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Nitration of the Low Molecular Weight Neurofilament Is Equivalent in Sporadic Amyotrophic Lateral Sclerosis and Control Cervical Spinal Cord

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To determine the extent to which enhanced nitration of the low molecular weight neurofilament subunit protein (NFL) is of pathogenic significance in sporadic ALS, we isolated the neurofilament (NF) from the cervical spinal cord of 15 cases of sporadic ALS and 11 age-matched control cases. Of the three NF subunits, only NFL demonstrated consistent nitrotyrosine immunoreactivity on immunoblots against mouse monoclonal anti-nitrotyrosine antibodies. Regardless of whether the NFL was isolated from the Triton X-100 soluble or insoluble cytoskeletal fractions, the extent of NFL nitration did not differ between ALS and control tissue. Similarly, no differences were observed on either two dimensional isoelectric focusing or NFL peptide maps. These findings suggest that NFL is particularly susceptible to peroxynitrite-mediated nitration *in vivo,* but reveal no significant qualitative or quantitative modifications in the nitration of NFL isolated from sporadic ALS cervical spinal cord tissue as compared to non-ALS controls. © 1998 Academic Press

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Amyotrophic lateral sclerosis (ALS) is amongst the most common of the adult-onset neurodegenerative disorders, affecting 1.0 - 1.8/100,000 individuals annually (1, 2). The life-time risk for the development of this fatal disorder has been estimated at 1:1,000 (3). However, the prevalence of ALS is increasing at a rate greater than increases in longevity of the population (4, 5). Although the etiology of ALS is unknown, mutations in the copper/zinc superoxide dismutase gene occur in a proportion of familial ALS (*f*ALS) pedigrees (6).

We have recently shown that the nitration of the low molecular weight neurofilament (NFL) by peroxynitrite is catalyzed *in vitro* by both wild-type and ALS mutant SODs (7). The reduced affinity of ALS mutant SODs for zinc will increase tyrosine nitration of NFL and other proteins by reduced ability of zinc deficient SOD to scavenge superoxide combined with an increased ability to catalyze nitration (8). Although SOD 1 mutations in sporadic ALS (*s*ALS) have not been observed, SOD-catalyzed nitration may also be of significance in this more common variant of ALS. Observations in support of this common mechanism include the co-localization of nitrotyrosine, *c*NOS, citrulline and arginine immunoreactivity to the neurofilamentous aggregates in Betz cells (9) and intense nitrotyrosine and NOS immunoreactivity in spinal motor neurons in *s*ALS (10, 11). Both 3-nitrotyrosine and its metabolite 3-nitro-4-hydroxyphenylacetic acid levels are elevated in the lumbar spinal cords of ALS patients (12), reminiscent of similar elevations in transgenic mice harboring the familial ALS G93A SOD 1 mutation (13).

In the experiments reported here, we examined the extent to which NFL nitration occurs in *s*ALS. We have observed that nitrated NFL is found in both control and *s*ALS tissue, and that there is neither a quantitative nor a qualitative difference in the extent of this nitration in *s*ALS. This suggests that the co-localization of nitrotyrosine immunoreactivity with neurofilamentous aggregates in ALS is reflective of the nitration of NFL but that NFL nitration is not specific to ALS.

EXPERIMENTAL PROCEDURES

Case ascertainment. Sporadic ALS cases utilized in this study fulfilled the criteria of clinically definite ALS and were confirmed at autopsy (14). In total, 15 cases of sporadic ALS were studied with a mean disease duration from symptom onset of 33.7 ± 22.3 months (range 2.3-65.4). The mean postmortem interval was 8.5 ± 5.2 hours (range 1.6-23) and the mean age at death 63.6 ± 11.3 years (range 46-88). Control spinal cord was also obtained from 11 cases in which the mean postmortem interval was 10.2 ± 6.3 hours (range 4-23) and the mean age at death 59.0 ± 22.5 years (range 17-82). The control cases consisted of the following disease states: ischemic heart

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disease (2), intracerebral hemorrhage (2), dilated cardiomyopathy (1), Alzheimer's disease (1), glioblastoma multiforme (1), meningitis (1), cerebrovascular disease (1) and a myeloproliferative disorder (1). Neuropathological examination of the cervical spinal cords of these cases showed no focal abnormalities.

Neurofilament isolation. Spinal cord tissue was initially homogenized in Laemmli buffer (15) and 65 μg of protein was separated on 6-12% SDS. PAGE gels for immunoblot studies to determine the major species undergoing nitration. To assay for individual neurofilament subunit protein nitration, cervical spinal cord neurofilament (NF) was isolated by homogenizing frozen samples in M1 buffer and concentrating the Triton X-100 insoluble fraction (P1) by centrifugation (30,000 $\times$ g $\times$ 60 min, 4°C) as previously described (16; 17). The supernatent (S1) was immediately processed as described below. P1 was resuspended in M30 buffer containing 30% sucrose, centrifuged to remove myelin (30,000 $\times$ g $\times$ 60 min., 4°C), and the subsequent pellet (P2) retained as the NF-enriched isolate for studies of nitration. The initial supernatent (S1) was centrifuged to remove any residual insoluble matter (100,000 $\times$ g $\times$ 60 min., 4°C) and the resulting supernatent concentrated using Nanospin filter units with a 30,000 m.wt. cutoff as per the manufacturer's instructions (Gelman Sciences, Quebec, Canada) to yield the concentrated supernatent for subsequent studies. This fraction has been previously shown to contain newly synthesized NF (18). For the generation of peptide maps, individual NF subunit proteins were purified by electroelution from the P2 fraction and concentrated supernatent isolates using previously described techniques (19).

Protein electrophoresis and Western blotting. The protein concentration was determined by the technique of Lowry (20). For studies of nitrotyrosine immunoreactivity by Western blot analysis, 60 μg of either the P2 or concentrated supernatent protein was electrophoresed, whereas 10 μg was loaded for silver staining or immunoblots of either NFL or a-internexin. Samples were electrophoresed under constant current on 6-12% SDS polyacrylamide gels and then electrophoretically transferred to either nitrocellulose or PVDF membrane (100 V, constant voltage, 60 min.).

The immunoblotting technique varied according to the technique of antigen:antibody localization required. Following electrophoretic transfer, NFL immunoblots were blocked with 5% milk in borate saline buffer (BSB) for 30 minutes, incubated with a mouse monoclonal anti-68 kDa NF antibody (clone NR 4, Boehringer Mannheim) at a dilution of 1:4,000 overnight at room temperature. Separate immunoblots were performed using a polyclonal antibody for a-internexin (1:500, overnight incubation, room temp., Chemicon) using both the P2 and concentrated supernatent isolates. Antigen/antibody complex was localized with a peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories Canada, Mississauga, Ont), at a dilution of 1:2000, for 3 hrs at RT and the immunoblots developed with 1 mg/ml 3′,3′ diaminobenzidine and 3% H_2O_2.

Nitrotyrosine immunoreactivity was detected using either of two mouse monoclonal antibodies recognizing different nitrotyrosine epitopes (clones 1A6 and 7A2) at titers of 1:100 with an overnight incubation at room temp (21). The immunoblots were developed using enhanced chemiluminescence as per the manufacturer's protocol with the exception of a 3 hour incubation in the secondary antibody and the use of TBS in all buffers (DuPont NEN Renaissance, Mandel Scientific, Guelph, Ont.) and exposed to HyperFilm MP (Amersham, Mississauga, Ont.). The abolition of immunoreactivity by the simultaneous incubation of the blots with the clone 1A6 monoclonal antibody (mAb) and a nitrated tripeptide (15.0 μM) confirmed antibody specificity.

Analysis of nitration. For the analysis of the nitrotyrosine immunoreactivity in the P2 samples, known quantities of nitrated BSA (50, 100 and 200 ng NT-BSA) were included in each gel as an internal reference and the gels processed using enhanced chemiluminescence as described above. The intensity of the nitrotyrosine immunoreactivity was measured as the absorbance (O.D.) using the volume analysis program of a Bio-Rad GS 700 densitometer. In all instances, the maximum O.D. values of the P2 samples were less than the maximum value for the NT-BSA samples. To control for the variability of NFL quantity in each P2 isolate, a calibration curve was generated with 0-1.0 μg bovine NFL (ICN, Montreal, Canada) and immunostained using the mouse monoclonal anti NFL antibody. Within the range of 0-300 ng NFL, the O.D. values obtained showed a linear response (O.D. of 0-0.40). The NFL O.D. values obtained from the electrophoresis of 10 μg of P2 were within the linear range of the standardized bovine NFL curve for all samples, allowing quantitation of the NFL within the P2 isolate. The final estimate of NT-NFL thus became [NT-NFL intensity (O.D. value)]/[P2-NFL quantity]. This technique allowed for a relative comparison of the intensity of NFL nitrotyrosine immunoreactivity across several different gels.

The intensity of NFL nitrotyrosine immunoreactivity within the concentrated supernatent was expressed in a fashion similar to that described above. However, NFL isolated from this fraction did not consistently immunoreact with the anti NFL mAb. Calibration curves on silver-stained gels were thus generated with BSA and used to quantitate the NFL in 10 μg of concentrated supernatent. The NFL O.D. fell within the linear range of the BSA quantity. The expression of the extent of NFL nitrotyrosine immunoreactivity was similar to that described above, with the denominator consisting of the NFL quantified from the silver-stained gels.

Immunoprecipitation. To determine whether the immunostaining observed was specific for NFL, we immunoprecipitated NFL from homogenates of both control (n = 3) and ALS (n = 3) spinal cord P2 isolates. 60 μg of the protein in the homogenate was solubilized in immunoprecipitation buffer (1mM Pefabloc, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM TRIS, pH 8.0, 150 mM NaCl) and incubated with 15 μl of anti-NFL mAb and 100 μl Tachisorb immunoadsorbent (Calbiochem, La Jolla, CA) for 1 hr. at RT. Following centrifugation (1,500 $\times$ g $\times$ 20 min.), the pellet was suspended in Laemmli buffer and electrophoresed. The gel was electrophoretically transferred to nitrocellulose membrane and immunostained for nitrotyrosine as described above with clone 1A6.

Two dimensional isoelectric focusing. 7.5 μg of P2 was equilibrated in IEF buffer (9.2 M urea, 2.0% NP-40, 5% β-mercaptoethanol, 1.6% Ampholine 4-6.5 ampholytes (Pharmacia, Baie d'Urfé, Quebec), 0.4% Ampholine 3.5-9.5 ampholytes) for 2 hours at RT, briefly centrifuged (13,000 $\times$ g, 5 min., RT) to remove particulate matter, and then loaded onto minigel IEF gel tubes (Bio-Rad Mini-Protean II 2-D cell system). Pre-equilibrated IEF gels contained 9.2 M urea, 1.6% 4-6.5 ampholytes, 0.4% 3.5-9.5 ampholytes, 4% acrylamide (total monomer), 2.0% NP-40, 0.01% ammonium persulfate, 0.1% TEMED. 2.5 μg of 2-D SDS.PAGE standards (Bio-Rad) were loaded onto each gel, and the samples electrophoresed (500 V $\times$ 15 min., 750 V $\times$ 4.5 hrs). IEF gels were then equilibrated in second dimension buffer (0.0625 M Tris HCl, pH 6.8, 2.3% SDS, 5.0% β-mercaptoethanol, 10% glycerol), loaded onto a 6-15% slab gel and electrophoresed (200 V, constant voltage, 35 min.). Gels were either silver-stained for estimation of pI values, or transferred to nitrocellulose membrane and immunoblotted using the anti-NFL mAb (1:4,000). Substrate localization was as described with 3,4 diaminobenzidine and 3% H_2O_2.

Peptide maps. NFL peptide maps were generated using 5 μg of electroeluted NFL with either chymotrypsin (50 ng) or V8 protease (30 ng) (Sigma). All protease stocks were corrected for purity and suspended in protease buffer (125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM Na-EDTA, 5% glycerol, 0.01% phenol red). NFL samples were loaded onto a 10 well minigel and overlaid with 10 μl of protease stock solution. The NFL and protease were stacked at 25 mA constant current until focused at the interface of the stacking gel and separating gel (15% SDS PAGE), and then the current turned off for 30 minutes. Electrophoresis was then resumed. Silver-stained gels were scanned using the profile analysis program of the GS-700 densi-

tometer, and both ALS and control tissue peptide maps compared to lanes in which only the protease was contained. Profiles were then compared to determine if novel fragments had been induced in the ALS samples. In separate experiments, peptide maps were immunoblotted using the clone 1A6 antibody. However, for both proteases, significant nitrotyrosine immunoreactivity of the protease and its fragments was observed, rendering it impossible to determine if the NFL digests yielded unique nitrotyrosine immunoreactive fragments (data not shown).

Statistical analysis. Statistical analysis of the intensity of nitration was carried out using the median values of the corrected nitration intensity using the Kruskal-Wallis one way analysis of variance (SigmaStat program, Jandel Scientific).

RESULTS

Homogenates of cervical spinal cord, immunostained with clone 1A6 following transfer to nitrocellulose membrane, demonstrated intense nitrotyrosine immunoreactivity of two prominent protein bands with R_f values consistent with NFL and GFAP (Figure 1A). A series of smaller molecular weight bands were also observed, although with less consistent staining. The NFL immunoreactivity was blocked using 15 μM of the nitrated peptide although faint GFAP nitrotyrosine immunoreactivity could still be observed (Figure 1B). Immunoprecipitation of the NFL from the homogenate with subsequent immunoblotting with clone 1A6 confirmed the identity of the NFL protein as the primary species undergoing nitration (Figure 1C). Alpha-internexin migrated more rapidly than did the NFL, and was not observed in the concentrated supernatent fraction.

Both the P2 isolate and the concentrated supernatent demonstrated NFL nitrotyrosine immunoreactivity, although the intensity of the immunostaining varied with the membrane utilized in the transfer (Figure 2). In order to understand this difference in staining intensity, nitrated BSA (NT-BSA) was used as an internal control. BSA nitrotyrosine immunoreactivity with monoclonal antibody clone 1A6 was observed only when nitrocellulose was used as the transfer membrane and was abolished when PVDF was used. In contrast, NT-BSA immunoreactivity was readily observed on both nitrocellulose and PVDF membrane when monoclonal antibody clone 7A2 was used as the primary antibody. Triton X-100 insoluble NFL (P2), transferred to nitrocellulose membrane, demonstrated NFL nitration with clone 1A6 for both sALS and control isolates. When the studies were replicated using P2 on PVDF membrane, NFL immunoreactivity was abolished, although the GFAP immunoreactivity remained. In concert with the NT-BSA studies, these observations imply that one or more sites of P2 nitration recognized by clone 1A6 could be "hidden" by the PVDF/protein interactions. Thus, all comparative analysis of the intensity of P2 NFL nitrotyrosine immunoreactivity was performed using nitrocellulose as the transfer membrane.

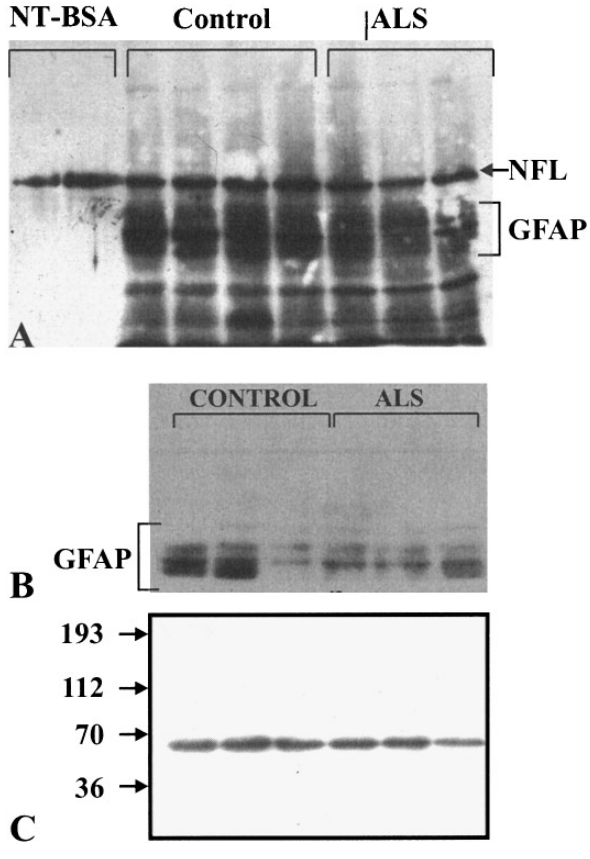

FIG. 1. Homogenates of cervical spinal cord, electrophoresed on 6–12% SDS polyacrylamide gels and transferred to nitrocellulose membrane, demonstrated intense nitrotyrosine immunoreactivity of two prominent protein bands with R_f values consistent with NFL and GFAP (A). Faint immunoreactivity in the range of 160 kDa was additionally observed, consistent with NFM nitration. A series of smaller molecular weight bands were also observed, although with less consistent staining. No differences in the patterns of nitrotyrosine immunoreactivity were observed (proteins transferred to nitrocellulose membrane, immunostained with clone 1A6, 1:100, overnight, room temp., antigen:antibody localized by enhanced chemiluminescence). Lanes 1 and 2 contain 100 and 250 ng nitrated BSA (NT-BSA), respectively. Following incubation with 15 μM nitrated peptide, NFL immunoreactivity was abolished, while faint GFAP immunoreactivity remained (B). The specificity of NFL nitrotyrosine immunoreactivity was confirmed by immunoprecipitating NFL followed by immunoblotting with clone 1A6 (C).

In contrast, the concentrated supernatent NFL was immunoreactive against both clones 1A6 and 7A2, regardless of the membrane utilized. This difference in immunoreactivities implies one or more different sites of tyrosine nitration, recognized by both the clone 1A6 and 7A2 antibodies, which are unique to NFL in the Triton X-100 soluble fraction. For purposes of this analysis, concentrated supernatent NFL immunoreactivity was analyzed using data from both the nitrocellulose

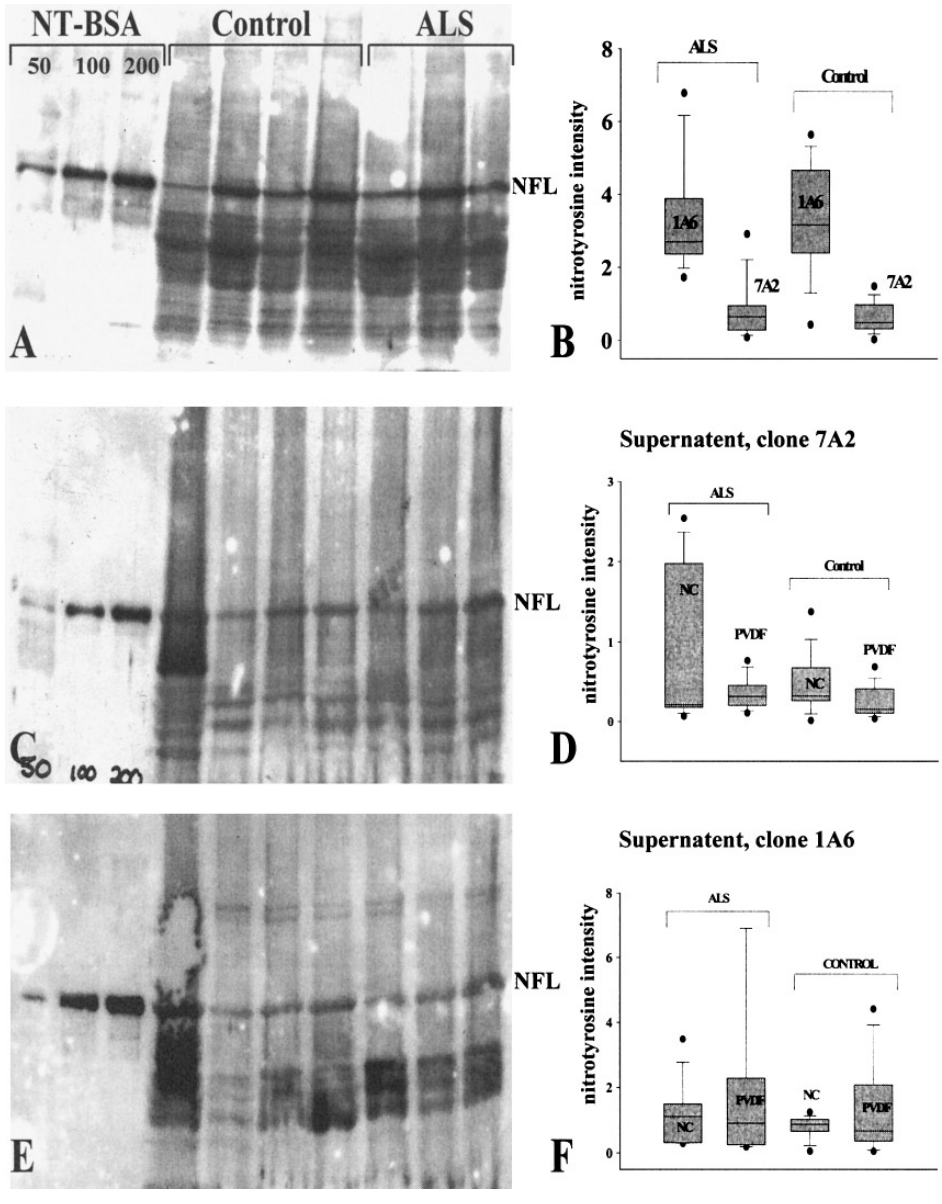

FIG. 2. Nitrotyrosine immunoreactivity of the Triton soluble (concentrated supernatant) and insoluble fractions (P2). Box plots (median value, 25th and 75th percentile at margins, error bars of 5th and 95th percentile) are provide for P2 transferred to nitrocellulose membrane (B); concentrated supernatent immunostained with clone 7A2 (D); and concentrated supernatent immunostained with clone 1A6 (F). Both control and ALS P2 isolates demonstrated intense NFL immunoreactivity to clone 1A6 when transferred to nitrocellulose membrane (A). Although the median values of the intensity of nitration were less for immunoblots performed with the clone 7A2 antibody, there was no significant difference observed between control and ALS cases (B). When the studies were replicated on PVDF membrane, NFL immunoreactivity was abolished, although the GFAP immunoreactivity remained (data not shown). Intense nitrotyrosine immunoreactivity was observed in the Triton soluble concentrated supernatent transferred to PVDF membrane for clone 7A2 (C) and clone 1A6 (E). Regardless of whether nitricellulose of PVDF membrane was utilized, no differences were observed in the median values of the nitrated NFL immunoreactivity intensity (D & F).

and PVDF membranes, and with both antibodies. Regardless of these specific patterns of immunoreactivity, no significant differences in the intensity of the NFL nitrotyrosine immunoreactivity was observed between control and ALS cases in either the P2 isolates transferred to nitrocellulose using clone 1A6 ($p = 0.0750$) or clone 7A2 ($p = 0.2711$) (Fig. 2B); or concentrated supernatant fractions ($p = 0.4490$ for clone 7A2; $p = 0.9081$ for clone 1A6) (Figure 2D & F, resp.).

The 2D IEF patterns did not differ significantly between the samples (Figure 3). In general, two major IEF patterns of NFL were observed, differing in the

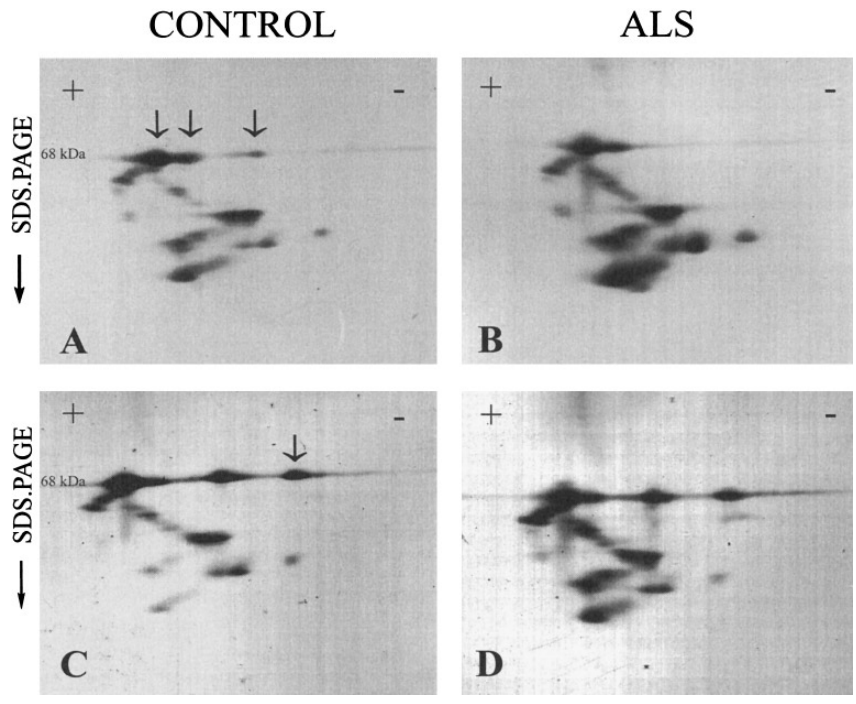

FIG. 3. 2D IEF patterns did not differ significantly between the samples. In general, two major IEF patterns of the 68 kDa NFL were observed, differing in the pI values of the 68 kDa isoforms. The first, and most common pattern, consisted of 2 or 3 isoforms of NFL ranging in pI values from 5.1 to 5.6 (A & B), with the second pattern consisting of an additional isoform with a pI value of approximately 6.3 (C & D). Smaller, more rapidly migrating NFL fragments were also observed in both the ALS and control cases, which are likely NFL degradative products. No differences in patterns of migration of the 68 kDa NFL isoforms were observed when the control and ALS cases were compared.

pI values of the 68 kDa isoforms. The first, and most common pattern, consisted of 2 or 3 isoforms of NFL ranging in pI values from 5.1 to 5.6, with the second consisting of an additional isoform with a pI value of approximately 6.3. Smaller, more rapidly migrating NFL fragments were also observed. As with the nitrotyrosine immunoblots, we observed no significant differences in the 2D IEF gels when control and ALS cases were compared. Attempts at utilizing the 2D IEF gels for nitrotyrosine immunoblots were unsuccessful due to the limitations for protein loading onto mini-IEF gels. Similarly, the peptide maps did not differ between ALS and control samples (Figure 4).

DISCUSSION

These findings suggest that no significant differences exist in either the extent or sites of nitration of NFL in sALS compared to that of control tissue. The observation of NFL nitration in both the Triton X-100 insoluble (P2) and soluble cytoskeletal (concentrated supernatent) fractions suggests that nitrated NFL is present regardless of whether the NFL has been incorporated into the stable NF triplet polymer, and is in agreement with previous *in vitro* observations (7). However, the masking of clone 1A6 nitrotyrosine immunoreactivity on PVDF membrane, unique to nitrated NFL in the Triton-insoluble fraction, does imply differences in one or more sites of tyrosine nitration between these two NFL isolates. NFL isolated from the Triton X-100 soluble fraction has been previously shown to contain newly synthesized NFL (18). Whether this Triton soluble NFL requires dephosphorylation at Ser2 prior to being incorporated in the NF triplet, and the effect of nitration upon this, remains to be determined (22). However, *in vitro* studies, using nitrated murine NFL or triplet bovine NF, suggest that NFL nitration is disruptive of NF assembly and that this process favors the Triton soluble fraction (7).

Our observations confirm and extend those of Bruijn et al (23) in which both sporadic (n = 4) ALS and familial ALS (SOD1 A4V mutation bearing, n = 2) whole spinal cord extracts or neurofilament-enriched extracts were shown by immunoblot analysis to have equivalent NFL nitrotyrosine immunoreactivity to control tissue (n = 3). It is not known the extent to which the latter cases were matched for postmortem interval and age. Although our studies suggest that there may be differences in the sites of nitration between the Triton X-100 soluble and insoluble NFL isolates, it seems less likely that there are differences in the sites of NFL nitration between ALS and control cases as demon-

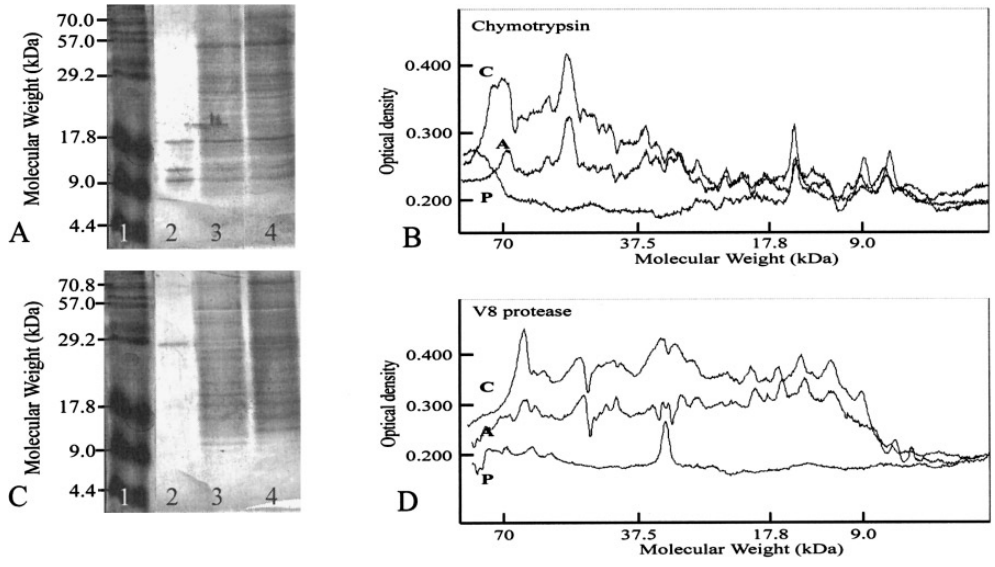

FIG. 4. Peptide mapping. Purified NFL was incubated with either chymotrypsin or V8 protease and then separated on 15% SDS polyacrylamide gels. Gels were silver-stained and scanned with a GS 700 densitometer. Silver stained gels of chymotrypsin digests (A) or V8 protease digests (C) failed to show novel proteolytic fragments (lane 1-molecular weight stds., lane 2-protease alone; lane 3-control NFL digest; lane 4-ALS digest). Densitometric scans similarly showed no evidence for novel proteolytic fragments (representative scanning of chymotrypsin (B) and V8 protease (D); C = control; A = ALS; P = protease).

strated by the failure to observe a difference on NFL 2D IEF studies. Nitration would be predicted to confer a net negative charge to the NFL isoforms and, given the molecular mass of NFL, should alter one or more of their pI values. Because of the physical limitations of the capillary tubes, we were unable to load sufficient protein to each gel in order to perform immunoblots using the anti-nitrotyrosine antibodies. Hence, it is possible that there are specific isoforms of nitrated NFL unique to the *s*ALS samples that are not recognized by the two monoclonal antibodies utilized in this analysis. To test this, we performed peptide mapping studies with the anticipation that nitration could potentially modify protease accessibility to the protein and generate novel peptide maps. As presented, no differences were observed when ALS and control NFL peptide maps were compared. This further suggests that nitrated NFL isoforms unique to *s*ALS do not exist. Future studies using mass/mass spectroscopy will be of benefit in providing more definitive data regarding the state of NFL nitration in ALS.

Both increased protein carbonyl content and nitrotyrosine immunoreactivity have been observed by immunohistochemical techniques in Betz cells and spinal motor neurons in ALS (10; 24; 25). More recently, nitrotyrosine immunoreactivity has been shown to co-localize with neurofilamentous inclusions in *s*ALS tissue, suggesting a link between the induction of neurofilamentous aggregates and nitration (9; 11). The observation of elevated levels of 3-nitrotyrosine and 3-nitro-4-hydroxyphenylacetic acid in lumbar spinal cords suggests an increased rate of formation of reactive nitrating species generation in the ALS population (12). However, while the process of reactive nitrating species generation is likely to be of significance to the disease process, the conclusion that this specifically affects NFL in sporadic ALS may be premature. Rather, the co-localization of NFL and nitrotyrosine immunoreactivity in sALS may not be due to the induction of aggregates by nitrated NFL, but to the aggregation of a protein that is particularly susceptible to nitration.

To date, NF proteins have been thought to function primarily as an integral determinant of axonal caliber and neuritic length. However, a NF-deficient mutant strain of Japanese quails, while developing hypotrophic axons, develops only quivering in the absence of a more profound motor neuron disease (26; 27). In transgenic mice lacking NF, no overt phenotype is present (28). Hence, the exact function(s) of NF remains to be elucidated. Our results suggest that one potential role is that of a biological sink for reactive nitrating species, thereby limiting unregulated peroxynitrite-mediated neuronal injury.

Normally, the simultaneous production of superoxide anion (O_2^-) and nitric oxide yields, as a reaction product, the oxidant peroxynitrite ($ONOO^-$) (29; 30). Superoxide (O_2^-) is generated by the reduction of O_2 and can spontaneously dismutate to H_2O_2. However, in the presence of $NO^{\bullet}$, near diffusion rate reaction kinetics renders the formation of $ONOO^-$ the favored pathway of O_2^- metabolism (31; 32). Although $NO\cdot$ can also be cytotoxic, its toxicity is mainly mediated through the

formation of $ONOO^-$ (reviewed in Szabó, (33)). Peroxynitrite toxicity can mediated by several pathways, including lipid peroxidation, sulfhydryl oxidation, the generation of hydroxyl radicals ($ROH\cdot$), and by peroxynitrite-mediated nitration of phenolic residues (34). At physiological pH, $ONOO^-$ reacts with the copper of the active site of Cu/Zn superoxide dismutase 1 (SOD 1) to yield a reactive nitrating species. This reactive nitrating species will nitrate aromatic ring structures-in particular tyrosine residues, for which NFL is a rich source (35-38).

We have recently demonstrated that spinal motor neurons constitutively express type I NOS (39), while Bergeron and colleagues have demonstrated that spinal motor neurons are a rich source of SOD 1 (40). Hence, the required elements for the production of $ONOO^-$ and its subsequent catalysis to a reactive nitrating species are indigenous to spinal motor neurons. Our observations open the possibility that NFL serves the role of a biological sink for reactive nitrating species generated in spinal motor neurons. In this regard, it is of note that NFL is a potent chelator of zinc and can compete with the copper/zinc binding site of SOD1 for zinc (41). As recently demonstrated, Zn-deficient SOD1 demonstrates an enhanced rate of tyrosine nitration (8) and recent *in vitro* studies in spinal motor neurons demonstrate that motor neurons under stress can produce sufficient peroxynitrite to induce apoptosis (42). Whether such a mechanism leading to motor neuron death is present in ALS is the subject of ongoing studies.

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