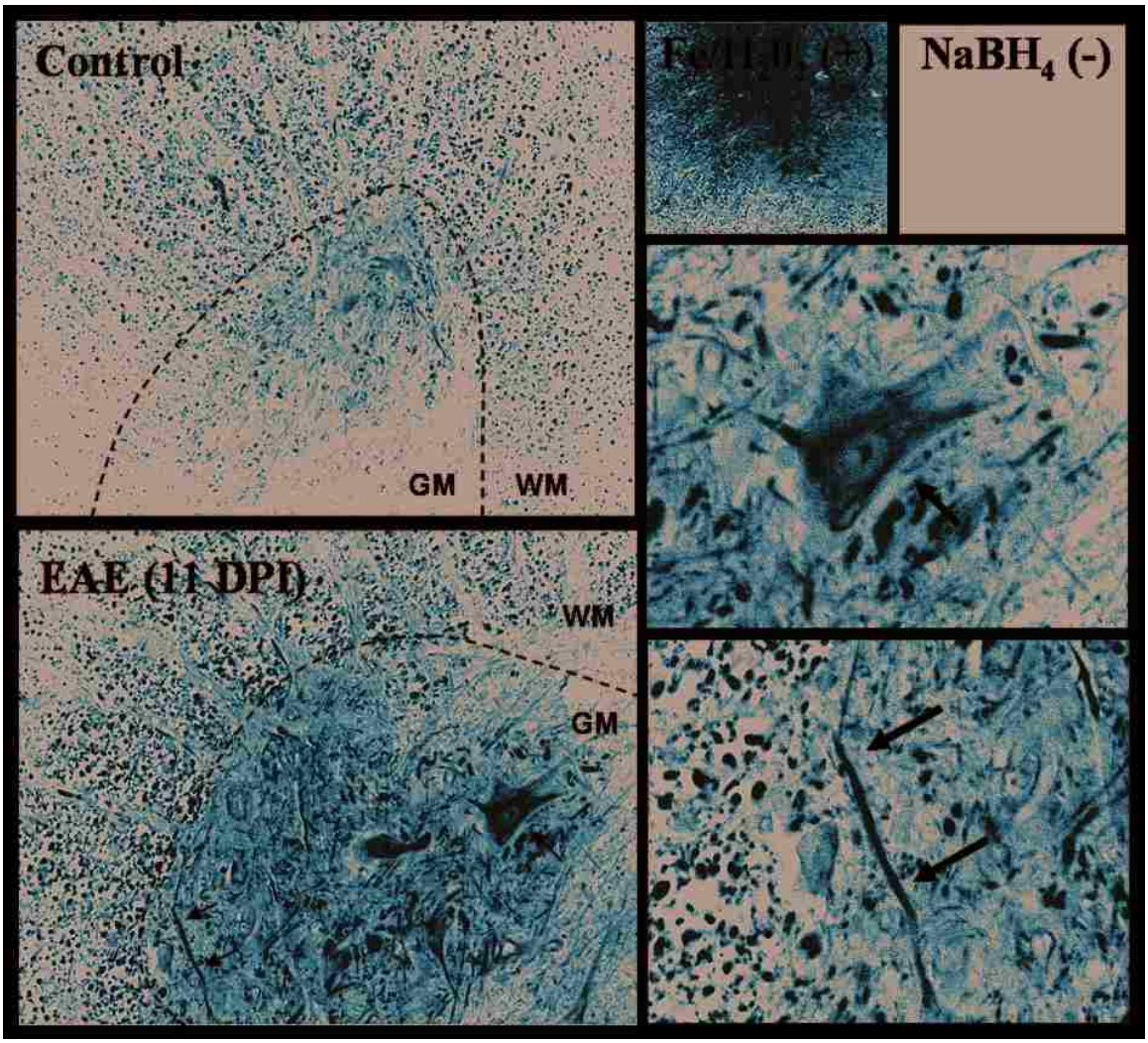
(A) Free glutathione (GSH) levels in EAE spinal cord homogenates through disease course measured using DTNB (see Materials and Methods). Data is represented as a percent of average control GSH levels (8.8 ± 1.3 nmol GSH/mg protein). (B) Lipid peroxidation was assessed by measuring TBARS as described in Materials and Methods in spinal cord homogenates through disease course. EAE values are expressed as a percentage of the average control value (0.48 ± 0.07 nmol TBARS/mg protein). GSH and TBARS data were analyzed for statistical significance using a one-way ANOVA with Dunnett's multiple comparison post-test to determine statistical significance from control, (**) $p<0.01$, (***) $p<0.001$. Lower glutathione at 10 -11 DPI points to considerable oxidative stress in the CNS during onset of neurological symptoms. Increase in TBARS at 7-15 DPI indicates carbonyl stress throughout the disease course.

Figure 2.2 Protein carbonyls in the spinal cord of EAE rats increase during disease course



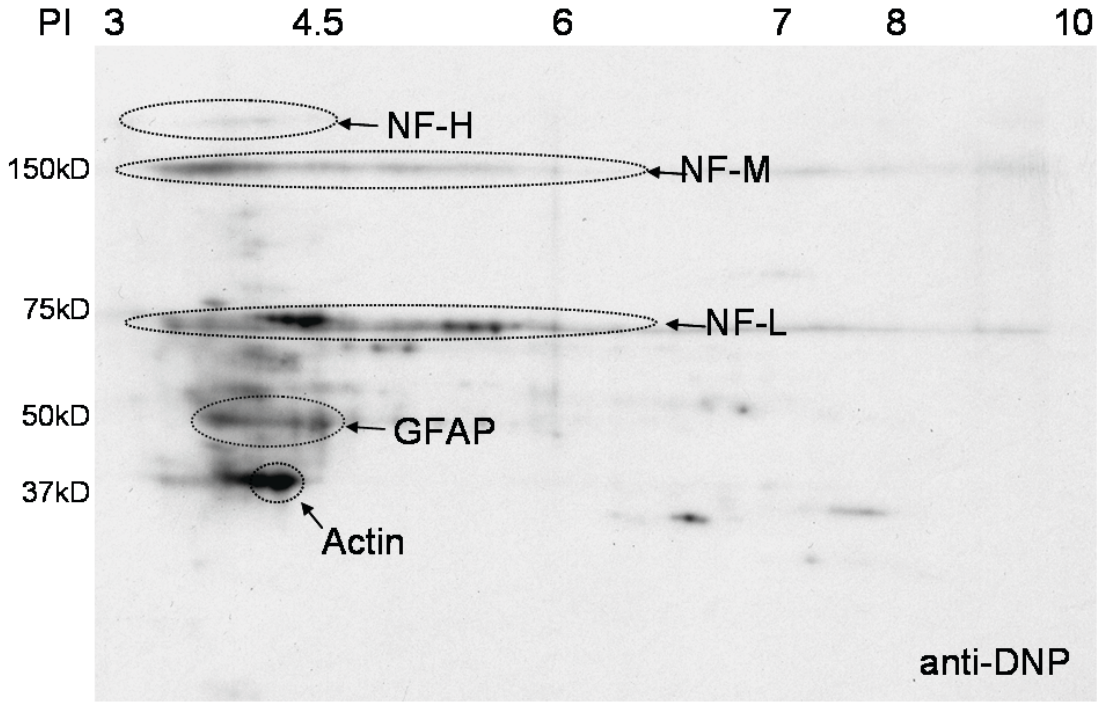
Spinal cord homogenates from control and EAE animals were derivatized with 2,4 dinitrophenylhydrazine then separated by SDS-PAGE. Carbonylated proteins were determined using western blotting as described in Materials and Methods. (A) Representative blot showing one control and 5 EAE animals sacrificed at a range of days post injection (DPI). (B) Protein carbonyls (PCO) were tracked throughout the disease course and are expressed as a percentage of the average carbonylation in control spinal cords. Mean carbonyl levels (line) and the value for individual animals are shown at various DPI. Data was analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparison post-test to determine statistical significance from control, (**) $p<0.01$. Carbonylated protein species accumulate in the spinal cord of animals with acute EAE at 11 and 13 DPI and return to control levels at 15 DPI indicating a clearance mechanism for removal of oxidized species.

Figure 2.3 Immunohistochemical localization of protein carbonyls in the rat spinal cord of EAE animals



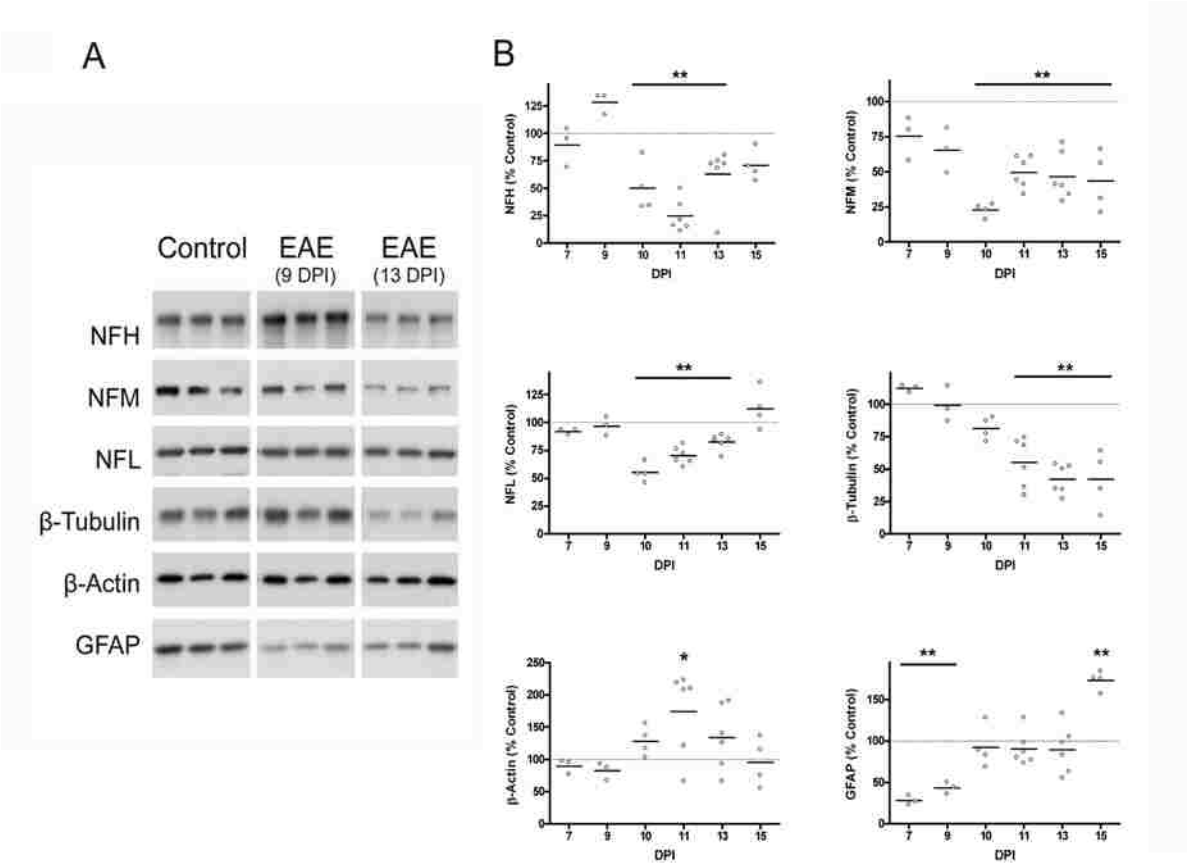
Carbonyls in thoracic spinal cord sections of a control and an EAE rat (score = 3.0) were detected by immunohistochemistry as described in Materials and Methods. Images show carbonyls the spinal cord ventral horn. Both neuronal cell bodies and axons are heavily stained in the EAE sample (arrows and insets). The carbonyls increase is seen throughout the spinal cord tissue, not localized around lesions. Tissue sections were treated with Fe/H2O2 to generate carbonyls (positive control) or NaBH4 to block endogenous carbonyls (negative control).

Figure 2.4 Two-dimensional Oxyblot analysis of EAE spinal cord proteins



Spinal cord homogenates from EAE animals 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 re-probed with antibodies against the major cytoskeletal proteins. Cytoskeletal proteins on anti-DNP blots were identified by spot matching using BioRad PD-Quest software. The major carbonylated targets in EAE include β-actin, GFAP and the neurofilament proteins.

Figure 2.5 Levels of cytoskeletal proteins in EAE spinal cord



Spinal cord homogenates from control and EAE animals (5 μg protein) were separated by SDS-PAGE followed by western blotting. Membranes were immunostained with antibodies against six cytoskeletal proteins (3 neurofilament chains, β-tubulin, β-actin, and GFAP). (A) Representative blot with 3 control and 3 EAE samples prepared from animals at 9 and 13 DPI are shown. (B) Levels of each cytoskeletal protein were tracked throughout the disease course and values are expressed as a percentage of the average protein level in control spinal cords. Values for individual animals as well as the mean value (line) are shown at each DPI. Data were analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparison post-test to determine statistical significance from control, (*) $p<0.05$, (**) $p<0.01$. Neurofilaments and β-tubulin are degraded in the disease, which is possibly a consequence of oxidative damage to these proteins.

3. Role of calpain and the proteasome in accumulation of protein carbonyls and cytoskeletal degradation in experimental autoimmune encephalomyelitis

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3.1 Abstract

Recent work from our laboratory has shown that various CNS cytoskeletal proteins are major targets of protein carbonylation in acute EAE. We also demonstrated that several of these oxidative targets, including all three neurofilament chains and β-tubulin, are degraded in the spinal cord during the peak of neurological disease. We hypothesized that oxidative damage could be targeting these cytoskeletal proteins to get degraded by one of two mechanisms responsible for the clearance of carbonylated proteins, the calcium-dependent cysteine protease calpain or the 20S proteasome. The present study was designed to investigate the role of calpain and the proteasome in the removal of these cytoskeletal proteins and to test whether oxidative modification of the proteins made them more susceptible to degradation. Calpain or proteasome activity was inhibited in EAE Lewis rats by intrathecal injection of calpeptin or epoxomicin, respectively, during the peak of clinical disease. Biochemical analyses of spinal cord homogenates investigated the effect of inhibition on total protein carbonylation, degradation of the neurofilaments and β-tubulin, and changes in the percentage of these cytoskeletal proteins that were carbonylated. After inhibiting calpain, we found significant increases in total protein carbonylation and in the amount of neurofilament proteins and β-tubulin that were spared from degradation, but no changes were seen in the specific oxidation of any of these protein species. Inhibition of the proteasome did not affect total protein carbonylation or degradation. These results suggest that calpain-mediated proteolysis plays a role in the increased clearance of cytoskeletal proteins seen in acute EAE and that oxidized and non-oxidized NF proteins are equally susceptible to digestion by this calcium-activated protease.

3.2 Introduction

A significant body of experimental evidence has accumulated recently demonstrating that oxidative stress is a major player in the pathogenesis of both MS and EAE (Gilgun-Sherki *et al.* 2004, LeVine 1992, Bizzozero 2009). Carbonylation, the non-enzymatic addition of aldehydes or ketones to specific amino acid residues, is one of the primary modifications to proteins under severe or prolonged oxidative stress conditions (Berlett & Stadtman 1997, Dalle-Donne *et al.* 2003, Nystrom 2005). Protein carbonyls are an irreversible form of protein damage (Dalle-Donne *et al.* 2003) occurring in many neurodegenerative disorders including Alzheimer's disease (Aksenov *et al.* 2001), Parkinson's disease (Floor & Wetzel 1998), amyotrophic lateral sclerosis (Ferrante *et al.* 1997) and multiple sclerosis (Bizzozero *et al.* 2005).

Concentrations of carbonylated proteins in cells are normally very low, but even small changes in the percentage of oxidized proteins or the number of oxidative modifications on a molecule can lead to the formation of protein aggregates, alter their ability to function, or influence their metabolic stability. A number or proteolytic systems that preferentially degrade oxidized proteins operate in cells in order to keep the cellular levels of protein carbonylation low. The core 20S proteasome degrades oxidized proteins in an ATP- and ubiquitin-independent manner, and is believed to be the main mechanism clearing carbonylated proteins in cells (Grune *et al*. 1997). Reduced activity of the 20S proteasome does lead to accumulation of protein carbonyls in chronic EAE (Zheng & Bizzozero 2010b). There is evidence that other proteases preferentially hydrolyze oxidized proteins as well. The calcium-dependent cysteine protease calpain has been shown to preferentially degrade oxidized neurofilament over non-oxidized protein

in a cell-free system (Troncoso *et al.* 1995). Calpain has also been shown to be an important proteolytic pathway for cytoskeletal proteins in the CNS (Banik & Shields 2000) and its activity is elevated in both chronic (Hassen *et al.* 2008) and acute EAE (Shields *et al.* 1998).

EAE shares a number of clinical and pathological features with MS, and is routinely employed to study the mechanistic bases of disease and to test therapeutic approaches (Gold *et al.* 2000). MBP-induced EAE in the Lewis rat, the animal model used in this study, is an acute monophasic clinical disease that resolves spontaneously within 5 days of onset. At the peak of disease there are perivenular infiltrates of inflammatory cells within the spinal cord. There is, however, no demyelination of CNS axons, making this an ideal model to study the neuroinflammatory aspects of MS. Previous studies in our laboratory have shown accumulation of protein carbonyls during the peak of neurological symptoms in acute EAE (Smerjac & Bizzozero 2008). Protein carbonyl levels return to normal levels after neuroinflammation and neurologic deficits subside, indicating that proteolytic mechanisms are working to clear these damaged proteins from CNS tissue. The peak of neurologic disease in acute EAE also coincides with damage to the cytoskeletal protein network as seen by the significant degradation of all three neurofilament chains and β-tubulin.

The present study was designed to identify the proteolytic activity leading to cytoskeletal protein digestion in EAE and whether oxidative damage to those species targets them for specific degradation. Our focus was on cytoskeletal proteins because they are targets of oxidation in MS (Bizzozero 2007) and EAE (Smerjac & Bizzozero 2008, Zheng & Bizzozero 2010a). The specific oxidation of certain cytoskeletal proteins

in acute EAE changes during the course of the disease, suggesting preferential targeting of the oxidized species for degradation (Smerjac & Bizzozero 2008). Results presented herein show that calpain plays a critical role in the proteolysis of neurofilament and tubulin proteins during acute neuroinflammation. Inhibition of calpain activity during the peak of disease leads to accumulation of oxidized protein species, but does not change the specific oxidation levels of any of the neurofilament proteins or β-tubulin. Reducing the activity of the 20S proteasome does not affect total protein carbonylation or cytoskeletal protein levels, but does lead to accumulation of oxidized NFM. These results suggest that while the proteasome may contribute to removal of oxidized proteins, calpain is the main protease involved in degradation of the cytoskeleton and does not preferentially target oxidized cytoskeletal proteins species in acute EAE. To the best of our knowledge this is the first study using inhibition of calpain or the 20S proteasome to investigate mechanisms of preferential degradation of oxidized proteins *in vivo*.

3.3 Materials and Methods

3.3.1 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. Seven-week-old male Lewis rats were purchased from Harlan Bioproducts (Indianapolis, IN) and housed in the UNM-animal resource facility. EAE was induced in these animals as described previously (Smerjac & Bizzozero, 2008). Rats were weighed and examined daily for the presence of neurological signs. Animals were euthanized by decapitation 48 hours after i.t. injection. Spinal cords segments L1-L3 were dissected and homogenized in HEN

buffer (25 mM HEPES 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 -80˚C until use. Protein concentration was assessed with the Bio-Rad DCT protein assay (Bio-Rad Laboratories; Hercules, CA) using bovine serum albumin as standard.

3.3.2 Acute lumbar puncture and intrathecal drug delivery

Intrathecal (i.t.) drug injections were conducted when rats displayed the first signs of clinical disease using the technique described by Milligan *et al.* (2005). An injection catheter was inserted under brief isoflurane anesthesia (1–2% in oxygen). Here, a 25 cm PE-10 catheter (attached by a 30-gauge sterile needle to a sterile, 50 µL glass Hamilton syringe) was marked 7.7 cm from the open end. Under light anesthesia, the dorsal pelvic area was shaved and cleaned with 70% alcohol. An 18-gauge sterile needle with the plastic hub removed was inserted between lumbar vertebrae L5 and L6. The open end of the PE-10 catheter was inserted via the 18-gauge needle and threaded to the 7.7 cm mark allowing for intrathecal PE-10 catheter-tip placement at the T12 – L1 junction. Injections consisted of 4 µL of DMSO containing either 4µg of calpeptin (Enzo Life Sciences, Inc.), 4 µg of epoxomicin (Cayman Chemical Company, Ann Arbor, MI) or no drug (vehicle), with a 5 µl post 0.9% sterile, isotonic saline flush. The PE-10 catheter was immediately withdrawn and the 18-gauge needle was removed from the L5-L6 inter-vertebral space. Rats were then placed in their home cage and observed during recovery from anesthesia. This acute injection method took 2–3 min to complete, and rats showed full recovery from anesthesia within 10 min.

3.3.3 Calpain activity assay

Calpain activity was determined in EAE spinal cords homogenates using a fluorescence assay (Hassen *et al.* 2006). Spinal cord homogenates (100 µg protein) were diluted to 2 mg/mL in HEN buffer and membrane-bound calpain was removed prior to the assay by centrifugation at 10,000 xg for 25 min. Next, 25 µL of the soluble fraction was incubated with the substrate Suc-Leu-Leu-Val-Tyr-AMC in 100mM KCl, 10mM $CaCl_2$, 25mM HEPES, pH 7.5. The incubation was carried out in the absence and presence of 0.4 µg/µL calpeptin. Calpain activity was calculated as the difference in fluorescence intensity at 460nm between the samples with and without inhibitor using an excitation wavelength of 380nm.

3.3.4 Proteasome activity assay

Proteolytic activity of the 20S proteasome was determined in EAE spinal cord homogenates using a similar fluorescence assay (Rodgers & Dean 2003). Briefly, 50µg of protein were incubated for 1h at 25˚C with 50µM of the 7-aminomethyl-4-coumarin (AMC)-labeled peptide Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like activity) in the absence or presence of 10µM clasto-lactacystin-β-lactone (Enzo Life Sciences, Plymouth Meeting, PA). The activity of the 20S proteasome was calculated as the difference in fluorescence intensity at 460nm between the samples with and without inhibitor using an excitation wavelength of 380nm.

3.3.5 Assessment of protein carbonylation by western blotting

Protein carbonyl groups were measured with the OxyBlot™ protein oxidation detection kit (Intergen Co., Purchase, NY), following the protocol provided by the manufacturer. In

brief, proteins (5μg) were incubated with 2,4-dinitrophenyl-hydrazine to form the 2,4-dinitrophenyl (DNP) hydrazone derivatives. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride (PVDF) membranes. DNP-containing proteins were then immunostained using rabbit anti-DNP antiserum (1:500) and goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:2000). Blots were developed by enhanced chemioluminescence (ECL) using the Western Lightning ECL™ kit from Perkin-Elmer (Boston, MA). The developed films were scanned in a Hewlett Packard Scanjet 4890 and the images quantified using the Scion Image for Windows, version Alpha 4.0.3.2 (Scion Corporation, Frederick, MD).

3.3.6 Assessment of cytoskeletal protein levels by western blotting

Aliquots (5 μg) from spinal cord homogenates were separated by SDS-PAGE on 10% polyacrylamide gels and blotted against PVDF membranes. Blots were probed with antibodies against NFH (1:1000, rabbit polyclonal; Chemicon, Temecula, CA), NFM (1:4000, rabbit polyclonal, Chemicon), NFL (1:1000, mouse monoclonal, Chemicon), and β-tubulin (1:1000, mouse monoclonal; Sigma), followed by incubation with the appropriate HRP-conjugated secondary antibody. Blots were developed by ECL and quantified as described above.

3.3.7 Identification of carbonylated proteins

Tissue proteins (1mg), dissolved in 200 μl 2% SDS, were incubated at room temperature with 5 mM biotin-hydrazide (Sigma). After 60 min, proteins were precipitated with 1 mL of acetone at -20˚C and collected by centrifugation at 10,000 xg for 10 min. Pellets were

washed three times with 1 mL of acetone-water (3:1, v/v) and dissolved in 100 µL of 50 mM Hepes pH 7.5 containing 1% SDS and 100 mM NaCl. The solutions were diluted 20-fold with 100 mM NaCl and centrifuged at 10,000 xg for 10 min to remove any aggregated material. The supernatants were then incubated for 1 h at 20˚C with 50µL of streptavidin-agarose (Sigma) previously equilibrated in 50 mM Hepes pH 7.5 containing 0.05% SDS and 100mM NaCl (buffer A). The resin was washed 5-times with 500 µL of buffer A, 4-times with buffer A containing 1M NaCl, and once with buffer A without NaCl. Bound-proteins were eluted from the resin with 100 µL of SDS-sample buffer containing 1% 2-mercaptoethanol. Aliquots from the total and bound fractions were separated by SDS-PAGE on 10% polyacrylamide gels and blotted against PVDF membranes. Blots were probed with antibodies against NFH, NFM, NFL, and β-tubulin followed by incubation with the appropriate HRP-conjugated secondary antibody. Blots were developed by ECL and quantified described above.

3.3.8 Statistical Analysis

Results were analyzed for statistical significance with Student's unpaired *t*-test or one-way ANOVA using IBM® SPSS® Statistics program (version 19.0.0).

3.4 Results

3.4.1 Neurological symptoms of EAE rats are not altered by intrathecal administration of inhibitors

EAE in male Lewis rats was induced 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; 5,

moribund. Following immunization with MBP, Lewis rats experienced an acute and monophasic inflammation of the spinal cord resulting in clinical disability. As in previous studies in our lab, the onset of neurological symptoms began at 9-11 days post immunization (DPI) (Smerjac & Bizzozero 2008). The first day animals started exhibiting neurological disease, clinical score 1 or greater, an i.t. injection was performed as described in "Materials and Methods." Animals injected with antigen that did not show symptoms on 11 DPI were excluded from our analysis. Rats were treated with DMSO (vehicle), the calpain inhibitor calpeptin, or the 20S proteasome inhibitor epoxomicin. Animals were monitored for clinical disease for 48 hours after i.t. injection and then sacrificed. Clinical disease at time of i.t. injection for the 3 groups did not show any statistical difference, [$F(2, 32) = 0.783$, $p = 0.466$]. The disease course after i.t. injection was not altered by inhibitor treatment (average clinical score at sacrifice, [$F(2, 32) = 0.602$, $p = 0.554$]). EAE rats were sacrificed on days 11 and 13 post EAE induction, 48 hours after i.t. injection. Spinal cord sections from vertebrae L1 – L3 were removed and homogenized for biochemical analysis.

3.4.2 *In vivo* inhibitor treatment reduces calpain or proteasome activity in spinal cords of EAE rats

The aim of this study was to investigate the mechanism of cytoskeletal protein degradation in the spinal cord of EAE Lewis rats and determine whether oxidative damage in the form of protein carbonyls led to preferential degradation. This question was approached by inhibiting two potential degradation pathways: the calcium-dependent cysteine protease calpain and the 20S proteasome. Calpeptin, a membrane-permeable inhibitor that targets both m-calpain and μ-calpain (Saito & Nixon 1993), was used to

inhibit calpain activity in the EAE spinal cord. We chose a dose of 4-µg of calpeptin, or 20-23 µg/kg, per animal, based on preliminary studies in our laboratory showing that i.t. injections of this dosage to inhibit calpain activity by 29 – 33% in non-EAE animals. Inhibition levels were reached 4 hours after i.t. injection and remained steady up to 48 hours post injection. A 2 µg/kg dose showed no significant inhibition of calpain activity and a 200 µg/kg did not offer significantly higher inhibition than the chosen dose (data not shown). Epoxomicin has been used for proteasome inhibition in the spinal cord in a chronic pain model (Ossipov *et al.* 2007). It is quite specific for the 20S proteasome inhibiting the chymotrypsin-like activity and reducing the trypsin-like and caspase-like catalytic rates (Meng *et al.* 1999). Preliminary studies with a dose of 4 µg epoxomicin/animal yielded inhibition of proteasome activity in the range of 33-43% from 4 hours after i.t. injection remaining steady at 48 hours post injection. A 10x higher dose did not show significantly higher proteasome inhibition (data not shown).

EAE animals were injected intrathecally with epoxomicin, calpeptin or the vehicle DMSO alone at 9 – 11 DPI. Forty-eight hours after i.t. injection animals were sacrificed and the L1 – L3 segments of the spinal cord were dissected and homogenized for biochemical analysis. Proteasome activity was measured in the spinal cord homogenates from each animal using a fluorescence proteasome activity described in "Materials and Methods" and is expressed as a percentage of the average proteasome activity in the vehicle-treated EAE animals (Figure 3.1A). Proteasome activity in epoxomicin-treated EAE rats was significantly reduced to 76.4% ± 8.7% (Mean ± SEM) of that in vehicle-treated animals while calpeptin treatment did not affect proteasome activity (111.8% ± 21.5%). Calpain activity was measured in the supernatants of the

spinal cord homogenates as described in "Materials and Methods." As shown in Figure 3.1B, calpain activity was significantly reduced in calpeptin-treated animals to 78.3% ± 8.3% of the levels of vehicle-treated EAE animals, while epoxomicin treatment did not affect calpain activity (106.3% ± 12.4%). It should be noted that inhibition of both the proteasome and calpain in EAE animals was not as effective as the inhibition seen in non-EAE animals from pilot drug studies. It is noteworthy that proteasome activity in EAE animals is increased 1.6X over that in the aged-matched naïve Lewis rats, and that calpain activity augments 5.5X over naïve rats (dashed lines in Figure 3.1).

3.4.3 Calpain inhibition increases oxidative damage to proteins in EAE

We recently reported that protein carbonylation increases in EAE Lewis rats during the peak of disease (Smerjac & Bizzozero 2008). Previous studies investigating degradation of oxidized protein *in vitro* suggested that both the proteasome and calpain show preference for degrading carbonylated proteins (Grune *et al.* 1997, Troncoso *et al*. 1995). We wanted to investigate whether either of these mechanisms played a role in removal of carbonylated proteins in the spinal cords of EAE animals. The presence of protein carbonyls in the spinal cord tissue in i.t. injected EAE rats was investigated using the oxyblot technique. A representative western blot detecting protein carbonyls (i.e., oxyblot) of 4 vehicle-, 3 calpeptin-, and 3 epoxomicin-injected EAE spinal cords is shown in Figure 3.2A. There is a build-up of protein carbonyls in all EAE animals compared to naïve animals, and in the calpeptin-injected EAE animals there is a clear increase in the carbonyl content of several protein bands relative to vehicle-treated EAE. Quantitative analysis of the oxyblots revealed that total protein carbonyl levels in calpeptin-treated EAE spinal cords increased by 251% relative to vehicle-treated EAE

spinal cords (Figure 3.2B). No significant increase in total protein carbonyls was seen in epoxomicin-treated EAE, although the intensity of some bands are clearly higher. The above results suggest that calpain acts as a clearance mechanism to eliminate these damaged proteins in the disease tissue.

3.4.4 Intrathecal calpeptin injection reduces degradation of cytoskeletal proteins during the peak of EAE

Previous studies in our lab showed that the integrity of CNS neuronal cytoskeletal network in the spinal cord of EAE rats is greatly compromised. There are significantly lower levels of NFH, NFM, NFL, and β-tubulin in EAE compared to CFA-treated controls. This study investigated whether inhibition of calpain or the proteasome could prevent this degradation. We used western blotting to measure the levels of these proteins in spinal cord homogenates. Figure 3.3A shows representative western blots of the three neurofilament chains and β-tubulin in the spinal cord of i.t. injected EAE rats. Figure 3.3B depicts the level of these proteins in each of the i.t. treatment groups relative to the levels in 4 non-EAE control animals (dotted line at 100%). As previously reported in our lab the disappearance of cytoskeletal proteins, which are metabolically very stable (Li & Black 1996, Nixon & Logvinenko 1986), points toward increased degradation of these proteins rather than diminished synthesis. NFL degradation has been reported by other groups and is believed to be indicative of increased calpain activity in EAE spinal cord. (Shields & Banik 1998). In this study we found that a single i.t. injection with the calpain inhibitor calpeptin at the onset of neurologic symptom significantly prevents degradation of all three neurofilament chains and β-tubulin in the spinal cord of EAE

animals. In contrast, proteasome inhibition does not appear to prevent the clearance of these proteins.

3.4.5 Oxidation levels of cytoskeletal proteins are not altered by partial inhibition of proteasome or calpain

We next set out to investigate whether there are changes in the specific oxidation (percentage of total protein modified by carbonylation) in EAE animals treated with calpain or proteasome inhibitors. An increase in specific oxidation upon treatment with an inhibitor would support previous *in vitro* studies that suggest calpain (Troncoso *et al*. 1995) or the proteasome (Grune *et al*. 1997, Poppek & Grune 2006) preferentially target oxidized proteins for degradation. Quantification of the extent of oxidation of these species was performed by the pull-down/western blot procedure. To this end, protein carbonyls from i.t.-injected EAE spinal cord homogenates were first converted into biotinylated residues by reaction with biotin-hydrazide. Biotin-containing proteins were then isolated with streptavidin-agarose and analyzed by western blotting employing antibodies against the three neurofilament chains and β-tubulin. Specific oxidation (percentage of total protein modified by carbonylation) for the individual proteins were in the following ranges: NFH, 0.48% - 3.78%; NFM, 0.06% - 0.81%; NFL, 0.004% - 0.14%; β-tubulin, 0.04% - 0.63%. Although the percentage of carbonylated protein is low, even moderate increases in the carbonylation can have tremendous effects on protein function, aggregate formation, or metabolic stability. Figure 3.4 shows specific oxidation of the neurofilaments and β-tubulin in the three injection groups expressed as a percentage of the average vehicle-injected EAE animals. We found no significant differences in the specific oxidation of any of these proteins after treatment with

calpeptin compared to vehicle-injected EAE animals. Epoxomicin inhibition of the proteasome only showed a significant increase in the percentage of oxidized NFM. Thus, although calpain is the major degradation pathway for these cytoskeletal proteins, these *in vivo* studies do not support the idea that oxidized cytoskeletal proteins are preferentially targeted toward degradation by calpain in the spinal cord at the peak of EAE disease. Although the proteasome is not the primary pathway for degradation of cytoskeletal proteins, it may contribute to the degradation of oxidized NFM.

3.5 Discussion

We have previously shown that cytoskeletal proteins are degraded in the spinal cords of EAE Lewis rats and the proportion of carbonylated NFL decreases throughout the disease course (Smerjac & Bizzozero 2008). We hypothesized there could be a clearance mechanism for these cytoskeletal proteins that specifically targets the oxidized proteins for degradation. To investigate this possibility we chose to inhibit two protein degradation pathway that have been proposed to specifically target carbonylated proteins: calpain and the 20S proteasome. The specific inhibitors calpeptin and epoxomicin were delivered directly into the spinal cord at the T12 – L1 junction using intrathecal injection. Pilot studies proved this drug delivery method to be far superior to i.p. injections used by other groups at inhibiting calpain or the proteasome for a sustained period after a single injection. Using this method we were able to reduce calpain activity by ~22% using calpeptin and proteasome activity by ~24% using epoxomicin, as measured with fluorescence-based protease activity assays in the spinal cord tissue after sacrifice. Although calpain and proteasome inhibitors have been used to improve clinical disease in EAE Lewis rats (Hassen *et al*. 2006, Hosseini *et al.* 2001, Vanderlugt *et al.* 2000), we

found that acute treatment with inhibitors starting at the first sign of clinical disease did not alter the disease course. It was critical to our experimental paradigm that we were able to inhibit these proteases without altering the disease course, so differences seen in protein oxidation and cytoskeletal degradation could be attributed to the differences in enzyme activity and not disease severity.

We then showed that this modest inhibition of calpain early in the clinical disease led to increased accumulation of total protein carbonyls, as measured by oxyblot. Degradation of the neurofilament proteins and β-tubulin was also significantly reduced in the animals with inhibited calpain activity. However, we were unable to detect significant changes in the specific carbonylation of these particular cytoskeletal proteins. Thus, we conclude that while calpain is an important degradation mechanism for cytoskeletal proteins, it does not preferentially remove carbonylated neurofilaments or tubulin at a faster rate than it degraded the non-oxidized cytoskeletal elements. Approaching the question of the role of the proteasome in cytoskeletal degradation and oxidation, we found proteasomal inhibition did not lead to changes in total carbonylation or degradation, but does increase the specific oxidation of NFM proteins. The increase seen in total protein oxidation after treatment with calpain inhibitors is most likely due to the lower amount of overall protein degradation. With decreased proteolysis there are more neurofilament and tubulin, which are major oxidized species, and the overall increase in carbonylation reflects the increased presence of these, and potential other unidentified, carbonylated proteins molecules. The increase seen in oxidized NFM after proteasome inhibition supports the idea that the proteasome may contribute to removal of carbonylated neurofilament even though calpain is clearly the principal degradation

mechanism of this protein. This is the first study, to our knowledge, to investigate the specific targeting of carbonylated proteins for degradation by the proteasome or calpain *in vivo*.

We have previously reported that there is a change in the specific oxidation of NFL throughout the disease course and hypothesized that this was due to preferential degradation of oxidized proteins by either calpain or the proteasome (Smerjac & Bizzozero, 2008). Another potential explanation for the lower percentage of oxidized NFL molecules later in the disease course is that cytoskeletal digestion could trigger synthesis of new NFL proteins to replace the degraded molecules, which aids in the neurological recovery seen in this acute EAE model. Oxidative damage accumulates in organisms as they age (Stadtman & Berlett 1998), and therefore proteins with slow turnover rates are more likely to have oxidative modifications like carbonylation. Newly synthesized NFL molecules would be less likely to have oxidative modifications, thereby decreasing the specific carbonylation of the particular protein. The presence of newly synthesized, non-oxidized proteins diluting the total protein pool is a reasonable explanation for the decrease in specific oxidation of certain cytoskeletal proteins seen during the EAE disease course.

Our findings suggest that preferential degradation of carbonylated proteins is not a contributing factor in cytoskeletal removal. It is, however, important to consider the limitations of our experiment, which approached this question in an *in vivo* system by treating animals with inhibitors. We achieved a 22% decrease in calpain activity and a 24% decrease in proteasome activity in spinal cord tissue after direct intrathecal injection. These reductions in enzyme activity are significant, and led to considerable biochemical

changes in protein oxidation and cytoskeletal degradation. However, in this system we could not achieve higher levels of inhibition even with 10x doses of calpeptin or epoxomicin, or using other proteasome and calpain inhibitors (data not shown). It must be considered that the remaining active calpain or proteasome could be sufficient to preferentially remove oxidized proteins from the cell. Additionally, we chose to focus on neurofilaments and tubulin, which we demonstrated previously to be some of the major targets of oxidation and degradation in the cell, but it is possible that investigating specific oxidation levels of other carbonylated proteins after proteasome or calpain inhibition could identify proteins that do have increased susceptibility to degradation.

3.6 Acknowledgements

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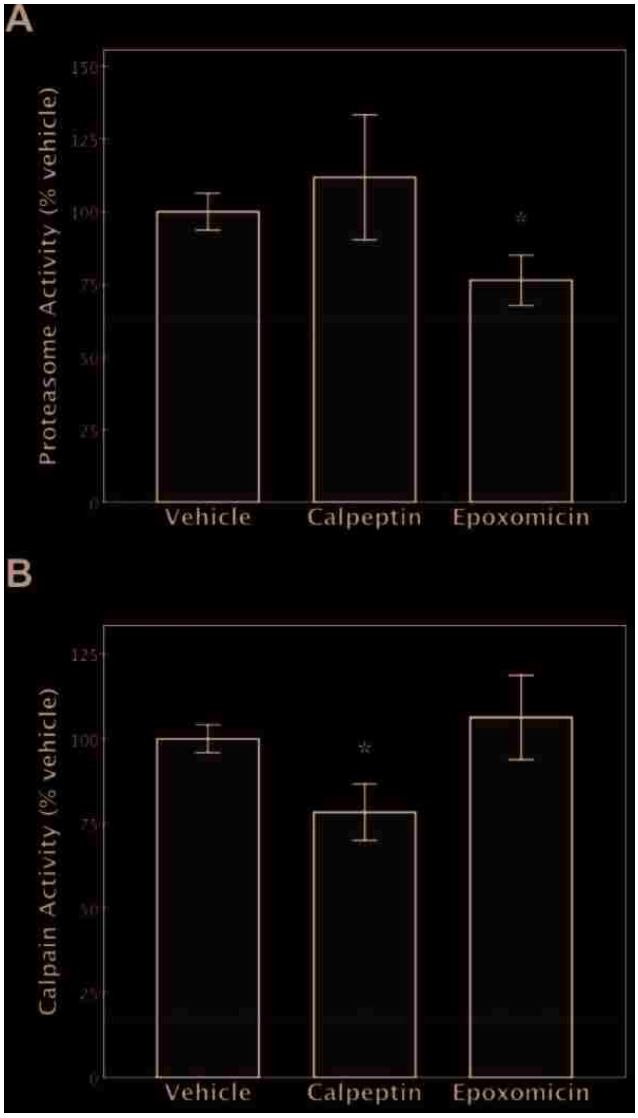
3.8 Tables and Figures

Table 3.1 Characteristics of disease in EAE animals in inhibitor treatment groups

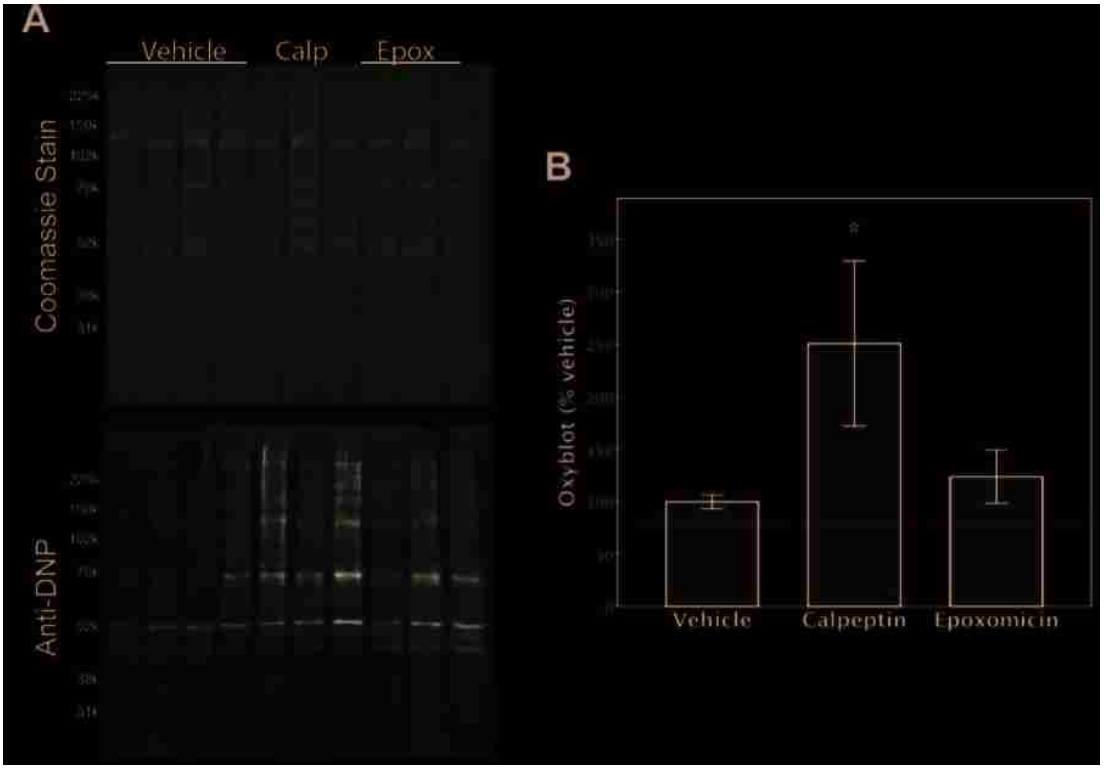
Treatment	*Number of EAE animals*	*DPI at sacrifice*	*Clinical Score at injection*	*Clinical Score at sacrifice*
Vehicle (4µL DMSO)	n = 15	7 = 11 DPI 8 = 13 DPI	8 = Grade 1 1 = Grade 2.5 5 = Grade 3 1 = Grade 3.5 --- Mean 1.8 ± 1.1	1 = Grade 2.5 8 = Grade 3 1 = Grade 3.5 3 = Grade 4 2 = Grade 4.5 --- Mean 3.4 ± 0.6
Calpeptin (4µL of 1mg/mL in DMSO)	n = 11	7 = 11 DPI 4 = 13 DPI	3 = Grade 1 2 = Grade 2.5 5 = Grade 3 1 = Grade 3.5 --- Mean 2.3 ± 1.0	0 = Grade 2.5 3 = Grade 3 4 = Grade 3.5 3 = Grade 4 1 = Grade 4.5 --- Mean 3.6 ± 0.5
Epoxomicin (4µL of 1mg/mL in DMSO)	n = 9	6 = 11 DPI 3 = 13 DPI	4 = Grade 1 2 = Grade 2.5 3 = Grade 3 0 = Grade 3.5 --- Mean 1.9 ± 1.1	1 = Grade 2.5 3 = Grade 3 3 = Grade 3.5 2 = Grade 4 0 = Grade 4.5 --- Mean 3.3 ± 0.5

Figure 3.1. Proteasome and calpain activity in inhibitor-treated EAE spinal cord.



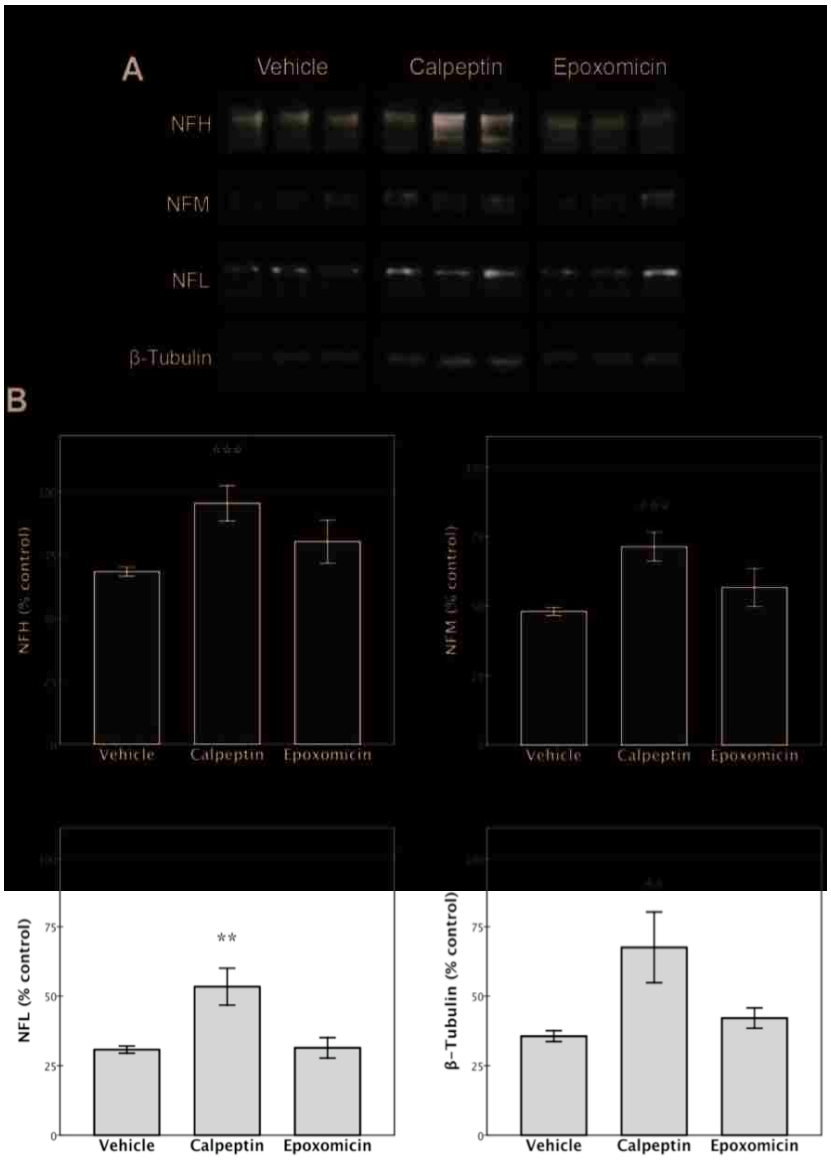
EAE was induced in Lewis rats; upon first signs of clinical disease (9-11DPI) animals were injected intrathecally with the proteasome inhibitor epoxomicin (n = 9) dissolved in 4 µL DMSO, the calpain inhibitor calpeptin (n = 11) dissolved in 5 µL DMSO, or with the vehicle DMSO alone (n = 15). Spinal cord segments L1 - L3 were dissected and homogenized in HEN buffer 48 hours after i.t. injection. (A) Chymotrypsin-like activity of the 20S proteasome was measured as described in "Materials and Methods." (B) Calpain activity in spinal cord tissue was measured as described in "Materials and Methods." Values are expressed as a percentage of the average vehicle-treated value. Proteasome and calpain activity was analyzed for statistical significance to determine difference from vehicle-injected EAE animals using unpaired *t*-test, (*) $p<0.05$. There was a significant reduction in calpain activity for calpeptin- compared to vehicle- injected rats; $t(24)=2.530$, $p=0.018$. Epoxomicin injection significantly reduced proteasome activity; $t(22)=2.233$, $p=0.036$. Dotted lines represent the average proteasome or calpain activity from 4 age-matched uninjected non-EAE Lewis rats.

Figure 3.2. Protein carbonyls in the spinal cord of EAE rats accumulate after treatment with a calpain inhibitor.



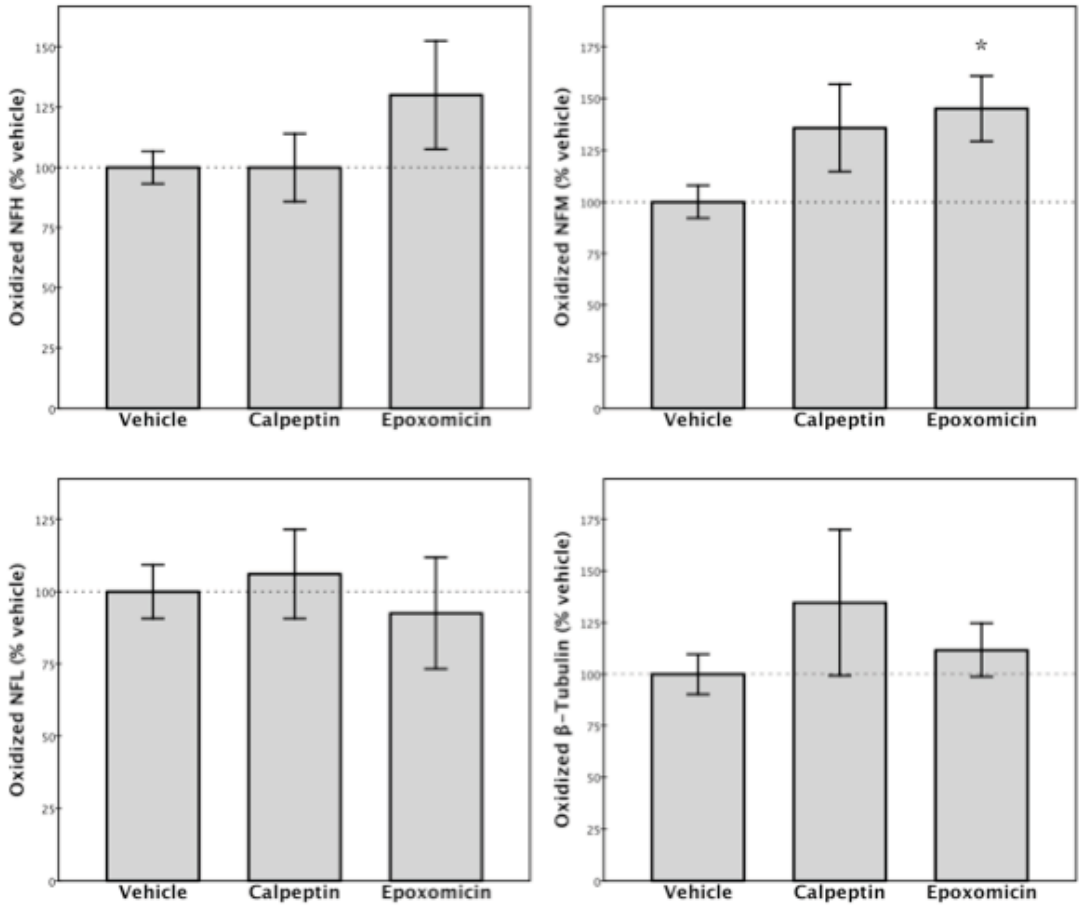
Spinal cord proteins (5 µg) from i.t. injected EAE animals were separated by SDS-PAGE. Carbonylated proteins were determined using western blotting as described in "Materials and Methods". (A) Representative oxyblot showing four vehicle-, three calpeptin-, and three epoxomicin-injected EAE animals sacrificed 48 hours after i.t. injection. (B) Protein carbonyl values, epoxomicin-injected, n = 9 and calpeptin-injected, n = 11, are expressed as a percentage of the average carbonylation in vehicle-injected EAE spinal cords (n = 15). Dotted lines represent the average protein carbonylation from 4 age-matched uninjected non-EAE Lewis rats. Data was analyzed for statistical significance from that of vehicle-injected EAE animals using unpaired *t*-test, (*) $p<0.05$. There was a significant difference in overall protein carbonylation for calpeptin- and vehicle-injected conditions; $t(24)=2.240$, $p = 0.035$.

Figure 3.3. Levels of cytoskeletal proteins in i.t.-injected EAE spinal cords.



Spinal cord proteins (5 μg) from i.t.-injected EAE animals were separated by SDS-PAGE followed by western blotting. Membranes were immunostained with antibodies against four cytoskeletal proteins (3 neurofilament chains and β-tubulin). (A) Representative western blot samples from 3 vehicle-, 3 calpeptin- and 3 epoxomicin-treated EAE rats. (B) Mean levels of each cytoskeletal protein were tracked throughout the disease course, epoxomicin-, n = 9, calpeptin-, n = 11, and vehicle-injected, n = 15. Values are expressed as a percentage of the average protein level compared to 4 uninjected non-EAE control spinal cords (dotted line at 100%). Data was analyzed for statistical significance from that in vehicle-injected EAE animals using unpaired *t*-test, (**) $p<0.01$, (***) $p<0.001$. Calpain inhibition significantly prevented the degradation of NFH; $t(24)=4.299$, $p=0.000$, NFM; $t(24)=4.939$, $p=0.000$, NFL; $t(24)=3.875$, $p=0.001$, β-tubulin; $t(24)=2.879$, $p=0.008$, compared to treatment with the DMSO vehicle. Treatment with epoxomicin did not affect neurofilament or β-tubulin levels.

Figure 3.4. Specific oxidation of cytoskeletal protein in i.t.-injected EAE spinal cords.



Spinal cord proteins were derivatized with biotin hydrazide and carbonylated proteins were isolated with streptavidin-agarose as described in Materials and Methods. Total and bound fractions were analyzed by SDS-PAGE followed by western blotting for four cytoskeletal proteins (3 neurofilament chains, and β-tubulin). Specific oxidation (percentage of total protein modified by carbonylation) for the individual proteins are reported for the individual treatment groups, epoxomicin-, n = 9, calpeptin-, n = 10, and vehicle-injected, n = 13, and expressed as a percentage of the vehicle-injected EAE animals. Data was analyzed for statistical significance using unpaired *t*-test, (*) $p<0.05$. A significant increase the amount of oxidized NFM; $t(20)=2.785$, $p=0.011$, was seen upon treatment with the proteasome inhibitor epoxomicin. No changes in specific oxidation of these four proteins were observed upon treatment with calpain inhibitors.

4. General Discussion

4.1 Oxidative stress and its role in metabolic stability of proteins in EAE

Multiple sclerosis (MS) is an inflammatory disease of the CNS leading to demyelination and axonal damage (Trapp *et al*. 1998). EAE is a widely employed animal model, which has a number of clinical features analogous to MS (Gold *et al*. 2000). A significant body of experimental evidence has accumulated suggesting that oxidative stress is a major player in the pathogenesis of inflammatory demyelination. Generation of ROS *in vivo* has been inferred from (1) the presence of the lipid peroxidation products malondialdehyde (Hunter *et al*. 1985, Calabrese *et al.* 1994, Naidoo & Knapp 1992) and isoprostanes (Greco *et al.* 1999) in the cerebrospinal fluid (CSF) and plasma of MS patients, (2) reduced plasma levels of antioxidants (e.g., ubiquinone, vitamin E) (Shukla *et al.* 1977) and antioxidant enzymes (e.g., catalase and glutathione peroxidase) (LeVine 1992) in MS, (3) low levels of antioxidants like glutathione, α-tocopherol and uric acid in MS plaques (Langemann *et al*. 1992), and (4) the damage to mitochondrial DNA in active chronic plaques (Vladimirova *et al.* 1998). In addition to accumulation of oxidized macromolecules and reduction of antioxidants, MS and EAE are characterized by dysregulation of transition metals like iron (LeVine 1997) and copper (Melo *et al.* 2003), which promote oxidative damage via the Fenton and Haber-Weiss reactions (Lewen *et al.* 2000). Additional evidence for a pathogenic role of ROS comes from the effectiveness of various antioxidant treatments at ameliorating EAE. For example, oral administration of the oxidant-scavenger N-acetylcysteine, which raises intracellular glutathione levels, inhibits the induction of acute EAE (Lehmann *et al.* 1994). Therapeutic success in the treatment of EAE has also been reported with other antioxidants such as α-lipoic acid

(Marracci *et al.* 2002), bilirubin (Liu *et al.* 2003), uric acid (Hooper *et al.* 1998) and flavonoids (Hendriks *et al.* 2004). Administration of antioxidant enzymes like metallothionein (Penkowa & Hidalgo 2003), catalase (Ruuls *et al.* 1995) and synthetic catalytic scavengers with combined superoxide dismutase and catalase activity (Malfroy *et al.* 1997) were found to be effective as well. Likewise, chelation of the pro-oxidant iron with deferoxamine (Pedchenko & LeVine 1998) and copper with N-acetylcysteine amide (Offen *et al.* 2004) has been shown to reduce tissue damage and improve the clinical course of EAE. Most relevant to the current study are the findings that protein carbonyls accumulate in brain tissue of MS patients (Bizzozero *et al*. 2005b) and that of EAE mice (Zheng & Bizzozero 2010a), and that cytoskeletal proteins are major targets of carbonylation in these diseases (Bizzozero 2007). The objectives of this thesis were to (1) characterize oxidative stress conditions in acute EAE, identifying the major protein targets of oxidation and (2) examine the proteolytic mechanisms responsible for clearance of oxidized proteins during the inflammatory phase of EAE.

In this study I have characterized oxidative stress conditions that occur in MBP-induced EAE in the Lewis rat by measuring established markers for oxidative damage throughout the disease course. I discovered that the concentration of free GSH in the spinal cord of EAE rats is greatly decreased at 10-11 DPI. GSH levels return to normal values as inflammation subsides and animals recover after 13 DPI. Acute depletion of GSH in brain slices leads to oxidative stress sufficient to induce protein carbonylation (Bizzozero *et al*. 2006).

TBARS assay, the most commonly used measurement for lipid peroxidation, detects dialdehydes (mostly MDA) resulting from breakdown of lipid hydroperoxides and

endoperoxides (Esterbauer *et al*. 1991). These dialdehyde lipid peroxidation products are reactive carbonyl species (RCS) and can react with proteins to induce protein carbonyls. In contrast to the reduction of GSH levels, which parallels disease activity, the concentration of TBARS in the EAE spinal cords is elevated at every time point measured (i.e., 7-15 DPI). This result indicates that the oxidative stress takes place prior to the appearance of inflammatory lesions in the spinal cord of EAE animals, a pathological process that correlates temporally with neurological symptoms (Schaecher *et al*. 2002). The presence of TBARS in 15 DPI animals, which are recovering neurologically, may be due to continued ROS generation or more likely to the persistence of dialdehydes in the tissue (MDA $t_{½}$ = 2 days) (Aldini *et al*. 2007).

One of the most important finding in this study is that several carbonylated protein species accumulate in the spinal cord of EAE rats at 11–13 DPI. The degree and distribution of protein carbonylation present during autoimmune neuroinflammation indicates that the oxidative stress conditions in this model are very severe. It is believed that the accumulation of carbonylated proteins is a multifaceted process that takes into account both the rate of oxidation (dependent on both ROS formation and antioxidant levels) and the rate of proteolysis of the damaged proteins (Sayre *et al*. 2005). In this acute animal model of MS there appears to be a mechanism that eliminates these damaged proteins and bring carbonylation to control levels as the inflammatory process subsides. Other work in our laboratory in female C57/BL6 EAE mice, which exhibit an acute and chronic phase of disease, also display increased protein carbonyls, localized primarily to cerebellar astrocytes in the vicinity of inflammatory regions, during the acute phase (Zheng & Bizzozero 2010a). Since ROS have variable but still relatively short

half-lives (10^{-9} s for hydroxyl radicals, 10^{-6} s for superoxide, 10^{-3} s for peroxynitrite, 10^{0} s peroxyl radicals and 10^{2} s for hydrogen peroxide) (Reth 2002), they normally damage targets that are in close proximity to their source. Thus, if ROS in EAE spinal cords were generated mostly by inflammatory cells, one would expect to find accumulation of protein carbonyls near the inflammatory foci, as seen in acute EAE mice cerebellum (Zheng & Bizzozero 2010a). Instead, I discovered that protein carbonyls are not circumscribed to inflammatory lesions but accumulate throughout the spinal cord. This more generalized carbonyl increase, staining multiple cell types including neurons, suggests that the origin of ROS is likely metabolic, although other cellular/subcellular sources of ROS cannot be excluded. Supporting this idea are the facts that (1) there is mitochondrial damage and augmented superoxide production in EAE and MS (Kalman *et al*. 2007) and (2) protein carbonyls do not increase until after the antioxidant glutathione is depleted. In this model, mitochondrial dysfunction and the resulting intracellular ROS production is likely triggered by glutamate excitotoxicity and calcium influx, which also activate calpain, found in this study to be the major proteolytic mechanism for cytoskeletal protein removal. The lack of protein carbonylation in the presence of sustained RCS production is noteworthy and suggests that carbonylation of proteins in EAE takes place via a direct mechanism and not indirectly by reaction with RCS (MDA, 4-HNE glyoxal, methyl glyoxal). In this regard, our group has recently found that, while dependent on lipid peroxidation, the carbonylation of cytoskeletal proteins during depletion of glutathione probably occurs via lipid hydroperoxides rather than RCS (Bizzozero *et al*. 2007). Furthermore, western blot analysis of CNS proteins using specific antibodies against RCS showed that the amount of MDA-, acrolein- and 4-HNE-

protein adducts does increase neither upon GSH depletion (Zheng & Bizzozero 2010c) nor during the course of MOG-induced EAE in C57/BL6 mice (Zheng & Bizzozero 2010a).

Another major finding in this study is the identification of neurofilaments, β-tubulin, β-actin and GFAP as targets of carbonylation in EAE. Interestingly, it was also observed that there is a gradient in the carbonylation of NF proteins with NFH being the most heavily oxidized and NFL having the lowest specific oxidation. This may be due to the structure of the intermediate filament in which NFL forms the filament core and NFH and NFM are more peripherally located making them more susceptible to attack by ROS (Tsuda et. al. 2000) as well as the presence of more carbonylation-susceptible amino acid sequences in NFH compared to NFM and NFL (see Appendix C). Cytoskeletal proteins are established targets of protein carbonylation in many diseases including several CNS disorders (Aksenov *et al*. 2001, Muntane *et al*. 2006), and oxidative damage to these proteins is known to have deleterious effects on the macromolecule structures. For instance, actin filaments and microtubules both destabilize and disassemble upon oxidation of their protein components (Banan *et al*. 2001, Banan *et al*. 2004, Dalle-Donne *et al*. 2001, Neely *et al*. 2005, Ozeki *et al*. 2005). The secondary structure of neurofilaments is also altered when the individual proteins become carbonylated (Gelinas *et al*. 2000), often leading to the formation of dense aggregates (Smith *et al*. 1995). I have demonstrated that neurofilament proteins and β-tubulin are extensively degraded in the EAE spinal cord, which likely causes alterations in the cytoskeletal network. I have also observed that the specific oxidation of NFL protein decreases in EAE, suggesting the possibility that oxidized neurofilament may be preferentially degraded in this disease. It

is well known that proteolysis of carbonylated proteins in cells under conditions of oxidative stress aids in preventing formation of large aggregates that could be cytotoxic (Davies & Shringarpure 2006, Shringarpure *et al*. 2001). Identifying the triggers for neurofilament degradation may help us to understand the mechanisms of axonal degeneration during neuroinflammation in acute EAE.

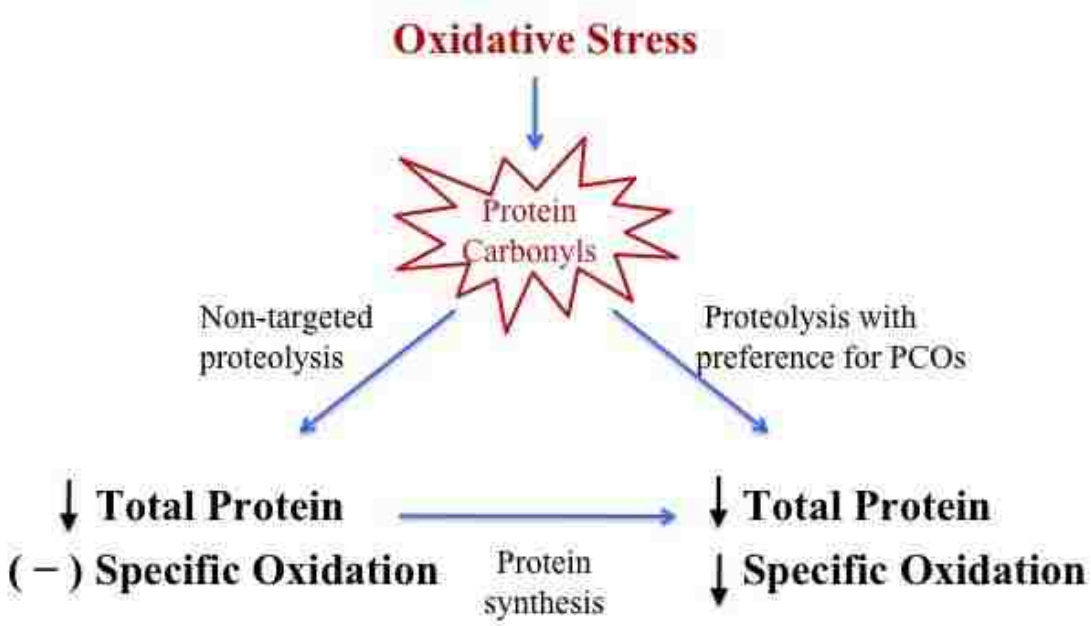
Whether calpain, the proteasome, or a combination of these proteolysis pathways is responsible for the degradation of carbonylated neurofilament proteins in EAE was the focus of chapter 3. The 20S proteasome has been shown to selectively recognize and degrade oxidized proteins (Grune *et al.* 1995, Pacifici *et al*. 1993, Rivett 1985), and upon its inhibition carbonylated proteins do indeed accumulate (Lee *et al*. 2001). Calpain is also believed to be responsible for neurofilament degradation in EAE (Shields & Banik 1998), but it is unclear if this calcium-activated protease has preference for oxidized over native polypeptides. Previous studies using proteasome and calpain inhibitors in EAE have primarily focused on abatement of neurological symptoms without looking at specific proteins that are protected from proteolysis (Guyton *et al*. 2005, Hassen *et al.* 2008, Hosseini *et al*. 2001, Vanderlugt *et al*. 2000).

In this study I used a single i.t. injection of the proteasome inhibitor epoxomicin or the calpain inhibitor calpeptin to inhibit these proteolytic pathways for 48 hours at the onset of clinical symptoms. I showed that a modest inhibition of calpain 48 hours early in clinical disease leads to increased accumulation of total protein carbonyls, as measured by oxyblot. Degradation of the neurofilament proteins and β -tubulin was also significantly reduced in the animals with inhibited calpain activity. However, I was unable to detect significant changes in the specific carbonylation of these particular

cytoskeletal proteins. Thus, I conclude that while calpain is an important degradation mechanism for cytoskeletal proteins, it does not preferentially remove carbonylated neurofilaments or tubulin at a faster rate than it degraded the non-oxidized cytoskeletal elements. Approaching the question of the role of the proteasome in cytoskeletal degradation and oxidation, I found proteasomal inhibition did not lead to changes in total carbonylation or degradation of these proteins. There was an increase in the percent of oxidized NFM with proteasome inhibition, indicating that the proteasome may contribute to removal of oxidized neurofilament even though calpain is clearly the primary mechanism for degradation of those proteins.

The increase seen in total protein oxidation after treatment with a calpain inhibitor is most likely due to the lower amount of protein degradation overall. With decreased proteolysis there are more neurofilament and tubulin, which are major oxidized species, and the overall increase in carbonylation reflects the increased presence of these, and potentially other unidentified, carbonylated proteins molecules. In chapter 2, I reported that there is a change in the specific oxidation of NFL throughout the disease course and hypothesized that this was due to preferential degradation of oxidized proteins by either calpain or the proteasome (Smerjac & Bizzozero 2008). Another potential explanation for the lower percentage of oxidized NFL molecules later in the disease course is that cytoskeletal digestion could trigger synthesis of new NFL proteins to replace the degraded molecules, which would contribute to the neurological recovery seen in this acute EAE model. Oxidatively damaged proteins accumulate in organisms as they age (Stadtman & Berlett 1998), and therefore proteins with slow turnover rates are more likely to have oxidative modifications like carbonylation. Newly synthesized NFL

molecules would be less likely to have oxidative modifications, thereby decreasing the specific carbonylation of the particular protein (Figure 4.1). The presence of newly synthesized, non-oxidized proteins diluting the total protein pool is a logical explanation



for the decrease in specific oxidation of certain cytoskeletal proteins seen during EAE. In contrast, during the chronic phase in the murine EAE model carbonylation levels are much higher than those seen in the acute rat model and there are 2-5 fold increases in the proportion of cerebellar GFAP, β-actin, and β-tubulin that are carbonylated in the chronic phase compared to the acute phase (Zheng & Bizzozero 2010a). This increase is believed to be a result of impaired proteasomal activity during chronic EAE (Zheng & Bizzozero 2010b). Proteasomal activity in this acute model in Lewis rats is increased during the disease. This disparity highlights the various factors contributing to carbonyl accumulation during different phases of these myelin-directed neuroinflammatory disorders.

The clinical implications of this study are twofold. First, the discovery that there are likely significant sources of metabolic ROS during acute neuroinflammation provides insights into mechanisms that contribute to oxidative cellular damage in MS. When carbonyls accumulate in the CNS the damage to proteins can result in cross-linking and the formation of high molecular weight aggregates that are resistant to proteolysis. This aggregate formation may impair neuronal signaling and contribute to neurological disability seen in advanced stages of the disease. Potential therapies focused on reducing mitochondrial oxidants may help to prevent oxidative protein damage improving or delaying progressive disease activity. Second, in this work I have identified calpain as the major proteolytic mechanisms for cytoskeletal degradation during neuroinflammation. Degradation of the neuronal cytoskeleton, especially neurofilaments, coincides with axonal damage, which is believed to be responsible for the progressive neurologic impairments seen in secondary-progressive MS. Acute treatment with calpain inhibitors during the peak of neuroinflammatory activity, even after the onset of symptoms, was able to significantly prevent degradation of the neuronal cytoskeleton. Reducing cytoskeletal proteolysis during inflammatory attacks may offer a therapeutic strategy to impede axonal damage, thereby reducing or delaying the onset of progressive neurologic disability seen in the later disease stages.

4.2 Limitations of the experimental model and future directions

Our findings suggest that preferential degradation of carbonylated proteins by calpain or the proteasome is not a contributing factor to the decrease in specific oxidation of NFL. It is, however, important to consider the limitations of our experiment, which approached this question in an *in vivo* system by treating animals with inhibitors. I achieved a 22%

decrease in calpain activity and a 24% decrease proteasome activity in EAE spinal cord tissue after direct intrathecal injection. This reduction in calpain enzyme activity is significant, and led to considerable biochemical changes in protein oxidation and cytoskeletal degradation. However, in this system I could not achieve higher levels of inhibition of either proteolytic system even with 10x doses of calpeptin or epoxomicin, or using other proteasome and calpain inhibitors (data not shown). It must be considered that remaining active calpain or proteasome could be sufficient to preferentially remove oxidized proteins from the cell. When considering that the proteasome activity is increased 1.6 fold and calpain is 5.5 times more active in EAE over naïve rats, it is possible that even with this reduction in activity there is still adequate amounts of active proteasome or calpain to clear damaged proteins from the cells.

The protocol that I utilized for i.t. injection of inhibitor used a single injection. It would be interesting to explore the effect of a sustained delivery of these calpain or proteasome inhibitors. One possibility would be to use an indwelling spinal catheter allowing for multiple inhibitor injections in the same animal. Indwelling spinal catheters for drug delivery in rats are routinely employed in a variety of experimental paradigms including MOG-induced EAE in Dark Agouti rats (Ramos *et al.* 2010). This approach would require surgery to place the catheter at least one week prior to disease onset. Extensive control experiments would be required to work out doses of inhibitors and determine whether spinal catheter placement influences oxidative stress and cytoskeletal proteolysis in EAE Lewis rats since i.t. catheterization alone is known to induce immune responses in the spinal cord, including cytokine induction, microglia and astrocyte activation (DeLeo *et al.* 1997).

Additionally, we chose to focus on neurofilaments and tubulin, which I demonstrated previously to be some of the major targets of oxidation and degradation in the cell, but it is possible that investigating specific oxidation levels of other carbonylated proteins after proteasome or calpain inhibition could identify proteins that do have increased susceptibility to degradation in this model. Two-dimensional oxyblots could be used to isolate protein spots that show significant changes in oxidation levels after treatment with inhibitors. After identification of the proteins by mass spectrometry, I could use the biotin-hydrazide pull-down followed by western blotting to measure changes in the specific oxidation of those proteins and to confirm changes seen in the 2D oxyblot.

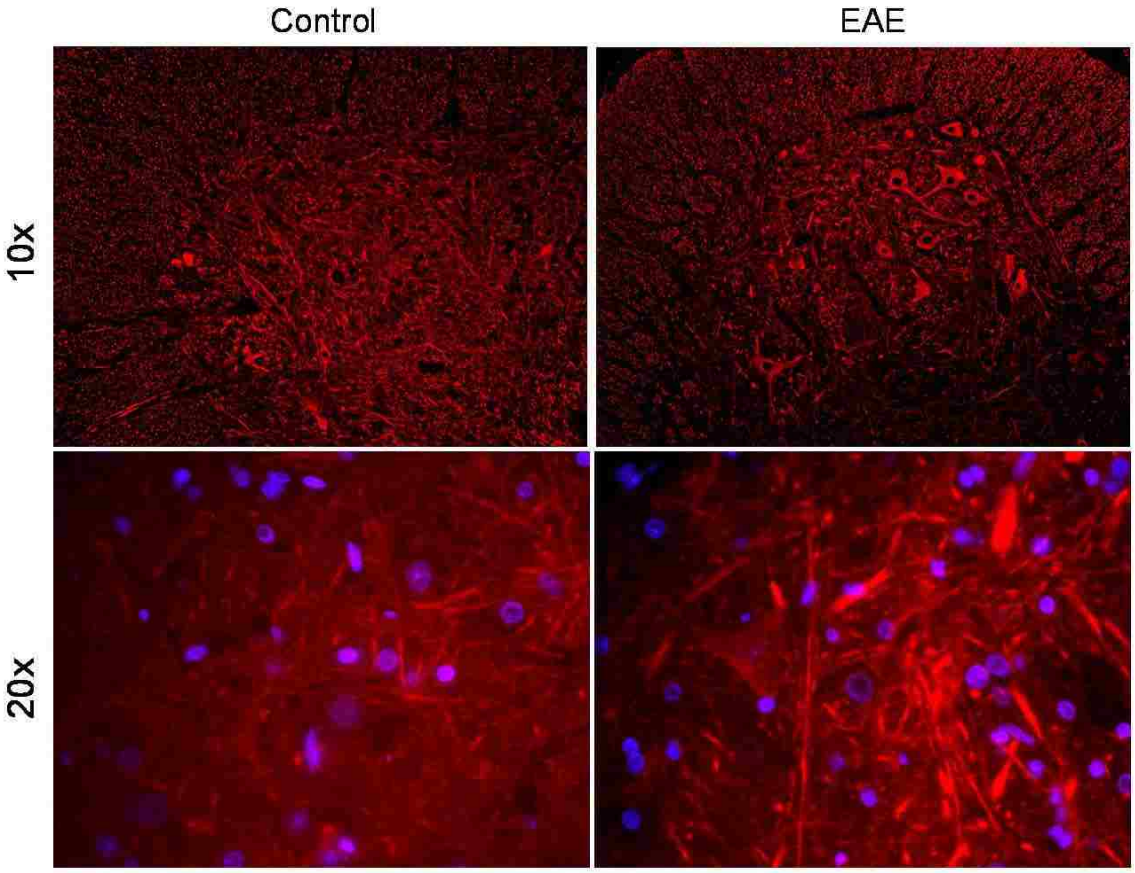
Finally, experiments are underway in our lab to confirm the *in vivo* findings in a cell culture system. These experiments are using glutathione depletion to trigger oxidative stress and induce protein carbonyls in cultured neurons, which will then be treated with calpeptin and epoxomicin to inhibit calpain or the proteasome. To date, degradation of oxidized neurofilament proteins has only been investigated in cell-free systems (Troncoso *et al*. 1995). Determining proteolytic mechanisms for removal of carbonylated neurofilaments in cell culture, combined with the results from this *in vivo* study, will help to clarify the contribution of the proteasome and calpain to proteolysis of neurofilament proteins and help elucidate the role of protein carbonylation in axonal damage.

4.3 Scientific impact

This study was the first to identify oxidation of the neuronal cytoskeleton during acute phase of autoimmune neuroinflammation. Furthermore, the ubiquitous accumulation of oxidized proteins in various cell types and far from inflammatory lesions suggests severe oxidative stress not just from monocyte-derived ROS, but from metabolic sources as well. The cytoskeletal proteins that are carbonylated in EAE are also major oxidized molecules observed in MS patients. Identifying this type of damage to these proteins in an animal model will allow elucidation of mechanisms contributing to axonal damage in MS. This is also the first study, to our knowledge, to investigate the specific targeting of carbonylated proteins for degradation by the proteasome or calpain *in vivo.*

Appendix A

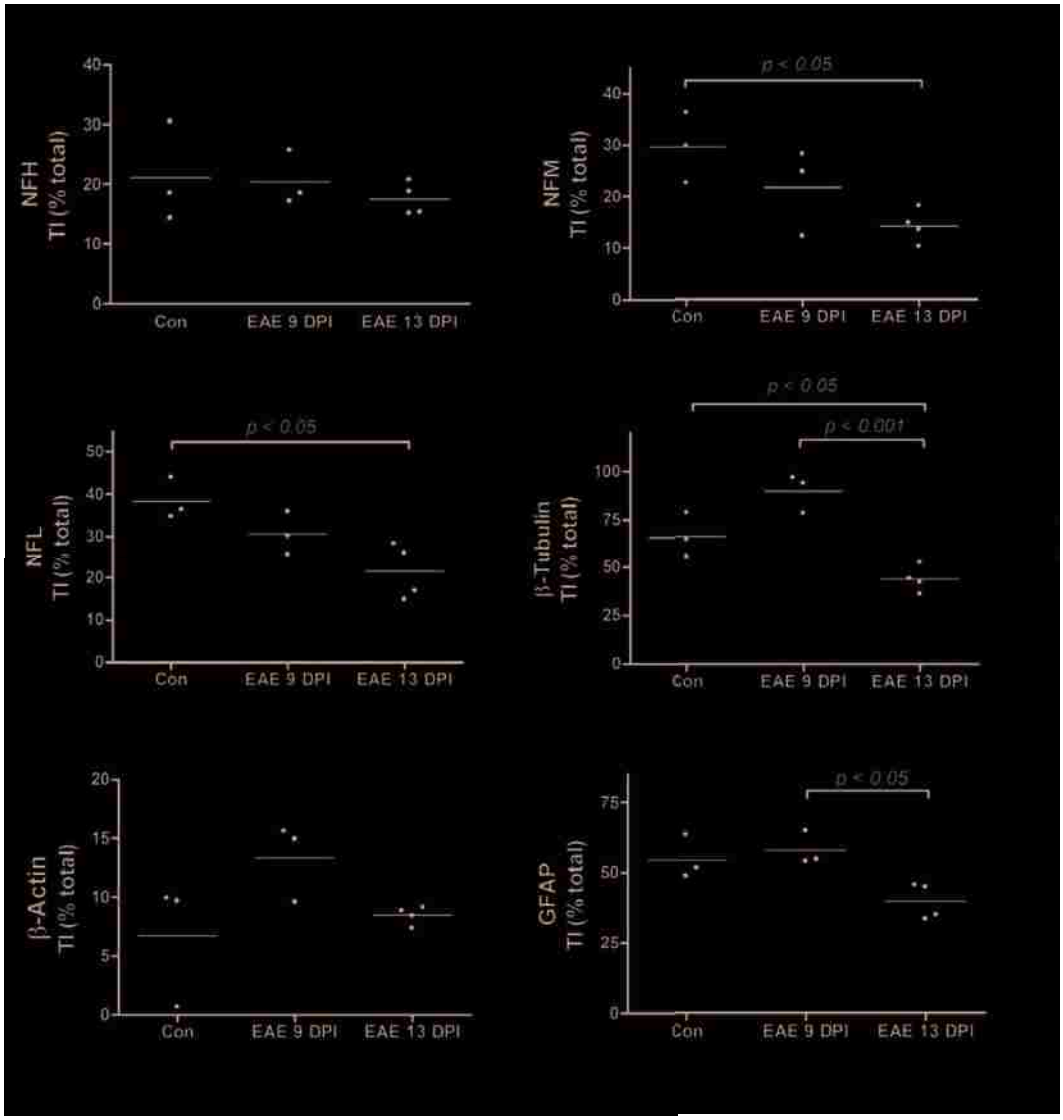
Figure A.1 NFM immunofluorescence in Control and EAE ventral horn region of lower thoracic spinal cord sections (10x and 32x) with DAPI counterstain.



Using immunofluorescence to look at the neurofilament network in EAE animals I was able to detect an altered staining pattern for NFM. This figure shows IHC for NFM in control and EAE spinal cord cross sections. In the 10x imaged you can see heavy staining in the cell bodies in EAE whereas the control animals show a more even neurofilament distribution. At 20x there NFM staining in EAE is more punctate than the normal filamentous staining seen in the control spinal cords, suggesting the possible formation of axonal ovoid bodies. Overall these images suggest alterations in the neurofilament network including changes in the axonal distribution of the proteins. The heavy staining in the cell body possibly points to an increase in newly synthesized neurofilament that has not yet been transported down the axons.

Appendix B

Figure B.1 Triton insoluble fraction of cytoskeletal proteins in CFA-injected control and EAE spinal cords.



Changes in the distribution (assembled vs. disassembled) of cytoskeletal protein during the course of EAE. Carbonylation can lead to depolymerization of the cytoskeletal structures, so lower proportions of specific proteins may be found in the Triton insoluble (assembled) cytoskeletal fraction. At 13 DPI there is significantly more disassembled NFM, NFL, β – tubulin. This suggests that at the peak of disease there is disassembly of the cytoskeleton and that damage may be contributing to the degradation of those proteins. Because there is also more disassembled GFAP, which is not degraded in EAE, at 13 DPI, there is likely to be increased synthesis of these proteins, as well, which have not yet become incorporated into filaments.

Appendix C

Direct metal-catalyzed oxidation attacks on the amino-acid side chains of proline (P), arginine (R), lysine (K) and threonine (T) form the majority of carbonyl derivatives. The rules governing sites that are highly susceptible to carbonylation are complex, but one predictive measure is the presence of an RKPT- enriched region, defined by the presence of at least 3 carbonylatable amino acids within a 4 amino acid sequence window (Maisonneuve *et al*. 2009). Mass spectrometry analysis of bovine serum albumin and 23 carbonylated proteins in *Escherichia coli* show that amino acids located within RKPT-enriched regions are approximately 4 times more prone to oxidation than those external to these regions. Sequence analysis of the three rat neurofilament chains for RKPT-enriched regions reveal that NFH contain 28-, NFM has 15-, and NFL has 5- RKPT-enriched regions (Figures C.1., C.2., and C.3.). This analysis supports my observation that the specific oxidation of the neurofilaments follows the gradient where carbonylation of NFH > NFM > NFL.

Figure C.1. Neurofilament heavy chain protein sequence with the 28 amino acid sequences that are highly susceptible to carbonylation underlined.

```
        10         20         30         40         50         60
MMSFGSADAL LGAPFAPLHG GGSLHYALSR KAGAGGTRSA AGSSSGFHSW ARTSVSSVSA
        70         80         90        100        110        120
SPSRFRGAAS STDSLDTLSN GPEGCVAAVA ARSEKEQLQA LNDRFAGYID KVRQLEAHNR
        130        140        150        160        170        180
TLEGEAAALR QQKGRAAMGE LYEREVREMR GAVLRLGAAR GHVRLEQEHL LEDIAHVRQR
        190        200        210        220        230        240
LDEEARQREE AEAAARALAR FAQEAEAARV ELQKKAQALQ EECGYLRRHH QEEVGELLGQ
        250        260        270        280        290        300
IQGCGAAQAQ AQAEARDALK CDVTSALREI RAQLEGHTVQ STLQSEEWFR VRLDRLSEAA
        310        320        330        340        350        360
KVNTDAMRSA QEEITEYRRQ LQARTTELEA LKSTKESLER QRSELEDRHQ VDMASYQDAI
        370        380        390        400        410        420
QQLDNELRNT KWEMAAQLRE YQDLLNVKMA LDIEIAAYRK LLEGEECRIG FGPSPFSLTE
        430        440        450        460        470        480
GLPKIPSMST HIKVKSEEKI KVVEKSEKET VIVEEQTEEI QVTEEVTEEE DKEAQGEEEE
        490        500        510        520        530        540
EAEEGGEEAA TTSPPAEEAA SPEKETKSPV KEEAKSPAEA KSPAEAKSPA EAKSPAEVKS
```

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        550         560         570         580         590         600
PAEVKSPAEA KSPAEAKSPA EVKSPAEVKS PAEAKSPAEA KSPAEVKSPA TVKSPGEAKS
        610         620         630         640         650         660
PAEAKSPAEV KSPVEAKSPA EAKSPASVKS PGEAKSPAEA KSPAEVKSPA TVKSPVEAKS
        670         680         690         700         710         720
PAEVKSPVTV KSPAEAKSPV EVKSPASVKS PSEAKSPAGA KSPAEAKSPV VAKSPAEAKS
        730         740         750         760         770         780
PAEAKPPAEA KSPAEAKSPA EAKSPAEAKS PAEAKSPVEV KSPEKAKSPV KEGAKSLAEA
        790         800         810         820         830         840
KSPEKAKSPV KEEIKPPAEV KSPEKAKSPM KEEAKSPEKA KTLDVKSPEA KTPAKEEAKR
        850         860         870         880         890         900
PADIRSPEQV KSPAKEEAKS PEKEETRTEK VAPKKEEVKS PVEEVKAKEP PKKVEEEKTP
        910         920         930         940         950         960
ATPKTEVKES KKDEAPKEAQ KPKAEEKEPL TEKPKDSPGE AKKEEAKEKK AAAPEEETPA
        970         980         990        1000        1010        1020
KLGVKEEAKP KEKAEDAKAK EPSKPSEKEK PKKEEVPAAP EKKDTKEEKT TESKKPEEKP
       1030        1040        1050        1060        1070
KMEAKAKEED KGLPQEPSKP KTEKAEKSSS TDQKDSQPSE KAPEDKAAKG DK
```

Figure C.2. Neurofilament medium chain protein sequence with the 15 amino acid sequences that are highly susceptible to carbonylation underlined.

```
        10          20          30          40          50          60
MSYTLDSLGN PSAYRRVPTE TRSSFSRVSG SPSSGFRSQS WSRGSPSTVS SSYKRSALAP
        70          80          90         100         110         120
RLAYSSAMLS SAESSLDFSQ SSSLLNGGSG GDYKLSRSNE KEQLQGLNDR FAGYIEKVHY
       130         140         150         160         170         180
LEQQNKEIEA EIHALRQKQA SHAQLGDAYD QEIRELRATL EMVNHEKAQV QLDSDHLEED
       190         200         210         220         230         240
IHRLKERFEE EARLRDDTEA AIRAVRKDIE ESSMVKVELD KKVQSLQDEV AFLRSNHEEE
       250         260         270         280         290         300
VADLLAQIQA SHITVERKDY LKTDISTALK EIRSQLECHS DQNMHQAEEW FKCRYAKLTE
       310         320         330         340         350         360
AAEQNKEAIR SAKEEIAEYR RQLQSKSIEL ESVRGTKESL ERQLSDIEER HNHDLSSYQD
       370         380         390         400         410         420
IQQLENELR GTKWEMARHL REYQDLLNVK MALDIEIAAY RKLLEGEETR FSTFSGSITG
       430         440         450         460         470         480
PLYTHRQPSV TISSKIQKTK VEAPKLKVQH KFVEEIIEET KVEDEKSEME DALTVIAEEL
       490         500         510         520         530         540
AASAKEEKEE AEEKEEEPEV EKSPVKSPEA KEEEEGEKEE EEEGQEEEEE EDEGVKSDQA
       550         560         570         580         590         600
EEGGSEKEGS SEKDEGEQEE EGETEAEGEG EEAEAKEEKK TEGKVEEMAI KEEIKVEKPE
       610         620         630         640         650         660
KAKSPVPKSP VEEVKPKPEA KAGKDEQKEE EKVEEKKEVA KESPKEEKVE KKEEKPKDVP
       670         680         690         700         710         720
DKKKAESPVK EKAVEEMITI TKSVKVSLEK DTKEEKPQQQ EKVKEKAEEE GGSEEEVGDK
       730         740         750         760         770         780
SPQESKKEDI AINGEVEGKE EEEQETQEKG SGQEEEKGVV TNGLDVSPAE EKKGEDRSDD
       790         800         810         820         830         840
KVVVTKKVEK ITSEGGDGAT KYITKSVTVT QKVEEHEETF EEKLVSTKKV EKVTSHAIVK

EVTQGD
```

Figure C.3. Neurofilament light chain protein sequence with the 5 amino acid sequences that are highly susceptible to carbonylation underlined.

```
       10         20         30         40         50         60
MSSFSYEPYF STSYKRRYVE TPRVHISSVR SGYSTARSAY SSYSAPVSSS LSVRRSYSSS
       70         80         90        100        110        120
SGSLMPSLEN LDLSQVAAIS NDLKSIRTQE KAQLQDLNDR FASFIERVHE LEQQNKVLEA
      130        140        150        160        170        180
ELLVLRQKHS EPSRFRALYE QEIRDLRLAA EDATNEKQAL QGEREGLEET LRNLQARYEE
      190        200        210        220        230        240
EVLSREDAEG RLMEARKGAD EAALARAELE KRIDSLMDEI AFLKKVHEEE IAELQAQIQY
      250        260        270        280        290        300
QISVEMDVS SKPDLSAALK DIRAQYEKLA AKNMQNAEEW FKSRFTVLTE SAAKNTDAVR
      310        320        330        340        350        360
AAKDEVSESR RLLKAKTLEI EACRGMNEAL EKQLQELEDK QNADISAMQD TINKLENELR
      370        380        390        400        410        420
STKSEMARYL KEYQDLLNVK MALDIEIAAY RKLLEGEETR LSFTSVGSIT SGYSQSSQVF
      430        440        450        460        470        480
GRSAYSGLQS SSYLMSARAF PAYYTSHVQE EQSEVEETIE ATKAEEAKDE PPSEGEAEEE
      490        500        510        520        530        540
EKEKEEGEEE EGAEEEEAAK DESEDAKEEE GGEGEEEDTK ESEEEEKKEE SAGEEQAAKK

KD
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Abbreviations Used

4-HNE	4-Hydroxynonenal
ANOVA	analysis of variance
CFA	complete Freund's adjuvant
CNS	central nervous system
DNP	2,4-dinitrophenyl
DMSO	dimethyl sulfoxide
DPI	days post immunization
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ECL	enhanced chemioluminescence
GFAP	glial acidic fibrillary protein
GM	gray matter
GSH	glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HRP	horseradish peroxidase
IHC	immunohistochemistry
i.p.	intraperitoneal
i.t.	intrathecal
MDA	malondialdehyde
MBP	myelin basic protein
MS	multiple sclerosis
NFH	neurofilament heavy (200kDa) protein
NFL	neurofilament light (69kDa) protein
NFM	neurofilament medium (150kDa) protein

OS	oxidative stress
PAGE	polyacrylamide gel electrophoresis
PCOs	protein carbonyls
PE	polyethylene
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
RCS	reactive carbonyl species
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TBARS	thiobarbituric acid reactive substances
WM	white matter

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