

Invited Review

A putative role for calpain in demyelination associated with optic neuritis

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Summary. Calcium activated neutral proteinase (calpain) is an endopeptidase present in the central nervous system which degrades myelin proteins. To examine the role of calpain in demyelination associated with optic neuritis, immunocytochemical expression of calpain was evaluated in Lewis rats with experimental optic neuritis. Calpain expression was increased in activated microglia, infiltrating macrophages, activated T cells, and reactive astrocytes in experimental optic neuritis compared to controls. Calpain activity and translational expression were also examined by Western blotting studies measuring the extent of myelin protein degradation, calpain-specific fodrin proteolysis, axonal neurofilament degradation, and calpain proenzyme content. Results showed myelin associated glycoprotein and 68 kD neurofilament protein levels were significantly decreased while calpain translational expression and calpain-autolyzed fodrin levels were significantly increased in experimental optic neuritis compared to controls. Thus, increased activity and translational expression of calpain in optic neuritis may be integral to the pathogenesis of this disorder.

Key words: Calpain, Calpastatin, Optic neuritis, Multiple sclerosis, Calcium

Introduction

Optic nerve degeneration is one of the most common features of optic neuritis (ON) which leads to impaired vision and possible blindness. This condition is also recognized as one of the first manifestations of multiple sclerosis (MS) although not all patients with ON subsequently develop MS. Thus, subtle differences between these two degenerative diseases may exist. Although optic neuritis is thought to be a T cell mediated autoimmune disease like MS, the etiological factor(s) and pathophysiological mechanisms responsible for optic nerve degeneration remain poorly understood.

Thus, detailed pathophysiological, biochemical and therapeutic studies in animal models of ON are necessary for development of successful therapeutic protocols.

The optic nerve is composed largely of myelinated axons and glial cells including astrocytes, oligodendrocytes, and microglia. Axonal degeneration, myelin protein degradation, and/or death of myelin-forming oligodendrocytes and retinal ganglion cells may contribute to impaired vision associated with ON. The factors involved in myelin protein degradation have not been completely delineated, but proteases (including lysosomal and neutral proteinases) that degrade axon and myelin proteins have been implicated in demyelinating diseases such as MS (Hallpike and Adams, 1969; Smith, 1977; Allen, 1983; Banik, 1992). Calpain, a calcium-activated neutral proteinase is one such enzyme which has recently been shown to play a role in tissue degeneration associated with CNS trauma, MS, and experimental allergic encephalomyelitis (EAE), the corresponding MS animal model (Saatman et al., 1996; Banik et al., 1997; Kampfl et al., 1997; Shields and Banik, 1998a). The ubiquitous form of calpain exists as microcalpain (μ calpain) and millicalpain (mcalpain) isoforms which require μ M and mM calcium levels, respectively, for activation.

The mechanism of optic nerve degeneration in ON has been studied in EAE, an animal model of ON as well. The disease is induced in animals after subcutaneous injection of emulsified whole myelin/myelin proteins, [e.g., myelin basic protein (MBP)] and complete Freund's adjuvant with the onset of clinical symptoms at 10-12 days following administration. Earlier histopathological studies of optic nerves from animals with EAE revealed features also observed in MS such as infiltration of inflammatory cells and degeneration of axons and myelin (Bullington and Waksman, 1958; VonSallman et al., 1967). Recent histopathologic and electron microscopic studies have demonstrated perivascular cuffing, degeneration, and phagocytosis of myelin in ON, an important feature of chronic experimental ON (Rao, 1981). While these studies clearly demonstrated cellular infiltration, gliosis and morphological changes, including ultrastructural

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destruction of axons, the biochemical/molecular mechanisms of axon-myelin degeneration remain unclear. Thus, the role of calpain in the pathophysiology of optic nerve degeneration in experimental ON has been examined recently in our laboratory.

EAE was induced in Lewis rats by subcutaneously injecting an emulsion of guinea pig MBP and complete

Freund's adjuvant. The optic nerves from adjuvant controls and animals with acute disease (grade 4, two limbs plegic) were examined for calpain activity and expression (at translational and transcriptional levels). The data indicated increased calpain immunoreactivity in activated glial/inflammatory cells, with elevated calpain activity, translational expression, and

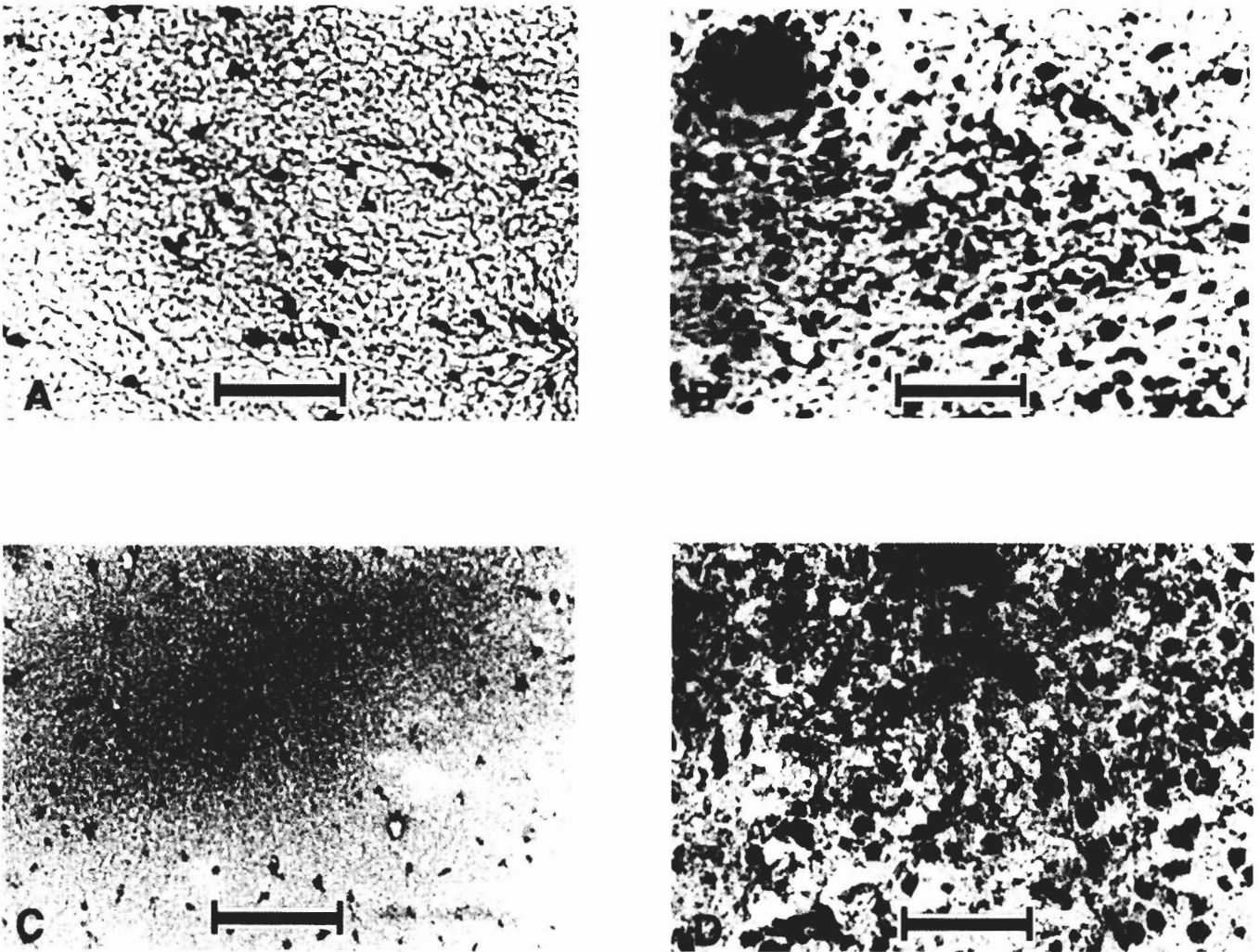
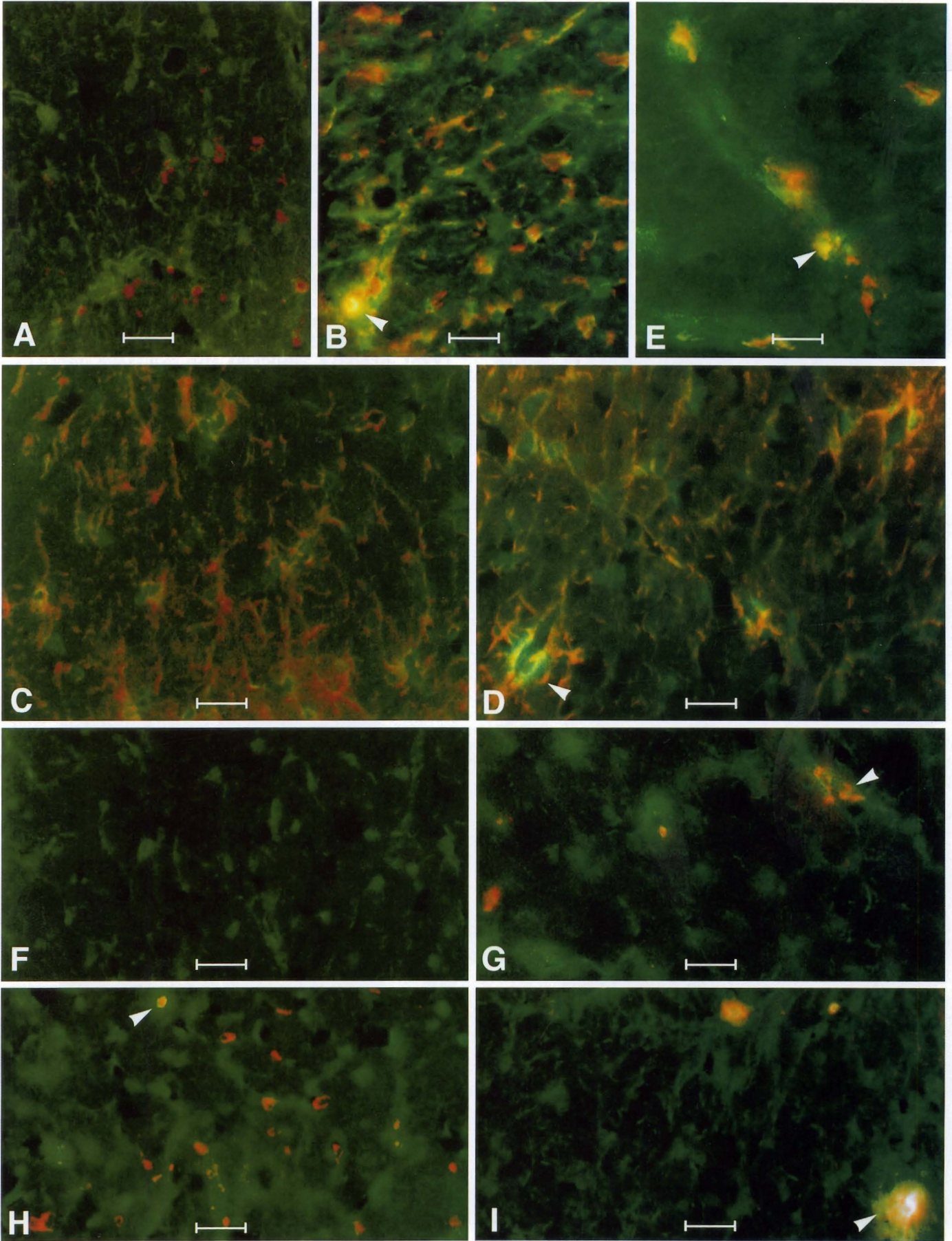


Fig. 1. H&E and calpain immunoperoxidase staining in Lewis rat optic nerve. x 200. **A.** Control Lewis rat optic nerve with H&E staining showed normal glial cells. **B.** Optic nerve from Lewis rat with acute EAE demonstrated perivascular cuffing and increased numbers of glial and inflammatory cells with H&E staining. **C.** Immunoperoxidase staining of normal optic nerve showed very little calpain expression in cell bodies. **D.** In optic nerves of rats with acute EAE, greater number of cells showed markedly increased calpain expression. Scale bars: 100 μ m.

Fig. 2. Double-immunofluorescent staining of Lewis rat optic nerves for calpain and cell-specific markers. x 200. **A.** Fluorescent labeling for calpain and OX42 (microglia) in normal optic nerve. **B.** Fluorescent labeling for calpain and OX42 in optic nerve at the same magnification showed calpain expression in reactive microglia (yellow) in acute EAE (arrow). **C.** Fluorescent labeling for calpain and GFAP (astrocytes) in normal optic nerve. **D.** Optic nerve of rat with acute EAE demonstrated increased calpain expression in astrocytic foot processes encasing inflammatory foci (arrow). **E.** Fluorescent labeling for calpain and ED2 (macrophages) in EAE rat optic nerve showed calpain expression (arrow). **F.** Fluorescent labeling for calpain and ED1 (specific for macrophage phagolysosome membrane protein) in normal rat optic nerve showed no phagocytic activity. **G.** Phagolysosomes (arrow) in calpain-positive macrophages were observed in optic nerves of EAE rats. **H.** Fluorescent labeling for calpain and CD2 (T cells) in EAE rats demonstrated increased calpain expression in some T cells (arrow). **I.** In EAE optic nerve, IFN- γ expression was colocalized with calpain expression (arrow), presumably by a cluster of activated T cells. Fig. 2F depicts normal controls for ED2, ED1, CD2, and IFN- γ expression. Scale bars: 50 μ m.



degradation of cytoskeletal and myelin proteins in optic nerves of animals with disease compared to controls (Shields et al., 1998; Shields and Banik, 1998b).

Histochemical and immunocytochemical studies

H & E studies of optic nerves from disease animals revealed a much greater number of glial and inflammatory cells compared to controls. These cells were clustered along blood vessels - consistent with mononuclear cell infiltration (Fig. 1) as previously observed (Bullington and Waksman, 1958; VonSallman et al., 1967; Raine, 1976; Rao, 1981). Subsequent single immunoperoxidase staining for calpain in optic nerves of disease animals revealed increased numbers of calpain-positive cells with markedly increased calpain immunoreactivity compared to those of control optic nerves. This suggested both glial and infiltrating inflammatory cells were capable of upregulating calpain expression during the autoimmune inflammatory response. In order to delineate a role for calpain in optic nerve degeneration, we attempted to identify specific cell type(s) expressing increased levels of calpain in experimental ON. Thus, double immunofluorescent labeling procedures using a polyclonal calpain antibody and monoclonal cell-specific marker antibodies were employed to identify cells in optic nerves of experimental ON and controls (Fig. 2).

Since reactive gliosis and inflammatory infiltrates are hallmarks of demyelinating diseases including optic neuritis, OX-42 and anti-GFAP antibodies were used for identification of calpain expression by microglia and astrocytes, respectively. Marker antibodies were also employed for macrophages (ED 1, 2) and T cells (CD 2). Staining with calpain and antibodies specific for T cells

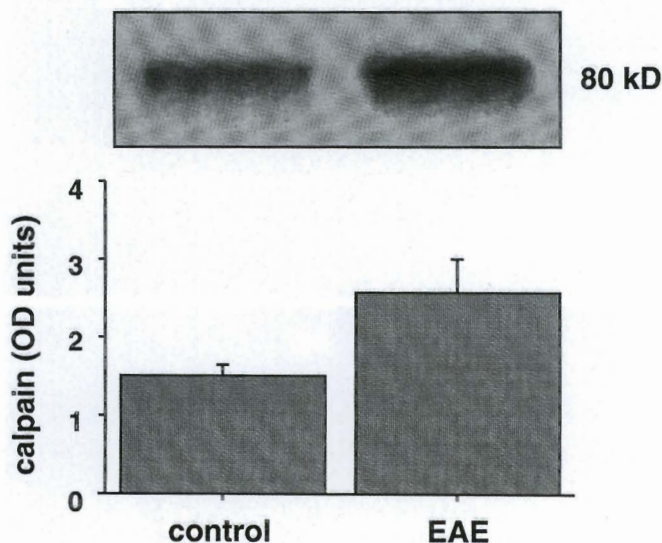


Fig. 3. Calpain translational expression in rats with experimental optic neuritis compared to adjuvant controls. Western blots (top) of samples using a polyclonal m-calpain antibody were quantified via densitometry and analyzed by one way ANOVA (\pm SEM).

showed relatively little calpain immunoreactivity in normal optic nerve. In contrast to controls, calpain expression was markedly increased in both activated microglia and macrophages in disease.

The findings of increased calpain staining in infiltrating macrophages and activated microglia suggested these cells, with elevated calpain activity and translational expression, may play a role in axon/myelin destruction. Since activated microglia secrete other enzymes capable of degrading MBP, calpain (active at extracellular calcium levels) may also be released. Previous *in vitro* studies found activated T cells and macrophages secrete calpain capable of digesting exogenous MBP (Deshpande et al., 1995a; Smith et al., 1998). Reactive astrocytes also expressed markedly increased levels of calpain in astrocytic processes surrounding inflammatory foci. However, this increased calpain expression in astrocytes was not uniformly present throughout the optic nerve of disease animals. Since optic neuritis is believed to be an autoimmune disease mediated by activated T cells, calpain levels in these cells were also examined (Bullington and Waksman, 1958; Raine et al., 1980; Rao, 1981). Just as T cells stimulated by cytokines upregulate calpain expression *in vitro*, labelling of optic nerve with an anti-IFN- γ monoclonal antibody revealed increased calpain expression in activated T cells in disease animals compared to those of control optic nerves (Deshpande et al., 1995b; Shields et al., 1998).

The findings of increased calpain expression in

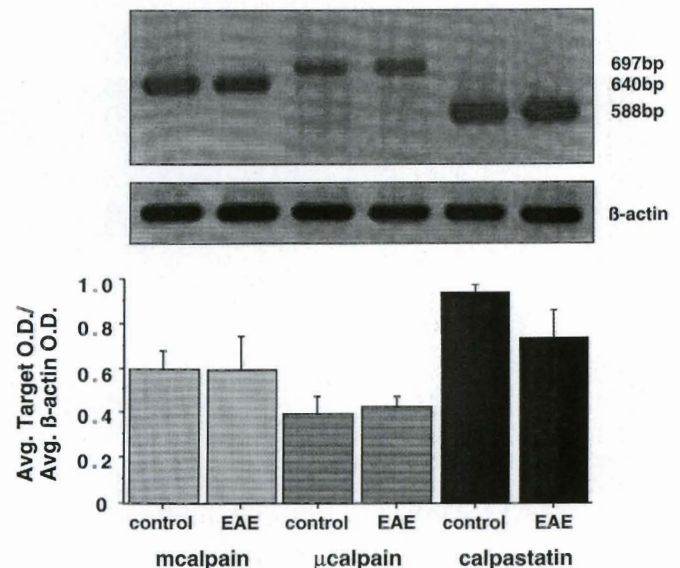


Fig. 4. Transcriptional expression of m-calpain, μ -calpain, and calpastatin in animals with experimental optic neuritis compared to adjuvant controls. β -actin (538bp) served as an internal control for each product (μ -calpain: 697bp; m-calpain: 640bp; calpastatin: 588bp) using RT-PCR (top). The ratios of target band optical densities with that of β -actin were calculated after quantification by densitometry and analyzed by one way ANOVA (\pm SEM) as depicted graphically. The ratios of m-calpain ($p=0.98$), μ -calpain ($p=0.76$), and calpastatin ($p=0.15$) were not significant in disease animals compared to controls.

Role of calpain in optic neuritis

activated glial and infiltrating inflammatory cells suggested up-regulation of calpain translational expression and possible secretion of this and other proteolytic enzymes (Deshpande et al., 1995a; Smith et al., 1998). Activated human T cell lines have been found to release cathepsins and neutral proteinases, including calpain (Deshpande et al., 1995a,b). Thus, calpain may be one of the proteinases released during inflammation in ON. The released enzyme may then degrade axonal proteins, contributing to granular degeneration of axons, and also digest myelin proteins such as MBP to produce novel immunogenic peptides implicated in epitope spreading. Immunogenic peptides used for induction of EAE in Lewis rats are present among calpain-cleaved fragments (residues 50-97 and 71-170) of MBP (Hashim et al., 1986; Tsubata and Takahashi, 1989; Banik et al., 1994).

Biochemical studies

Immunocytochemical studies demonstrating increased calpain expression in ON were confirmed by quantitative biochemical studies designed to evaluate calpain activity and expression at transcriptional and translational levels.

Calpain has been shown to degrade various cellular proteins (including cytoskeletal and myelin proteins) in brain, retina, lens and optic nerves (Nixon, 1986; Blomgren and Karlsson, 1990; Shearer and David, 1990; Banik et al., 1992; Shields et al., 1997). Increased degradation of these proteins has been demonstrated in CNS trauma, cerebral ischemia, cataract formation, retinal degeneration, and demyelinating diseases. Calpain also degrades the specific endogenous inhibitor, calpastatin, when the calpain:calpastatin ratio is increased in pathological conditions (Mellgren, 1987;

Suzuki et al., 1995). While the role of calpain in optic neuritis has not been previously examined, calpain-mediated degradation of cytoskeletal neurofilament proteins (NFPs) and myelin proteins has been demonstrated in optic nerve in vitro in the presence of increased calcium levels (Blomgren and Karlsson, 1990; Shields et al., 1997). These studies have also been correlated with structural alterations in axons and myelin in the presence of calcium ionophores (Schlaepfer and Zimmerman, 1990). The findings of increased calpain expression in activated glial and inflammatory cells in experimental optic neuritis as demonstrated by double immunofluorescent labeling suggested upregulation of calpain translational expression upon cell activation. Accordingly, Western blotting studies of optic nerves from animals with experimental optic neuritis revealed proenzyme calpain content was increased by 72% compared to controls (Fig. 3). In contrast, transcriptional expression of both μ - and m-calpain isoforms, as determined by RT-PCR, was not significantly altered compared to controls (Fig. 4). Thus, calpain translational expression may be post-transcriptionally regulated, as recently demonstrated with cathepsin B, another cysteine proteinase (Yano et al., 1997). This phenomenon has been observed in other animal models and may be regulated by mRNA binding proteins (Li et al., 1998).

Although increased proteolytic enzyme activity concomitant with axon and myelin protein degradation has been described in demyelinating diseases, no such correlation has been made in ON thus far (Einstein et al., 1970; Smith, 1977; Newcombe et al., 1982). Nevertheless, the upregulation of calpain translational expression suggests calpain activity may be increased in ON. Since calpain degrades axonal and myelin proteins of optic nerve in vitro, the degradation of cytoskeletal and myelin proteins, as a measure of calpain activity,

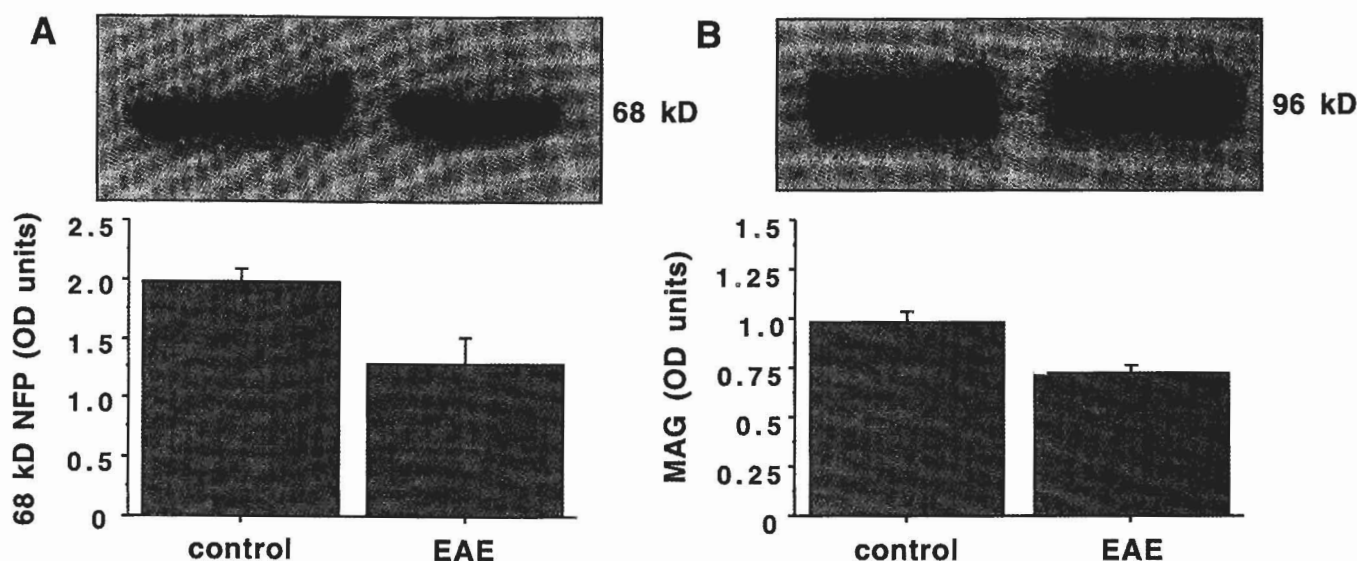


Fig. 5. Loss of (A) 68 kD neurofilament protein and (B) myelin associated glycoprotein (MAG) in animals with experimental optic neuritis compared to adjuvant controls. Western blots (top) of samples from both groups were quantified via densitometry and analyzed by one way ANOVA (\pm SEM).

was examined in experimental ON. Axonal proteins (68 kD NFP) (36%) and myelin associated glycoprotein (MAG) (26%) were significantly degraded in experimental ON compared to controls (Fig. 5). The findings of cytoskeletal protein loss also correlates with recent demonstrations of axonal degeneration in demyelinating diseases such as MS (Newcombe et al., 1982; Trapp et al., 1998). Although NFP and MAG degradation is prevented by calpain-specific inhibitors *in vitro* and *in vivo* (spinal cord injury), their complete degradation *in vivo* may not be entirely due to calpain activity since they are also substrates of cathepsins and cathepsin L-like proteinases (Sato and Miyatake, 1982;

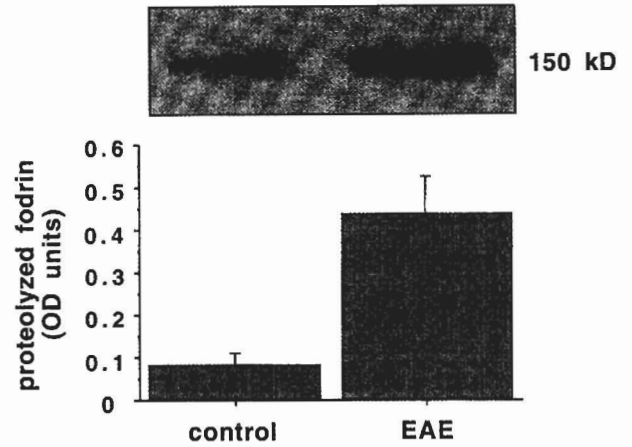


Fig. 6. Calpain-cleaved fodrin fragments (150 kD) in animals with experimental optic neuritis compared to adjuvant controls. Western blots (top) of samples from both groups were quantified via densitometry and analyzed by one way ANOVA (\pm SEM).

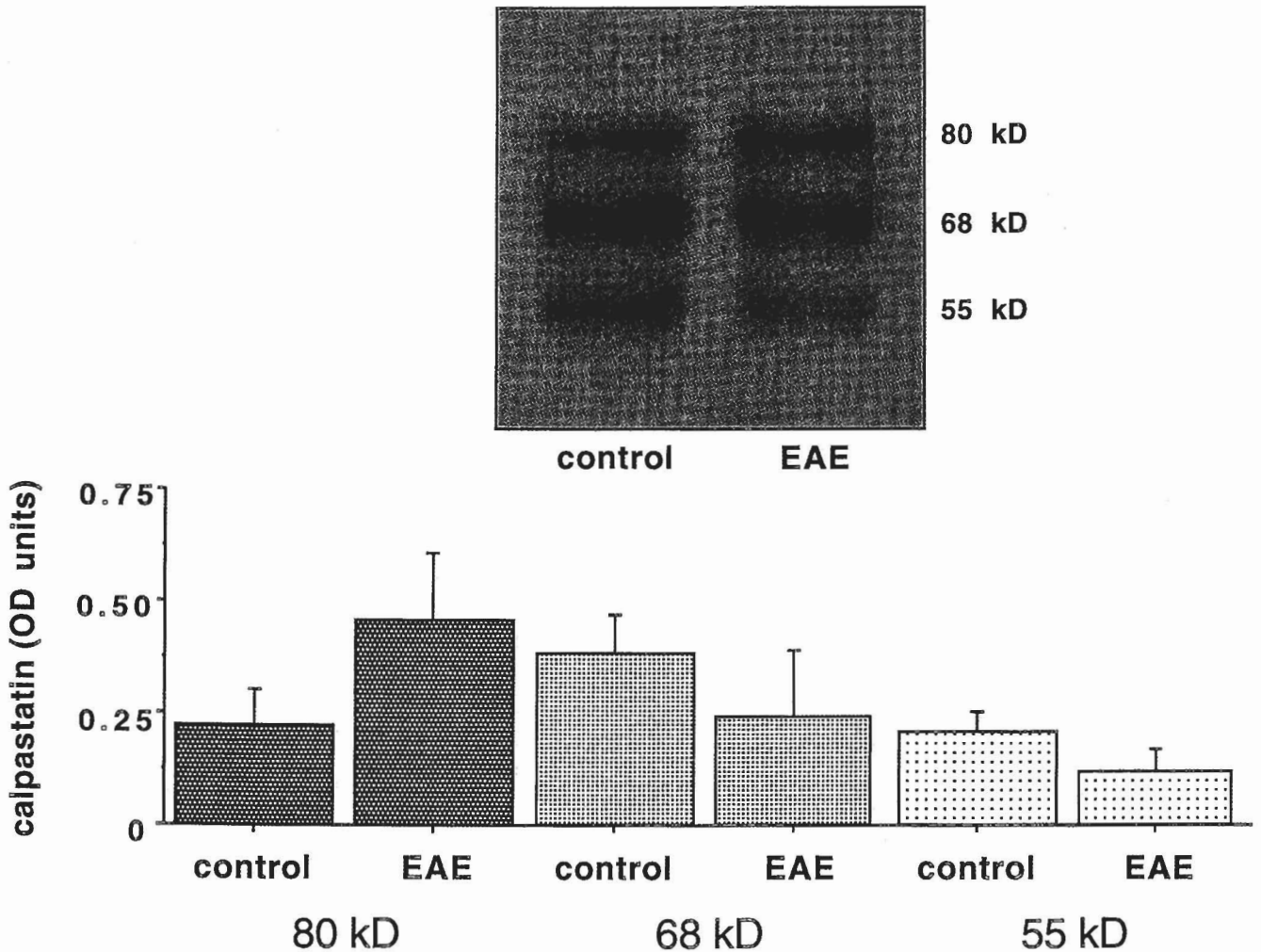


Fig. 7. Calpastatin translational expression in animals with experimental optic neuritis compared to adjuvant controls. Western blots (top) of samples from both groups were quantified via densitometry and analyzed by one way ANOVA as described in the text. There were no significant differences in 80 kD, 68 kD, and 55 kD isoforms in disease animals compared to controls.

Moller, 1996; Banik et al., 1998). Thus, in order to confirm increased calpain activity in ON, the formation of a 150 kD calpain-specific cleavage product of fodrin was measured. This study demonstrated a 463% increase in the formation of calpain-autolyzed product, indicating markedly increased calpain activity in ON compared to controls (Fig. 6, Shields and Banik, 1998b).

In disease, calpain present in glial/inflammatory cells may be activated by increased intracellular calcium concentrations. Likewise, calpain present in oligodendrocytes may be activated as a result of complement fixation and/or perforin damage (Ozawa et al., 1989; Scolding et al., 1990). Also, calpain released from activated glial and inflammatory cells may degrade myelin proteins since the enzyme is active at extracellular calcium levels (Deshpande et al., 1995a,b). In addition to calcium levels, the endogenous inhibitor calpastatin is also an important regulator of calpain activation. Calpastatin expression at transcriptional and translational levels in experimental ON was not significantly increased compared to controls (Fig. 7). Thus, with increased calpain activity/expression and without simultaneous calpastatin upregulation, calpain activity appears to be poorly regulated in ON since calpain also degrades the inhibitor upon activation (Mellgren, 1987).

In conclusion, immunocytochemical and biochemical studies have shown increased calpain expression in activated glial and inflammatory cells in experimental ON. Subsequently, active calpain released from these cells is capable of degrading axonal and myelin proteins, resulting in destabilization of the myelin sheath. Calpain may also degrade another substrate, MBP, into possible immunogenic peptides which can contribute to the inflammatory response through epitope spreading. These various studies indicate a crucial role for calpain in the pathogenesis of ON and suggest the use of calpain-specific inhibitors may delay or prevent disease development.

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