Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis

Ekaterina V. Ilieva,1,* Victória Ayala,1,* Mariona Jové,1 Esther Dafó,2 Daniel Cacabelos,1 Mónica Povedano,3 Maria Josep Bellmunt,1 Isidre Ferrer,2,4 Reinald Pamplona1 and Manuel Portero-Otín1

1Fisiopatologia Metabólica, IRBLLEIDA, Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida, Lleida, Spain, 2Institut de Neuropatologia, Servei Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, 3Servei de Neurologia, Hospital Universitari de Bellvitge and 4Facultat de Medicina, Universitat de Barcelona, Hospitalet de Llobregat, Spain

*These authors contributed equally to this work.

Correspondence to: Manuel Portero-Otín, MD, PhD, IRBLLEIDA, Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida, C/Montserrat Roig, 2, 25008 Lleida, Spain
E-mail: manuel.portero@cmb.udl.es

The occurrence of endoplasmic reticulum (ER) stress in the sporadic form of amyotrophic lateral sclerosis (ALS) is unknown, despite it has been recently documented in experimental models of the familial form. Here we show that spinal cord from patients with sporadic ALS showed signs of ER stress, such as increased levels of ER chaperones such as protein-disulfide isomerase, and increased phosphorylation of eukaryotic initiation factor 2α (eIF2α). Among the potential causes of such ER stress proteasomal impairment was confirmed in the same samples by demonstrating increased ubiquitin immunoreactivity and increased protein lipoxidative (125%), glycoxidative (55%) and direct oxidative damage (62%) over control values, as evidenced by mass-spectrometry and immunological methods. We found that protein oxidative damage was strongly associated to ALS-specific changes in fatty acid concentrations, specifically of n-3 series (as docosahexaenoic acid), and in the amount of mitochondrial components as respiratory complexes I and III, suggesting a mitochondrial dysfunction leading to increased free radical production. Oxidative stress was also evidenced in frontal cortex, suggesting that this region is affected early in ALS. As those events were partially reproduced by threohydroxyaspartate exposure in organotypic spinal cord cultures, we concluded that changes in fatty acid composition, mitochondrial function and proteasome activity, which may be driven by excitotoxicity, lead to oxidative stress and finally contribute to ER stress in sporadic ALS.

Keywords: Proteasome; glycation; lipoxidation; mitochondria; motor neuron

Abbreviations: ER = endoplasmic reticulum; GSA = glutamic semialdehyde; AASA = aminoadipic semialdehyde

Introduction

Amyotrophic lateral sclerosis is a multifactorial disease whose pathophysiological mechanisms include decreased availability to neurotrophic factors, disturbances in calcium metabolism, increased neuroinflammatory status, cytoskeletal changes and oxidative stress (Dupuis et al., 2004; Rao and Weiss, 2004; Strong et al., 2005; Boilee et al., 2006). Recent data indicate (Atkin et al., 2006; Turner and Atkin, 2006) that endoplasmic reticulum (ER) stress may be also involved in the familial form of the disease. ER stress, a complex pattern involving highly specific signalling pathways, ensures through the so-called unfolded protein response that protein folding capacities of ER are not overwhelmed. However, prolonged ER stress could contribute to cell death, both by mitochondria-dependent and independent pathways (Lindholm et al., 2006). In contrast with the familial form, no data was available on the occurrence of ER stress in the more common, sporadic form of the disease.

Some of the pathological hallmarks of ALS, such as increased ubiquitinated bodies, neuronal and astrocytic hyaline inclusions as well as axonal spheroids are protein aggregates that may be related to ER and oxidative stress. This fact is based on the relationship between protein oxidative damage and proteasomal activity following an inverted U shape, i.e. while moderate oxidative
modification of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity (Grune et al., 1996; Sitte et al., 2000; Grune et al., 2004). Such a decreased proteasomal activity has been previously recognized in other neurodegenerative processes, and it may explain ALS-characteristic increased ubiquitination and presence of proteinaceous aggregates. The disruption of the ER-associated degradation, a pathway which helps to clear misfolded protein species from ER, is a potential consequence of such proteasomal impairment, finally contributing to ER stress (Marciniak and Ron, 2006; Oyadomari et al., 2006; Zhang and Kaufman, 2006).

Despite these data suggesting the importance of protein oxidative damage in ALS, its study by using selective, chemically characterized markers has not been reported, though immunohistochemical evidences support ALS-induced increased oxidative damage (Ferrante et al., 1997; Pedersen et al., 1998; Shibata et al., 1999; Kato et al., 2000; Kikuchi et al., 2000; Shibata et al., 2000; Kikuchi et al., 2002). Such molecular dissection, allowing quantitative analyses of oxidative pathways should be useful for diagnostic and therapeutic approaches. The use of highly selective mass spectrometry-based techniques could help to further delineate the potential pathogenic role of oxidative stress in ALS. Several markers could be used, such as glutamic semialdehyde (GSA), which derives from the metal-catalysed oxidation of proline and arginine; or aminoadipic semialdehyde (AASA) which results from lysine oxidation (Requena et al., 1997, 2001, 2003; Dalfo et al., 2005). Besides these direct modifications of protein structures, the effects of free radical efflux in proteins could also involve third-party molecules which also give rise to increased damage, such as carbohydrates and/or lipids, in processes termed glycoxidation and lipoxidation, respectively (Requena et al., 1997; Baynes, 2003). Both carbohydrates and polyunsaturated fatty acids, when reacting with free radicals generate highly reactive dicarbonyl compounds, such as glyoxal, methylglyoxal, 4-hydroxynonenal and malondialdehyde, among others. These reactive carbonyl compounds can generate specific non-enzymatic adducts when reacting with proteins, such as Nε-carboxymethyl-lysine (CML), Nε-carboxyethyl-lysine (CEL) and Nε-malondialdehyde-lysine (MDAL) (Baynes and Thorpe, 2000). The high content of polyunsaturated fatty acid in central nervous system and its elevated oxygen consumption support the possible significance of lipid peroxidation-derived processes in neurodegeneration, including ALS. However, there is no chemical evidence for lipoxidative or glycoxidative damage of proteins in sporadic or familial ALS based on structural identification and supported by mass-spectrometry.

For these reasons, in this work we have studied the development of ER stress in sporadic ALS. We examined ER stress causal factors such as proteasome function, protein oxidative damage, fatty acid composition and potential disturbances in mitochondrial respiratory complexes (as the major sources of free radical efflux). These changes have been evaluated in human samples and in lumbar spinal cord organotypic cultures under chronic excitotoxicity, a well-supported model of the sporadic form of ALS (Rothstein et al., 1993).

**Patients and methods**

**Human spinal cord and brain specimens**

Brain and spinal cord samples were obtained from the Institute of Neuropathology Brain bank following the guidelines of the local Ethics Committee. The brains and spinal cords of seven men and five age-matched controls (four men and one woman) were obtained from 3 to 6 h after death, and were immediately prepared for morphological and biochemical studies. The agonial state was short with no evidence of acidosis or prolonged hypoxia. The pH of the post-mortem brain was between 7 and 7.4. All ALS patients had suffered from clinical signs and symptoms of lower and upper motorneuron disease, finally involving motor nuclei of the medulla oblongata. Importantly, none of these patients had cognitive impairment or dementia. Although variable from one case to another, the terminal stage of the disease was characterized by predominant bulbar failure manifested as impaired swallowing and usually complicated by aspiration pneumonia, or by dominant respiratory insufficiency. Age-matched controls did not show clinical and neuropathological anomalies. Frozen samples of the spinal cord (lumbar enlargement) and frontal cortex area 8 were used for biochemical studies. Samples of control and diseased spinal cords and brains were processed in parallel. Summary of the main clinical and neuropathological aspects is shown in Table 1.

**Organotypic cultures**

Spinal cord cultures were prepared from lumbar spinal cord of postnatal day 8 rat pups as previously described (Rothstein et al., 1993) and maintained in 50% minimal essential medium, 25 mM Heps, 25% Hanks balanced salt solution with d-glucose 25.6 mg/l, 25% heat-inactivated horse serum, 2 mM l-glutamine. Incubation at DIV7 of the slices with the glutamate transport inhibitor D.L-threo-hydroxyaspartate (THA) at 200 μM injures motorneurons with a morphology typical of excitotoxic degeneration after several weeks of treatment. In selected experiments, slices were also incubated with the ER stress inducers thapsigargin (32 ng/ml) and tunicamycin (500 and 5 ng/ml). After 15 or 30 days of treatment, cultures were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C and processed for immunocytochemistry. For western-blot and mass-spectrometric measurements slices were washed with PBS containing 1 mM diethylenetriaminepentaacetic acid and 1 mM butylated hydroxyl toluene, harvested and frozen at −80°C. Experiments for each condition (n=30 slices per experimental group) were repeated at least three times.

**Immunohistochemistry**

De-waxed sections 5-μm-thick of the spinal cord were processed for immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and H2O2 in PBS and normal serum, the sections were incubated with anti-phosphorylated eukaryotic initiation factor 2α (eIF2α) (1:100,
Table 1 Summary of clinical and pathological data in the present series

<table>
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<tr>
<th>Case</th>
<th>Age</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>Duration</th>
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<td>Neoplasia</td>
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Note: ALS: amyotrophic lateral sclerosis; p-m delay: post-mortem delay (in hours); age and duration are in years.

Confocal immunocytochemistry

The antibodies used are listed in the supplementary information (Table S1). Fluorescein-conjugated Bandeiraea simplicifolia lectin (type I) was used as a label for microglia. Appropriate secondary antibodies: Alexa Fluor 488 F(ab)2 fragment of goat anti-mouse IgG (1:500, Molecular Probes, USA) and Alexa Fluor 546 goat anti-rabbit IgG (1:500, Molecular Probes, USA) were used for immunofluorescence. Image analyses were carried out by a single investigator who was blinded to the experimental conditions. Large (>25 μm in diameter) SMI-32-immunopositive neurons were identified as motorneurons. Immunocytochemical controls were performed by the omission of the primary antibodies, resulting in a negative immunostaining in all cases studied. Mounted slices were examined under a FluoView 500 Olympus confocal laser scanning microscope (Hamburg, Germany).

Protein electrophoresis and western blot

Samples (spinal cord, frontal cortex or organotypic spinal cord cultures) were homogenized in a buffer containing 180 mM KCl, 5 mM 3-[N-morpholino]propanesulfonic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM diethylenetriaminepentaacetic acid and 1 mM butylated hydroxy toluene, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.3 with a Potter-Eljeveim device at 4°C. After a brief centrifugation (500 × g, 5 min) to pellet cellular debris, protein concentrations were measured in the supernatants using the Lowry assay (BioRad Laboratories, München, Germany).

For detection of protein carbonyls, and prior to electrophoresis, samples were derivatized with 2,4-dinitrophenylhydrazine (DNP) as previously described (Pamplona et al., 2005). Briefly, to 15 μl homogenates adjusted to 3.75 μg/μl protein SDS was added to a final concentration of 6%, and, after boiling for 3 min, 20 μl of 10 mM DNP in 10% trifluoroacetic acid were added. After 7 min at room temperature, 20 μl of a solution containing 2 M Tris base, 30% glycerol and 15% β-mercaptoethanol were added for neutralization and sample preparation for loading onto SDS-PAGE gels.

For immunodetection, after SDS-PAGE, proteins were transferred using a Mini Trans-Blot Transfer Cell (BioRad) to PVDF membranes (Immobilon-P Millipore, Bedford, MA). Immunodetection was performed using as primary antibodies those listed in supplementary information (Table S1): (i) for ER stress and proteasome function assessment: anti-KDEL, which recognizes KDEL-sequence containing proteins such as ER chaperones as protein disulide isomerase (PDI), GRP78 and GRP94; anti-eIF2α anti-GRP78, anti-phosphorylated eIF2α (S55), anti-PDI and anti-ubiquitin; (ii) for protein oxidative damage: anti-DNP antibody, anti-CML, anti-neuroketal and anti-MDAL; and (iii) for mitochondrial studies: anti-NDUFA 9 antibody for respiratory complex I, anti-core II antibody for respiratory complex III and anti-apoptosis inducing factor (AIF) antibody. For detection of primary antibodies, the following peroxidase-coupled secondary antibodies were used: sheep anti-mouse (1:30 000, Amersham, USA); anti-rabbit (1:40 000, Pierce, USA) and anti-goat (1:7500, Abcam, Cambridge, UK) antibodies. Luminescence was recorded and quantified in a Lumi-Imager equipment (Boehringer, Mannheim, Germany), using the Lumianalyist software. Control experiments showed that omission of primary or secondary antibody addition produced blots with no detectable signal.

Measurement of specific, protein-oxidation-derived markers: GSA, AASA, CML, CEL and MDAL

GSA, AASA, CML, CEL and MDAL concentrations in total proteins from spinal cord, frontal cortex or organotypic culture homogenates were measured by isotope-dilution gas chromatography/mass spectrometry (GC/MS) as previously described (Pamplona et al., 2005). Samples containing 500 μg of protein were diluted up with chloroform:methanol (2:1 v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM NaBH₄ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-froam reagent. Proteins were then precipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: [²H₈]Lysine (d₈-Lys; CDN Isotopes); [²H₁]CML (d₄-CML), [²H₂]CEL (d₄-CEL) and [²H₃]MDAL (d₈-MDAL); [²H₅] 5-hydroxy-2-aminovaleric acid (for GSA analysis) and [²H₆]6-hydroxy-2-aminopropionic acid (for AASA analysis). The samples were hydrolysed at 135°C for 30 min in 1 ml of 6 N HCl, and then dried in vacuo. The N,O-trifluoroacetic methyl ester derivatives of the protein hydrolysate were prepared and GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at...
275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally hold at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and d8-lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d5-5-hydroxy-2-aminobutyric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminobutyric acid and d6-6-hydroxy-2-aminobutyric acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and d4-CML, m/z 392 and 396, respectively; CEL and d4-CEL, m/z 379 and 383, respectively; and MDAL and d8-MDAL, m/z 474 and 482, respectively. The amounts of products were expressed as the ratio μmol GSA, AASA, CML, CEL or MDAL/mol lysine.

Fatty acid analysis
Distributional analysis of fatty acids was performed as previously described (Dalfo et al., 2005; Pamplona et al., 2005). Total lipids from spinal cord, frontal cortex or organotypic cultures were extracted with chloroform:methanol (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene to avoid artifactual oxidation. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2.5 ml of 0.25 mmol% Hexaenoic P/C14 C, then 5 mol% Pentaenoic P/C2 6) + (n Pentaenoic P/C2 8)

Statistical analyses
All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL). Once normality of distribution was assessed by Kolmogorov–Smirnov test, differences between groups (ALS samples versus control; THA treated versus vehicle) were analysed by the Student’s t-tests and correlations between variables were evaluated by the Pearson’s statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results
Samples from ALS patients evidence signs of ER stress: proteasomal impairment as a potential mechanism
Since ER stress has been implied in ALS experimental models we examined signs of ER stress by western-blot and immunohistochemistry in samples from ALS patients. The results demonstrated ER stress in ALS samples (Fig. 1). Thus, increased expression of chaperones PDI and KDEL-containing proteins were found in spinal cord from ALS patients (Fig. 1A), but not in frontal cortex samples (not shown). This was accompanied by a marked increase in eIF2α phosphorylation (a sign of protein synthesis control after ER stress), both in spinal cord lysates (Fig. 1A) and in remaining neuronal bodies in spinal cord (Fig. 1B).

As proteasome is responsible for degradation of ER misfolded proteins, we examined whether its function was preserved. Accounting that ubiquitin-modified proteins are degraded by the proteasome, its function can be inferred by western-blot and immunohistochemical analyses of ubiquitin-modified proteins. The results of such approach showing increased protein ubiquitination in spinal cord from ALS patients, but not in frontal cortex, are compatible with compromised proteasomal function (Fig. 1C). It should be also considered that increased protein turnover due to massive protein degradation could saturate the ubiquitin-proteasome system in spite of proteasome preserved activity.

Proteins from spinal cord and frontal cortex present structurally characterized oxidation products and the amount of these modifications increases with ALS, favoured by changes in fatty acid composition
While moderate oxidative modification of proteins increases their susceptibility for proteasomal clearance, higher rates of oxidative modification actually inhibit proteasome activity. Therefore, we evaluated protein oxidative damage in spinal cord and frontal cortex samples, to ascertain whether proteasomal dysfunction could be related to increased protein oxidative damage. GC/MS analyses demonstrated that those proteins contained oxidation-derived products resulting from metal-catalysed oxidation, glycoxidation and lipoxidation. The more abundant products were those derived from metal-catalysed oxidation, AASA and GSA assuming almost 95% of measured markers. GSA stood as the more frequent modification. While moderate oxidative modification of proteins increases their susceptibility for proteasomal clearance, higher rates of oxidative modification actually inhibit proteasome activity. Therefore, we evaluated protein oxidative damage in spinal cord and frontal cortex samples, to ascertain whether proteasomal dysfunction could be related to increased protein oxidative damage. GC/MS analyses demonstrated that those proteins contained oxidation-derived products resulting from metal-catalysed oxidation, glycoxidation and lipoxidation. The more abundant products were those derived from metal-catalysed oxidation, AASA and GSA assuming almost 95% of measured markers. GSA stood as the more frequent modification, with levels being 30-fold higher than of those of AASA. The concentrations of both GSA and AASA were significantly higher in proteins from spinal cord (P < 0.001) and frontal cortex (P < 0.01) samples of ALS patients than in control age-matched individuals (Fig. 2A). Similarly to specific oxidation products, the concentrations of CEL and CML, glycoxidation products, were also significantly higher in...
Fig. 1 ER stress and protein ubiquitination in spinal cords from ALS patients. (A) Representative western blot of spinal cord homogenates showing increased content of ER resident proteins containing KDEL motifs, PDI and phosphorylated eIF2α in samples from ALS patients. (B) Representative immunohistochemical image of phosphorylated eIF2α in the motor column of human lumbar spinal cords, showing increased staining in ALS samples. (C) Representative western blot of anti-ubiquitin, suggesting decreased ubiquitin degradation in spinal cord samples from ALS patients and control individuals, but not in frontal cortex. Right numbers of the blot indicate apparent molecular weight. Immunoblotting of actin is also shown. The lower panels shows the quantitation of those blots by densitometry, adjusted to actin content and differences were analysed respect to control group by Student’s t-test being *P < 0.01 and **P < 0.001.

Fig. 2 Proteins from ALS samples show significant increases in the amounts of oxidation markers and changes in fatty acid composition. A–C show GC/MS analyses of GSA and AASA (markers of MCO), CML and CEL (arising from glycoxidation and lipoxidation) and MDAL, originated from lipoxidation. (D) Changes in PI and DHA levels associated with ALS in spinal cord differ from those present in frontal cortex. (E) ALS also leads to organ-specific changes in desaturation indexes (18:1/18:0) and n6/n3 ratios. Values shown are% changes of mean±SE over values from control samples (in spinal cord GSA: 219.48 ± 4.19 μmol/mol lysine; AASA: 102 ± 7 μmol/mol lysine; CEL: 385 ± 57 μmol/mol lysine; CML: 521 ± 30 μmol/mol lysine and MDAL: 258 ± 48 μmol/mol lysine; PI: 116.24 ± 4.23; DHA: 10.89 ± 0.20%; 18:1/18:0 ratio:1.36 ± 0.025; n6/n3 ratio: 0.67 ± 0.04; in frontal cortex GSA: 205.12 ± 638 μmol/mol lysine; AASA: 82 ± 4 μmol/mol lysine; CEL: 249 ± 16 μmol/mol lysine; CML: 522 ± 37 μmol/mol lysine MDAL: 197 ± 6 μmol/mol lysine; PI: 155.31 ± 1.55; DHA: 12.35 ± 0.17; 18:1/18:0 ratio: 0.75 ± 0.01; n6/n3 ratio: 1.1 ± 0.034). *P < 0.01 and **P < 0.001 respect to control group by Student’s t-test.
proteins from spinal cord (P < 0.001) and frontal cortex (P < 0.01) of ALS patients than in control individuals (Fig. 2B). The concentration of MDAL, a lipoxidation product, was also increased in samples from ALS patients, both in spinal cord (P < 0.001) and in frontal cortex (P < 0.01) compared to control individuals (Fig. 2C). Nonetheless, the magnitude of difference between ALS and control samples was considerably higher in spinal cord (120%) than in frontal cortex (50%).

As fatty acid profile strongly influences membrane peroxidizability, and consequently protein lipoxidative damage, we analysed fatty acid content in ALS samples. Those analyses revealed significant differences associated with ALS in spinal cord and frontal cortex, both in individual fatty acids and in global indexes (Table 2).

The most remarkable change involves the highly peroxidizable docosahexaenoic acid (DHA), which showed a significant decrease in spinal cord samples with ALS (P < 0.01), contrasting with the significant increase observed in frontal cortex (P < 0.01; Fig. 2D). With reference to the fatty-acid-derived indexes, spinal cords from ALS patients showed significant decreases in the content of PUFA of the n-3 family (P < 0.001; Table 2), while increases of this parameter were detected in frontal cortex (P < 0.006; Table 2). Changes in fatty acid profile led to significant changes in double bond index (P < 0.004; Table 2), PI (P < 0.003; Fig. 2D), Δ9-desaturase estimation (P < 0.01; Fig. 2E) and n6/n3 ratio (P < 0.001; Fig. 2E).

Overall, these indexes could reflect the potential vulnerability of membranes to peroxidative damage. Since changes in double bond and PI in spinal cord were inverse to those observed in frontal cortex, these data suggest that spinal cord membranes are actively producing substrates for peroxidative modification of proteins, while as frontal cortex are not under this circumstance. Accordingly, analysis of MDAL/PI ratio, suggest that for a given PI, rates of MDAL formation are 3-fold higher in spinal cord than in frontal cortex from ALS patients.

**Different kinds of protein oxidative damage are correlated together and are associated to changes in fatty acid content**

After quantitations of protein oxidation and fatty acid analyses, several significant correlations were present among anatomically different locations (Fig. 3). GSA levels correlated significantly with AASA (r = 0.918; P < 0.0001; Fig. 3A), with MDAL (r = 0.865; P < 0.0001; Fig. 3B), with CEL (r = 0.716; P < 0.002) and with CML (r = 0.878; P < 0.0001). This suggests that protein carbonyl formation...
is also associated to glycoxidative and lipoxidative modifications in spinal cord and frontal cortex. Furthermore, peroxidizability index was inversely correlated with CEL \( (r = 0.816; P < 0.0001) \) and MDAL \( (r = -0.685; P < 0.003) \) concentrations, suggesting an association between lipid peroxidizability, glycoxidation and lipoxidation modifications. Interestingly, levels of MDAL display a second-order relationship with DHA levels \( (r = 0.961; P < 0.0001; \text{Fig. 3D}) \), suggesting an intimate interplay between these two factors.

**Protein oxidative damage, showing preferential targets in ALS spinal cord proteins can be associated to mitochondrial disturbances**

It is known that an important determinant for oxidative damage of proteins, besides fatty acid content, is the mitochondrial free radical production. Therefore, we analysed the expression of representative subunits of mitochondrial respiratory complexes I and III, whose activity is counted among the major sources for mitochondrial free radical production (Herrero and Barja, 2000; Chen et al., 2003), as well as the levels of AIF, shown recently to enhance the functional stability of complex I (Vahsen et al., 2004). These analyses demonstrated that both complex I and III concentrations are significantly decreased in spinal cord samples from ALS patients \( (P < 0.01; \text{Fig. 4A}) \) while AIF expression is not changed in ALS \( (\text{Fig. 4A}) \). Reinforcing an apparently different pace of ALS-induced changes between frontal cortex and spinal cord, these effects were not present in frontal cortex from ALS patients (data not shown).

Western-blot analyses of frontal cortex and spinal cord proteins showed differences in the distribution of oxidation (DNP-reactive), glycoxidation (anti-CML) and lipoxidation-derived (anti-neuroketal) protein modifications (Fig. 4B) supporting both diversity and specificity of protein oxidative damage. These findings agree with quantitative analyses by GC/MS as densitometric measurements revealed increased oxidative damage in ALS samples. Major targets of glycoxidation were proteins with apparent molecular weights ranging from 35 to 55 kDa, partially coincident with anti-DNP immunoreactivity (which was also evident for high-molecular weight bands). This pattern differs from targets of neuroketal formation (Fig. 4B), which showed more discrete targets (being targets of 40 and 60 kDa the more prominent). These differences were not present in frontal cortex samples (data not shown).

**Chronic excitotoxicity in organotypic spinal cord cultures leads ER stress, ubiquitin alterations, protein oxidative damage and changes in fatty acid profile**

Chronic excitotoxicity has been implied in the pathogenesis of ALS (Rothstein et al., 1993; Boillee et al., 2006). This can be reproduced \textit{in vitro} by treatment with the pre-synaptic...
glutamate transport inhibitor THA, leading to losses in the number of motorneurons, as assessed by SMI-32 immunoreactivity (data not shown). In accordance to findings in ALS samples, signs of ER stress were also found in spinal cord slices under chronic excitotoxicity after a 30-day period (Figs. S1A and S2A). Although eIF2α phosphorylation was present in neuronal bodies of vehicle-treated slices, increased immunoreactivity was found along neuritic processes as well as in small cellular populations morphologically compatible with glia on THA-treated slices. Furthermore, demonstration of a markedly increased intracellular ubiquitin immunoreactivity excluding nucleus (Fig. S1B) suggest a role of proteasomal dysfunction in this phenomena at an earlier stage. Furthermore, incubation with the ER stress inducing agents tunicamycin and thapsigargin led to increases in chaperone expression (Fig. S2A) and to a marked decrease in the number of motorneurons, suggesting its preferential sensitivity to ER stress (Fig. S2B).

Since those analyses revealed ER stress and proteasome dysfunction in chronic excitotoxicity, we analysed whether protein oxidative damage was also increased in these conditions. The results demonstrated that protein oxidative damage is increased by excitotoxicity (Fig. S3). All measured markers of oxidation, glycoxidation and lipoxidation, increased significantly after THA treatment ($P < 0.001$, Fig. S3A). As in the ALS cases, these increases in protein oxidative modifications were associated to changes in fatty acid composition (Table S2, Fig. S3B). Noteworthy, THA-induced changes resembled those present in frontal cortex, with 2-fold increases in the DHA content ($P < 0.0001$, Fig. S3B), PI increases—150% respect vehicle-treated slices—($P < 0.0001$, Fig. S3B) and decreased n6/n3 ratios ($P < 0.0001$, Fig. S3B). Confocal microscopy of the most THA-sensitive marker, CEL, revealed that proteins modified with this product were present, in increased amounts, throughout glial and neuronal populations (Fig. S3C). Neuropil and star-shaped cells resembling microglia were major distribution sites of CEL immunoreactivity in THA-treated slices. Co-localization studies with Bandairaea Simplicifolia lectin supported the microglial origin of some of those cells (Fig. S3C).
Nevertheless, chronic excitotoxicity also led to increased AIF immunoreactivity in a non-nuclear distribution, suggesting common basis for mitochondrial dysfunction between sporadic ALS and chronic excitotoxicity both in neuronal and glial cells (Fig. S1C).

**Discussion**

Cells facing protein misfolding in the ER initiate the so-called unfolded protein response. As a part of this unfolded protein response, the initiation process in global protein synthesis is repressed by eIF2α phosphorylation via PERK kinase. There is also increased expression of ER chaperones and, if ER stress cause is not corrected, apoptosis is induced (Marciniak and Ron, 2006; Zhang and Kaufman, 2006). The results reported here, showing both increased phosphorylation of eIF2α and increased expression of ER chaperones (PDI and KDEL-containing proteins), strongly support the participation of ER stress in ALS pathogenesis. The relevance of this pathway was also evident in the chronic excitotoxicity paradigm, where both motorneurons and glial cells were stained for anti-phosphorylated eIF2α. Noteworthy, the importance of this pathway is also demonstrated by immunohistochemical analyses, where remaining neuronal bodies of spinal cord ventral horns in ALS show intense anti-phosphorylated eIF2α. Our data suggest that ER stress arises from oxidative stress and from a mitochondrial disturbance. Based on correlative data, we propose that this is a late phenomenon, when compared with protein oxidative damage, as we are able to detect increased protein oxidative damage, increased DHA amount and other fatty acid changes in brain cortex, without increased ubiquitination or any other noticeable change. Recent data demonstrates the occurrence of ER stress in familial ALS paradigms, associating PDI and mutated SOD in motorneurons (Atkin et al., 2006; Furukawa et al., 2006), showing also caspase-12 activation (Wootz et al., 2004; Turner and Atkin, 2006). More interestingly, chronic excitotoxicity leads to protein aggregation in ER from motorneurons (Tarabul et al., 2005), without inducing an upregulation of heat-shock proteins, as those cells have a characteristically high threshold for heat-shock proteins induction (Batulan et al., 2003). This fact, when added to the increased chaperone expression and immunohistochemical data on organotypic cultures, strongly suggest that ER stress is also taking place in glial cells. Due to the important role of the proteasome in ER-directed disposal of misfolded proteins, ER stress could be caused by decreased ER-associated degradation (Bush et al., 1997; Obeng et al., 2006; Yamamuro et al., 2006).

In agreement with our findings, previous data indicate a potential loss of proteasome activity in ALS (Urushitani et al., 2002; Cheroni et al., 2005; Ahtoniemi et al., 2006; Basso et al., 2006; Kabuta et al., 2006; Koyama et al., 2006; Mendonca et al., 2006), specially in the familial forms of the disease. *In vitro* experiments and *in vivo* data show that mutated SOD is associated with decreased amount of specific proteasome subunits, particularly LMP7 (Allen et al., 2003). Furthermore, proteasome inhibition leads to the reproduction of the abnormal solubility properties shown by mutated SOD *in vivo* (Koyama et al., 2006). However, the role of proteasome in sporadic ALS has received less attention. Recent data demonstrate increased proteasome immunoreactivity both in glia and motorneurons from spinal cords in sporadic ALS (Mendonca et al., 2006). Accordingly, findings reported here, demonstrating increased amount of ubiquitinated proteins in spinal cord samples, is compatible to such proteasomal involvement. It should be recalled that ubiquitinated lesions are prominent in ALS morphology. Noteworthy, this increased ubiquitination was also evident under chronic excitotoxicity, being present both in neuronal and non-neuronal populations. This agrees with the previous works, showing that proteasome inhibition in organotypic cultures induce selectivity damage to motorneurons, as chronic excitotoxicity does (Tsuji et al., 2005). As chronic excitotoxicity leads to increased oxidative damage (Rao and Weiss, 2004; Rival et al., 2004), our data suggest that this condition could contribute to proteasome inhibition. Relating differences with frontal cortex, western-blot analyses did not reveal such changes in this latter location. Once a given protein is modified by oxidation, it is degraded by 20S proteasome in an ATP and ubiquitin-independent fashion (Shringarpure et al., 2003; Grune et al., 2005), as recently shown for metal-free SOD (Di Noto et al., 2005). Nevertheless, a high degree of oxidative modification could even decrease proteasome activity (Sitte et al., 2000).

For these reasons we evaluated whether, by using novel mass spectrometry measurements, ALS samples presented increases in protein oxidative damage, both in spinal cord and in frontal cortex. Nonetheless, the magnitude of changes with reference to control was higher in spinal cord than in frontal cortex. These results suggest an early selective involvement of oxidative stress in spinal cord during ALS pathogenesis. In addition, the involvement of frontal cortex agrees with previous reported data in other neurodegenerative diseases, such as Alzheimer disease or Parkinson disease, where pathologically preserved locations of nervous system show incipient increases in protein oxidative damage, thought at a lower extent than classical targets of disease (Dalfo et al., 2005; Pamplona et al., 2005). Cognitive dysfunction and dementia have been reported as complications in a subgroup of patients with ALS, the majority of them presenting atrophy, neuronal loss and reactive gliosis in the frontal and temporal lobes (Kato et al., 2003). Although the present study is not focused on ALS cases with frontotemporal dementia, these results point to the suggestion that frontal cortex is a vulnerable region to ALS. As a working hypothesis it can be proposed that modifications in the levels of certain lipids are predisposing factors to further cellular damage. Nevertheless, data presented here support the notion that...
lipid peroxidation-associated processes seem to be the more sensible cellular oxidative phenomenon, based on MDAL values. Previous immunohistochemical evidences demonstrate increased lipid peroxidation in ALS (Hall et al., 1998; Pedersen et al., 1998). Furthermore, recent data showing accumulation of 4-oxo-2-nonenal DNA etheno adduct in brain cortex from ALS patients support the importance of lipid peroxidation-derived pathways (Shibata et al., 2006). Interestingly, while PI is still increased in frontal cortex, basically due to increases in DHA content, a potential defensive response of nervous tissue (Akbar et al., 2005), these indexes were decreased in spinal cord, suggesting a functional collapse and/or a lower content of neurons. Thus, while brain cortex could produce n-3-derived anti-inflammatory resolwins and docosatrienes (Hong et al., 2003), spinal cord neurons would have decreased DHA availability due to increased lipoxidative consumption. Rather than being a general phenomena, the selectivity for changes in n-3 strongly suggest specific mechanisms of the disease depending on these fatty acids, maybe affecting biosynthetic pathways and/or membrane remodelling systems that deserve further studies. Nonetheless, long-chain unsaturated fatty acids contribute to the formation of cytotoxic aggregates of ALS-linked superoxide dismutase-1, thereby stressing the importance of fatty acid changes in ALS pathogenesis (Kim et al., 2005). To shed further light on these issues we analysed a chronic excitotoxicity paradigm. The results demonstrated—at an early stage of the excitotoxic paradigm—changes partially resembling those present in frontal cortex, e.g. increases in all oxidative markers and increases in DHA content and PI. This was present at a time when motoneuron death is still not evident. In line with this, DHA induces resistance against excitotoxic degeneration of cholinergic neurones in vivo, leading to higher survival, lower dendritic involution and decreased axon degeneration (Hogyes et al., 2003). Globally, these results suggest a pathological spectrum, driven by excitotoxicity, ranging between overt pathological manifestation (present in spinal cord) and more subtle changes, with variations in fatty acids as reactive changes and protein oxidative modifications as important signals of disease.

The significant correlations found between different types of protein oxidative damage suggest that ALS could influence general mechanisms determining protein oxidative modifications, such as free radical production (Pamplona and Barja, 2006). In most cells, the major sources of free radical production are mitochondrial respiratory complexes I and III (Herrero and Barja, 2000) although in brain some mitochondrial matrix enzymes also contribute to free radical production (Starkov et al., 2004; Tretter and Adam-Vizi, 2004). The analyses performed in spinal cords suggest decreases in both respiratory complexes, as well to qualitative changes in complex III distribution. Due to the role of complex I and III as free radical generators (Chen et al., 2003), the results may be compatible with increased free radical efflux by incorrect assembly of complexes or to metabolic reprogramming (Iuso et al., 2006). These data complements previous reports demonstrating decreased activities of respiratory chain complexes I + III, II + III and IV, suggesting a loss of mitochondria in spinal cords from ALS patients (Wiedemann et al., 2002). Moreover, specific decreases in the activities of complexes II are observed in a mutated SOD transgenic model (Jung et al., 2002). Furthermore, losses of complex IV activity are present in the Wobbler mouse (Xu et al., 2001). It may be suggested that ALS samples presented a defect in the assembly of mitochondrial respiratory complexes, that would lead to changes in free radical production (Rana et al., 2000; Sellem et al., 2005). It may be also hypothesized that this scenario could contribute to a more reduced state of Fe-S clusters, leading to increased free radical efflux (Herrero and Barja, 2000). In this line, the amount of AIF, a bifunctional flavoprotein with NADH oxidase activity involved in mitochondrial respiration and caspase-independent apoptosis, was unchanged in spinal cords. Besides an ALS-induced increased apoptotic rate in this tissue (Oh et al., 2006), AIF could be also considered as a part of an adaptation response to increased free radical production (Zhu et al., 2003; Cande et al., 2004) or to decreased respiratory activities. Changes in AIF are not evident in ALS samples when compared to data in the in vitro paradigm: AIF presenting increased non-nuclear staining in THA-treated slices suggests an important role of this protein in the response to excitotoxicity, that may be focus of future studies. Therefore, it could be suggested that increased free radical production, arising from complex I and III suboptimal assembly, together with unchanged or even increased AIF expression, contributes to protein oxidative damage specifically in spinal cords, being those changes absent in frontal cortex. Despite such an increased mitochondrial leak could lead to potentially extensive protein modifications, western-blot analyses revealed that there are specific targets for oxidative, glycoxidative and lipoxidative damage that will be the focus of future studies.

To summarize, the present data demonstrate that there is increased protein oxidative modification in spinal cords and frontal cortex from ALS cases, together with changes in lipid composition. Concerning cellular targets of those phenomena, we have demonstrated a motorneuron involvement, but it should be remarked that glial cells are the major contributors to protein mass in spinal cords. Therefore, it is feasible to assume that many of the changes observed here respond to changes in both (or mainly) glial cell and neuronal populations. In this line, it should be recalled the importance of glial support and trophic environment for motorneurons, so any given change to glial population could contribute to motorneuron loss. In spinal cords, where loss of motorneurons was evident, build up of oxidatively damaged proteins was linked to changes in mitochondrial respiratory complexes and to increased ubiquitination, potentially linked to impaired
proteasome function. More importantly, this was associated to changes in ER proteins suggesting the occurrence of ER stress. Since those changes were reproducible by an excitotoxic paradigm at an early stage, it can be suggested that excitotoxicity leads to increased protein oxidation, proteasomal dysfunction and ER stress in neuronal and non-neuronal cells, potentially contributing to motor-neuron death in ALS.

**Supplementary material**

Supplementary material is available at *Brain* online.

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**References**

Ahtoniemi T, Goldsteins G, Keksa-Goldsteine V, Malm T, Kanninen K, LSMH-CT-2004-503039) to I.F. Supported by the COST Sixth Framework Programme (BrainNet Europe II, and D.C.; and FIS grants PI04-0184 and PI05-1570, and support by the European Commission under the Sixth Framework Programme (BrainNet Europe II, LSHM-CT-2004-503039) to I.F. Supported by the COST B-35 Action.


Furukawa Y, Fu R, Deng HX, Siddique T, O'Halloran TV. Disulfide cross-linked protein represents a significant fraction of ALS-associated cu, zinc superoxide dismutase aggregates in spinal cords of model mice. Proc Natl Acad Sci USA 2006; 103: 7148–53.


