Original Contribution

Proteomic analysis of 4-hydroxy-2-nonenal-modified proteins in G93A-SOD1 transgenic mice—A model of familial amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is an age-related, fatal motor neuron degenerative disease occurring both sporadically (sALS) and heritably (fALS), with inherited cases accounting for approximately 10% of diagnoses. Although multiple mechanisms likely contribute to the pathogenesis of motor neuron injury in ALS, recent advances suggest that oxidative stress may play a significant role in the amplification, and possibly the initiation, of the disease. Lipid peroxidation is one of the several outcomes of oxidative stress. Since the central nervous system (CNS) is enriched with polyunsaturated fatty acids, it is particularly vulnerable to membrane-associated oxidative stress. Peroxidation of cellular membrane lipids or circulating lipoprotein molecules generates highly reactive aldehydes, among which is 4-hydroxy-2-nonenal (HNE). HNE levels are increased in spinal cord motor neurons of ALS patients, indicating that lipid peroxidation is associated with the motor neuron degeneration in ALS. In the present study, we used a parallel proteomic approach to identify HNE-modified proteins in the spinal cord tissue of a model of fALS, G93A-SOD1 transgenic mice, in comparison to the nontransgenic mice. We found three significantly HNE-modified proteins in the spinal cord of G93A-SOD1 transgenic mice: dihydropyrimidinase-related protein 2 (DRP-2), heat-shock protein 70 (Hsp70), and possibly \(\alpha\)-enolase. These results support the role of oxidative stress as a major mechanism in the pathogenesis of ALS. Structural alteration and activity decline of functional proteins may consistently contribute to the neurodegeneration process in ALS.

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Keywords: Amyotrophic lateral sclerosis; Oxidative stress; Lipid peroxidation; Neurodegeneration; 4-Hydroxy-2-nonenal; Free radicals

Abbreviations: ALS, amyotrophic lateral sclerosis; sALS, sporadic ALS; fALS, ALS as a familial disorder; mSOD1, mutant SOD1; HNE, 4-hydroxy-2-nonenal; DRP-2, dihydropyrimidinase-related protein 2; Hsp70, heat-shock protein 70; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; Chaps, 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate; SDS, sodium dodecyl sulfate; BCIP/NBT: DTT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; AD, Alzheimer’s disease.

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Introduction

Degeneration of cortical and spinal motor neurons is the typical feature of amyotrophic lateral sclerosis (ALS) [1], a progressive, fatal disease occurring both sporadically (sALS) and as a familial disorder (fALS), the latter accounting for about 10% of patients. fALS is caused by dominantly inherited mutations in the SOD1 gene encoding Cu/Zn superoxide dismutase [2]. fALS cases are indistinguishable from sALS on the basis of clinical and pathological criteria [3], suggesting that the SOD1-mutant animal model provides insight into similar and converging pathogenic mechanisms shared by both sporadic and familial forms.

Over 100 SOD1 mutations [4] have been identified in fALS patients. Most of the individual mutations result from substitution of one single amino acid, such as SOD1G85R, SOD1G93R, and SOD1G93A, which were intensely characterized in transgenic mouse models of ALS [5–8]. It is well established that SOD1-mediated toxicity in ALS is due to a “gain” of toxic properties that are independent of the levels of SOD1 activity [9].

However, the mechanism of fALS caused by mutant SOD1 (mSOD1) is currently unknown. Two hypotheses have been proposed to explain the toxicity of ALS mutant SOD1 proteins. The oligomerization hypothesis states that mSOD1 proteins are or become misfolded and consequently oligomerize to form intracellular aggregates [10,11], which diminish the availability of essential proteins for normal cellular function. The second, the oxidative damage hypothesis, proposes that toxicity is caused by aberrant chemistry of the active Cu/Zn sites of the misfolded enzyme [12]. The mSODs exhibit enhanced oxidative stress by acting as a peroxidase [11] or a superoxide reductase [13] or by producing $O_2^-$ [14]. Indeed, autooxidation of mutant SOD1 proteins increases the frequency of protein misfolding [15]. Elevated levels of reactive free radicals and the formation of insoluble protein complexes of mutant SOD1 protein have been detected in spinal cords of the G93A transgenic mice prior to motor neuron degeneration [16,17]. It is tempting to assume that these two phenomena are linked, since oxidative damage to the SOD1 was demonstrated in the G93A-SOD1 transgenic mice prior to motor neuron degeneration [18–20]. Moreover, the oxidative damage hypothesis is further supported by considerable evidence of increased ROS-mediated oxidative stress, such as malondialdehyde, 4-hydroxyl-2-nonenal (HNE), oxidized proteins, DNA, and membrane phospholipids, in sporadic and fALS [11].

Lipid peroxidation is a result of oxidative stress in living cells. Since the central nervous system consumes a large fraction of inspired oxygen, contains copious amounts of redox transition metal ions, and is enriched with polyunsaturated fatty acids, the brain is particularly vulnerable to lipid peroxidation. The lipid peroxidation end product, HNE, is an unsaturated aldehyde that can modify proteins by Michael addition to form covalent adducts with cysteine, lysine, or histidine [21]. HNE-modification initiates conformational and structural changes of proteins and results in neuronal death [22,23]. The HNE level and HNE-modified proteins are increased in the spinal cord and ventral horn motor neurons of ALS patients [24], indicating that HNE plays a critical role motor neuron degeneration in ALS [25]. In order to gain insight into the roles of HNE-modified protein in the neurodegenerative in fALS, we used a proteomic approach to identify the significantly HNE-modified proteins in the spinal cord of G93A-SOD1 transgenic mice when compared to nontransgenic mice.

Methods

Animals

Transgenic mice expressing human SOD1 gene with a G93A mutation (strain B6SJLTgN (SOD1-G93A)-2Gur) [5] were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained as hemizygotes by mating transgenic males with B6/SJL F1 hybrid females. Nontransgenic littermates were used as the primary control group. From 4 months of age G93A transgenic mice showed signs of hindlimb weakness and rapidly developed a paralysis resembling fALS both clinically and pathologically [5]; they eventually died at a mean of 11 days after paralysis. Behavioral tests were carried out between Postnatal Days 100 and 110 to evaluate advanced motor dysfunction. All the studies on isolated cord were performed on tissue isolated from 120-day-old animals and were performed according to the guidelines on the ethical use of animals overseen by the Institutional Animal Care and Use Committee (IACUC) of the Oklahoma Medical Research Foundation.

Sample preparation

The spinal cords were rapidly removed from the mice (n = 5 for both non-Tg and G93A Tg animals), dissected on a bed of ice, immediately frozen in liquid nitrogen, and stored at −80°C until time of analysis. Spinal cords were suspended in 10 mM Heps containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH2PO4 and proteinase inhibitors: leupeptin (0.5 mg/mL), pepstatin (0.7 μg/mL), type IIS soybean trypsin inhibitor (0.5 μg/mL), and PMSF (40 μg/mL). Homogenates were sonicated 20 s three times and then centrifuged at 14,000 g for 15 min to remove debris. Protein concentration in the supernatant was assayed by BCA method (Pierce, Rockford, IL).

Two-dimensional gel electrophoresis

Samples of spinal cord proteins were prepared according to the procedure of Levine et al. [26]. Proteins (200 μg) were precipitated by addition of ice-cold 100% trichloroacetic acid (TCA) to obtain a final concentration of 15% TCA. Samples were placed on ice for 10 min to allow precipitation of...
proteins. Precipitates were centrifuged at 15,800 g for 2 min. The pellets were washed with 0.5 mL of 1/1 (v/v) ethanol/ethyl acetate solution. After centrifugation and washing with ethanol/ethyl acetate solution three times, the samples were dissolved in 25 µL of 8 M urea (Bio-Rad). The samples were then mixed with 185 µL of rehydration buffer (8 M urea, 20 mM dithiothreitol, 2.0% (w/v) Chaps, 0.2% Biolytes, 2 M thiourea, and bromophenol blue).

For the first-dimension electrophoresis, 200 µL of sample solution was applied to a ReadyStrip IPG strip (Bio-Rad). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strip was then actively rehydrated in protean IEF cell (Bio-Rad) for 16 h at 50 V. The isoelectric focusing was performed at 300 V for 2 h at 50 V. The isoelectric focusing was performed at 300 V for 2 h at 50 V. The focused IEF strip was stored at −80°C until second-dimension electrophoresis was performed.

For second-dimension electrophoresis, thawed IPG Strips pH 3-10 were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then reequilibrated for 15 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. Linear Gradient (8–16%) Prestan criterion Tris-HCl gels (Bio-Rad) were used to perform second-dimension electrophoresis. Precision Protein Standards (Bio-Rad) were run along with the sample at 200 V for 65 min.

After electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min. Approximately 40 mL of SYPRO Ruby Gel Stain (Bio-Rad) was used to stain the gels for 2 h on a gently continuous rocker. The gels were placed in deionized water overnight for destaining.

Western blotting

The same amount of protein samples (200 µg) was used for 2D-immunoblotting analysis, and the electrophoresis was carried out in the same way as described above. The proteins from the second-dimension electrophoresis gels were transferred to nitrocellulose (Bio-Rad) using a Transblot-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at 15 V for 2 h. HNE-protein adducts were detected on the nitrocellulose paper using a primary rabbit antibody (Intergen) specific for HNE-bound protein (1:100), followed by a secondary goat anti-rabbit IgG (Sigma, St Louis, MO) antibody. The resultant stain was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution (Sigma-Fast tablets; Sigma).

Image analysis

The gels (5 for each group, G93A-SOD1 transgenic mice and nontransgenic mice) and nitrocellulose blots (n = 10) were scanned and saved in TIF format using a Scanjet 3300 C (Hewlett Packard). PDQuest 2-D Analysis software (Bio-Rad) was used for matching and analysis of visualized protein spots among differential gels and membranes to compare protein and HNE immunoreactivity content between G93A-SOD1 transgenic and nontransgenic mice. This sophisticated software offers powerful comparative analysis and is specifically designed to analyze many gels or blots at once. Powerful automatching algorithms quickly and accurately match gels or blots and sophisticated statistical analysis tools identify experimentally significant spots. The principles of measuring intensity values by 2-D analysis software were similar to those of densitometric measurements. The average mode of background subtraction was used to normalize intensity values, which represents the amount of protein (total protein on gel and HNE-bound protein on the membrane) per spot. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or membranes) was compared between groups using statistical analysis. Statistical significance was assessed by a two-tailed Student’s t test. P values <0.05 were considered significant for comparison between control (nontransgenic mouse) and experimental data (G93A-SOD1 transgenic mice).

Trypsin digestion

In-gel digestion on selected gel spots was performed according to Thongboonkerd et al. [27]. The selected protein spots were excised with a clean blade and transferred into clean microcentrifuge tubes. The protein spots were then washed with 0.1 M ammonium bicarbonate (NH4HCO3) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in a flood hood. The protein spots were incubated with 20 µL of 20 mM DTT in 0.1 M NH4HCO3 at 56°C for 45 min. The DTT solution was then removed and replaced with 20 µL of 55 mM iodoacetamide in 0.1 M NH4HCO3. The solution was incubated at room temperature in the dark for 30 min. The iodoacetamide was removed and replaced with 0.2 mL of 50 mM NH4HCO3 and incubated at room temperature for 15 min. Two hundred microliters of acetonitrile was added. After a 15-min incubation, the solvent was removed, and the gel spots were dried in a flood hood for 30 min. The gel pieces were rehydrated with 20 ng/µL methylated trypsin (Promega, Madison, WI) in 50 mM NH4HCO3 with the minimal volume to cover the gel pieces. The gel pieces were chopped into smaller pieces and incubated at 37°C overnight in shaking incubator.

Mass spectrometry

All mass spectra reported in this study were acquired at the Department of Pharmacology in the University of Louisville School of Medicine and VAMC. A Spec 2E MALDI-TOF (matrix-assisted laser desorption ionization-
time of flight) mass spectrometer operated in the reflectron mode was used to generate peptide mass fingerprints. Peptides resulting from in-gel digestion with trypsin were analyzed on a 384 position, 600 μm AnchorChip Target (Bruker Daltonics, Bremen, Germany), and prepared according to AnchorChip recommendations (AnchorChip Technology, Rev. 2, Bruker Daltonics, Bremen, Germany). Briefly, 1 μL of digest was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid (0.3 mg/mL in ethanol:acetone, 2:1 ratio) directly on the target and allowed to dry at room temperature. The sample spot was washed with 1 μL of a 1% TFA solution for approximately 60 s. The TFA droplet was gently blown off the sample spot with compressed air. The resulting diffuse sample spot was recrystallized (refocused) using 1 μL of a solution of ethanol:acetone:0.1% TFA (6:3:1 ratio). Reported spectra are a summation of 100 laser shots. External calibration of the mass axis was used for acquisition and internal calibration using either trypsin autolysis ions or matrix clusters was applied postacquisition for accurate mass determination.

The MALDI spectra used for protein identification from tryptic fragments were searched against the NCBI protein databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass fingerprinting used the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues [28–30]. Up to 1 missed trypsin cleavage was allowed. Mass tolerance of 150 ppm was the window of error allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as $-10 \times \log_{10}(p)$, where $p$ is the absolute probability. MOWSE score greater than 61 were considered significant ($P < 0.05$). All the protein identifications were in the expected size range based on position in the gel.

Enzyme activity assay

Enolase activity assay was performed as described previously [31] with modification. Enolase was added to 100 μL of assay mixture (20 mM Na₂HPO₄, pH 7.4, 400 mM KCl, 0.01 mM EDTA, 2 mM 2-phospho-D-glycerate) in a UV-transparent microtiter plate (Corning, MA). The enzymatic activity was determined by the change of absorption at $A_{240}$ for 5 min.

Results

Two-dimensional (2D) electrophoresis offers an efficient tool for screening for abundant protein changes in different disease states as well as differences in metabolic pathways [30,32]. Western blot and subsequent immunochemical

![Fig. 1. Two-dimensional SYPRO Ruby-stained gel from G93A-SOD1 transgenic mice and nontransgenic mice (Control). Positions of the three identified protein are shown on the gels. Expanded images of the spots in the gels are also shown. See text.](image-url)
detection of HNE allowed identification of HNE-modified proteins in the spinal cord tissue of transgenic G93A-SOD1 mice in comparison to nontransgenic mice. We used a parallel approach to quantify the protein levels by SYPRO Ruby staining and the extent of HNE binding by immunostaining (Figs. 1 and 2). SYPRO Ruby fluorescent stain achieves a linear and sensitive staining of gel slabs, and immunoblotting with HNE antibody allows specific detection of HNE adducts in the spinal cord tissue of G93A-SOD1 transgenic mice. We measured the specific HNE-bound levels by dividing the HNE level of a protein spot on the blotted membrane by the protein level of its corresponding protein spot on the gel. Figure 1 shows the representative 2D-electrophoresis gels of G93A-SOD1 transgenic mice (bottom) and nontransgenic mice (top) after SYPRO Ruby staining. Figure 2 shows the representative 2D Western blots of the spinal cord of a G93A-SOD1 transgenic mouse (bottom) and a nontransgenic mouse (top). The HNE-modified proteins identified in this study were dihydropyrimidinase-related protein 2 (DRP-2), heat-shock protein 70 (Hsp70), and possibly α-enolase (Table 1). Control experiments in which secondary antibody alone was used showed no nonspecific labeling of the 2D Western blot in both control and G93A-SOD1 samples (Fig. 3). One example of a mass spectrum corresponding to a tryptic digest of one of the identified proteins is shown in Fig. 4.

HNE-bound protein levels of the three identified proteins in G93A-SOD1 mice compared to nontransgenic mice are summarized in Table 2. The identity of α-enolase was not statistically significant, but the molecular weight and the pI value of the identified protein match the location of the spot on the 2D gel map. Supporting this identification and consistent with prior studies that demonstrated loss of

Fig. 2. Two-dimensional HNE Western blots from G93A-SOD1 transgenic mice and nontransgenic mice (Control). Positions of the three identified protein are shown on the blots. Expanded images of the selected proteins on the blots are also shown. Relative change in HNE immunoreactivity, after normalization of the immunostaining intensities to the protein content, was significant for three proteins. See text.

Table 1
Summary of HNE-modified proteins in G93A spinal cord identified by proteomics

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>gi</th>
<th>Accession No.</th>
<th>No. peptides matched</th>
<th>% Coverage matched peptides</th>
<th>pI, MrW (kDa)</th>
<th>Mowse scorea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydropyrimidinase-related protein 2 (DRP-2)</td>
<td>gi</td>
<td>13242237</td>
<td>18</td>
<td>36</td>
<td>5.95, 62.6</td>
<td>152</td>
</tr>
<tr>
<td>Hsp70</td>
<td>gi</td>
<td>40254595</td>
<td>26</td>
<td>51</td>
<td>5.37, 71.1</td>
<td>192</td>
</tr>
<tr>
<td>α-Enolase</td>
<td>gi</td>
<td>12963491</td>
<td>6</td>
<td>13</td>
<td>6.37, 47.7</td>
<td>41</td>
</tr>
</tbody>
</table>

a MOWSE scores greater than 61 were considered significant statistically.
enzymatic activity of oxidatively modified proteins [33], we found that activity of the HNE-modified enzyme, enolase, is significantly decreased (mean loss of activity was 39%, \( n = 5 \), \( P < 0.05 \)) in the spinal cords of the G93A-SOD1 transgenic mice when compared to that in nontransgenic mice (Fig. 5).

Discussion

Others previously reported that HNE-modified proteins are increased in the spinal fluid, the spinal cord, and ventral horn motor neurons of ALS patients compared with control patients [24, 25], but there is little evidence indicating which individual proteins are specifically affected by HNE modification. In the present study, we show the identification of three proteins (DRP-2, Hsp70, and \( \alpha \)-enolase) significantly modified by HNE in the spinal cord tissue of G93A-SOD1 mice.

The role of ROS-mediated oxidative stress in ALS has been proposed by many laboratories and supported by the evidence that typical oxidation products such as malondialdehyde, HNE, oxidized proteins, oxidized DNA, and membrane phospholipid peroxidation are elevated both in sporadic and fALS spinal cord (review in [11]). HNE is a reactive and cytotoxic by-product of \( \alpha \)-polyunsaturated fatty acid peroxidation. HNE can rapidly diffuse from the lipid bilayer to modify extracellular or intracellular membrane proteins which are necessary for normal cellular function. HNE is implicated in several neurodegenerative disease, including ALS [25]. In the present study, we have identified the proteins that were significantly modified by HNE in the spinal cord tissue of a model of fALS, G93A-SOD1 transgenic mice, in comparison to that of the nontransgenic mice. We found that increased amounts of HNE are significantly covalently bound to dihydropyrimidinase-related protein 2, to the heat-shock protein 70, and possibly to \( \alpha \)-enolase.

DRP-2 is a member of the DRP gene family involved in axonal outgrowth and pathfinding through the transmission and modulation of extracellular signals[34, 35]. Immunoreactivity of human DRP-2 was shown in the neurofibrillary tangles of Alzheimer’s disease (AD) human

Table 2
HNE-bound protein levels in G93A-SOD1 mice

<table>
<thead>
<tr>
<th>Identified protein</th>
<th>Nontransgenic mice (AU ± SE)</th>
<th>G93A-SOD1 transgenic mice (AU ± SE)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydropyrimidinase-related protein (DRP-2)</td>
<td>1.16 ± 0.55</td>
<td>283.48 ± 98.5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Hsp70</td>
<td>3.46 ± 1.52</td>
<td>30.6 ± 7.02</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>( \alpha )-Enolase</td>
<td>1.15 ± 0.25</td>
<td>10.7 ± 4.12</td>
<td>&lt;0.049</td>
</tr>
</tbody>
</table>

Fig. 3. No nonspecific labeling of proteins by the secondary antibody used in these studies. See text.

Fig. 4. Example of a MALDI mass spectrum corresponding to Hsp70 protein. The \( m/z \) values obtained for the protein were used to identify the protein by searching in protein databases. Identification of the protein was obtained with high probability-based score. See text.
brain, suggesting that DRP-2 is involved in the neuritic degeneration in AD [36]. Proteomic data obtained in our laboratory showed significantly increased specific protein carbonyls level of DRP-2 in AD brain [37], suggesting that DRP-2 is also essential in repairing and maintaining the plasticity of neuronal connection in aged brains. Dysfunction of the DRP-2-repairing activity in brain indicates that depletion of DRP-2 may result in neuronal abnormalities, thus accelerating the neuritic degeneration in many neurodegenerative disorders. Our finding of the increased oxidative modification of DRP-2 in G93A-SOD1 transgenic mice when compared to nontransgenic mice establishes a potential link between oxidation-mediated loss in protein function and neuritic regeneration and plasticity known to be altered in ALS [3].

It has been reported that mSOD1 is aggregated with heat-shock proteins 70 and 40 (Hsp70 and Hsp40) and α-crystallin in transfected cells [38]. The Hsp70 is a chaperone protein that helps newly synthesized proteins to be folded and transported across the membrane [39,40]. Besides the constitutive expression of Hsp70 in the mammalian cells, the protein is induced by a variety of stimuli including heat, cytotoxic drugs, imbalance in the cellular redox state, ionizing radiation, heavy metals, disease states, and injuries [39–41]. Pathologically, human SOD1-immunoreactive inclusions in spinal cord of ALS patients and of transgenic mice are frequently stained with antibody against heat-shock cognate Hsc70 [42]. Moreover, overexpression of Hsp70 leads to reduction of protein aggregates and enhanced viability of G93A-SOD1 overexpressed motor neurons [43]. A recent study reported a potential anti-ALS drug candidate acting as a coinducer of the Hsp family [44], suggesting that Hsp70 plays a role in the folding of SOD1 and prevents aggregate formation. Here, we propose that diminished degradation of mSOD1 is possibly due to inactivation of Hsp70, due to the oxidative modification of the latter. Once covalently modified by HNE, which alters the physical state of proteins [45], Hsp70 would not be able to facilitate mSOD1 degradation by the proteasome. Our data suggest that mechanisms regulating Hsp70 chaperone activity could play a crucial role in the pathophysiology of motor neurons disease, in particular in the context of mutations of SOD1.

In this study, we observed a trend toward increased levels of HNE-bound α-enolase. Enolase is a glycolytic enzyme that catalyzes the dehydration of 2-phospho-D-glycerate to form phosphoenolpyruvate. α-Enolase was identified as a specifically oxidized protein in AD brains [37]. Inactivation of this glycolytic enzyme, as a result of oxidative modification, might contribute to an impairment of glycolysis and leads to decrease in production of ATP for normal cellular function. Levels of glucose in cerebral cortex synaptic terminals were markedly decreased in mSOD1 mice when compared to nontransgenic mice [46]. Consistent with the notion that HNE modification of glucose transport protein (GLUT-3) impairs activity of glucose transport and metabolism [45,47], our findings indicate that the HNE binds to and inactivates α-enolase and thus contributes to energy metabolism disruption in motor neurons in fALS.

There is evidence for increased membrane lipid peroxidation in association with degenerating neurons in aging, AD, Parkinson’s disease, traumatic CNS injury, and stroke, and in animal and cell culture models of such disorders [48–54]. HNE levels and HNE-modified proteins are consistently elevated in the spinal cord motor neurons of ALS patients [24]. In the current study, we identified the proteins that are significantly modified by HNE in the spinal cord of G93A-SOD1 transgenic mice when compared to that of nontransgenic mice. The evidence of protein and DNA oxidation and membrane lipid peroxidation, together with elevated levels of ROS [17] in mSOD1 mice, supports the hypothesis that free radical-triggered oxidation of major cell components is a pathway for motor neuron damage in fALS. Protein oxidation is one of the most important causes of brain protein damage and dysfunction [53,54] and decreased activity of oxidized enzymes might contribute to the impairment of the metabolic pathway and cell abnormalities. The results presented in this report show the oxidative modification of three key enzymes in cellular metabolism: DRP-2 (neuronal development and repair), Hsp70 (stress response) and possibly α-enolase (energy metabolism). With potential relevance to ALS, our data demonstrate that oxidative stress in the form of lipid peroxidation is implicated as a pivotal event in the motor neuron degeneration processes.

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