PROTEIN NITRATION IN A MOUSE MODEL OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS: POSSIBLE MULTIFUNCTIONAL ROLE IN THE PATHOGENESIS*

Filippo Casoni1,2, Manuela Basso1,2, Tania Massignan1,2, Elisabetta Gianazza3, Cristina Cheroni2, Mario Salmona2, Caterina Bendotti1, Valentina Bonetto1,2

From the 1Dulbecco Telethon Institute, Milan, Italy, the 2“Mario Negri” Institute for Pharmacological Research, Milan, Italy, and the 3Proteomics and Protein Structure Study Group and CEND, Department of Pharmacological Sciences, University of Milan, Milan, Italy

Running Title: Protein nitration in FALS mice

Address correspondence to: Valentina Bonetto, Dulbecco Telethon Institute and “Mario Negri” Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy, Tel. +390239014548; Fax +39023546277; E-Mail: bonetto@marionegri.it

Multiple mechanisms have been proposed to contribute to amyotrophic lateral sclerosis (ALS) pathogenesis, including oxidative stress. Early evidence of a role for oxidative damage was based on the finding, in patients and murine models, of high levels of markers, such as free nitrotyrosine (NT). However, no comprehensive study on the protein targets of nitration in ALS has been reported. We found an increased level of NT-immunoreactivity in spinal cord protein extracts of a transgenic mouse model of familial ALS (FALS) at a presymptomatic stage of the disease compared to age-matched controls. NT-immunoreactivity is increased in the soluble fraction of spinal cord homogenates and is found as a punctuate staining in motor neuron perikarya of presymptomatic FALS mice. Using a proteomic-based strategy, we identified proteins nitrated in vivo, under physiological or pathological conditions, and compared their level of specific nitration. Alpha and gamma enolase, ATP synthase beta chain and heat shock cognate 71 kDa protein and actin were over-nitrated in presymptomatic FALS mice. We identified by MALDI mass spectrometry 16 sites of nitration in proteins oxidized in vivo. In particular, alpha enolase nitration at Tyr43, target also of phosphorylation, brings additional evidence on the possible interference of nitration with phosphorylation. In conclusion, we propose that protein nitration may have a role in ALS pathogenesis, acting directly by inhibiting the function of specific proteins and indirectly interfering with protein degradation pathways and phosphorylation cascades.

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease. It is sporadic in 90% and familial in 10% of cases, of which 20% are caused by mutations of the copper-zinc superoxide dismutase (SOD1) gene. Familial and sporadic ALS cases are indistinguishable on the basis of clinical and pathological criteria, suggesting that the two forms share similar or converging pathogenetic mechanisms. Several mechanisms have been proposed to contribute to ALS pathogenesis, including excitotoxicity, mitochondrial dysfunction, impaired proteasomal function, protein aggregation and apoptosis. However, it is not clear which is the primary event, or the temporal relations between these pathways. Recent investigations support the notion that these mechanisms may be coordinated by oxidative stress, which can activate pathways that lead to additional oxidative stress and amplify the disease (1-3).

Early evidence suggesting a role for oxidative damage in ALS came from the identification of markers of oxidative stress in the cortex and spinal cord of patients with sporadic and familial ALS (4-6). Among these markers, nitrotyrosine (NT) has attracted attention in view of Beckman’s theory, which suggests a greater propensity of SOD1 mutants to use peroxynitrite as an enzyme substrate, leading to tyrosine nitration (7). In fact, increased levels of free NT have been found in human patients and mouse models of ALS (6,8,9). However, in only very few studies protein-bound NT has been specifically characterized in connection with ALS.

Increased levels of free or protein-bound NT were observed in several neurodegenerative and inflammatory diseases (10) and were usually considered a marker of peroxynitrite formation causing irreversible protein damage (11). Immunohistochemistry studies have shown that nitrated proteins accumulate in Lewy bodies of a number of neurodegenerative synucleopathies (12). Using specific antibodies that recognized...
only nitrated alpha synuclein, it was seen that the majority of the Lewy bodies contained nitrated alpha synuclein, indicating that this modification may participate in their formation (13).

Recent findings have raised the question whether protein nitration might also be a cellular signaling mechanism (14). In fact, it has been demonstrated that protein nitration is a reversible and selective process, like protein phosphorylation (15,16). The dynamic nature of the nitration was revealed by de-nitration and re-nitration of proteins in mitochondria subjected to hypoxia-anoxia and reoxygenation cycles. Characterization of the putative tyrosine denitrase activity, which has been described in preliminary reports (17), would give conclusive evidence of the reversibility of this biological process and its role in signal transduction.

To further explore these concepts and to clarify the role of protein nitration in ALS pathogenesis, detailed and comprehensive studies of the target proteins are needed. However, from a technical point of view it is challenging to analyze nitrated proteins in vivo. The modification is rare and common biochemical procedures may lead to loss of NT through conversion to aminotyrosine (18). However, proteomic tools based on immunoblotting techniques have been recently adapted to the analysis of nitrated proteins and applied to investigate several pathological situations (19). We used a proteomic approach to analyze the nitrated proteins in spinal cord extracts of a murine model of a familial form of ALS (FALS): a transgenic (Tg) mouse over-expressing human SOD1 carrying G93A mutation, which develops progressive motor dysfunction leading to paralysis and death (20). Moreover, by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry we identified 16 sites of nitration in proteins oxidized in vivo. Materials and Methods

Transgenic mouse models—Tg mice originally obtained from Jackson Laboratories, expressing a high copy number of mutant human SOD1 with a Gly-93-Ala substitution or WT human SOD1 mice, were bred and maintained in a C57BL/6 mouse strain at the Consorzio Mario Negri Sud, S. Maria Imbaro (CH), Italy. Tg mice are identified by PCR (21). The mice were housed at a temperature of 21 ± 1°C with relative humidity 55 ± 10% and 12 h light. Food (standard pellets) and water were supplied ad libitum. Female Tg SOD1 G93A mice were killed at 9, 14, and 20 weeks of age, corresponding respectively to presymptomatic, early symptomatic, and late stages of the motor dysfunction (22). Age-matched female Tg SOD1 WT mice were used as controls. Non-Tg C57BL/6 mice were used as further controls for immunohistochemistry. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. No. 116, G.U. Suppl. 40, Feb. 18, 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 DEC.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). Sample preparation—Soluble proteins: spinal cords were suspended 1:4 (w:v) in a lysis buffer [10 mM Tris-HCl buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid, 10 mM dithiothreitol, 50 mM iodoacetamide, 1 tablet of Complete™ per 10 mL buffer, Mini Protease Inhibitor Cocktail (Roche), and 5 µM MG132 proteasome inhibitor (Sigma)], and sonicated. Homogenates were ultracentrifuged at 55000 g for 30 min at 4 °C and soluble proteins separated from the pellet. Triton-insoluble proteins or “aggregates”: the pellet was further processed following a published protocol with some modifications (23), to enrich the fraction of ubiquitinated proteins. Briefly, the pellet was resuspended in ice-cold 15 mM Tris-HCl buffer, pH 7.6, containing 0.25 M sucrose, 150 mM KCl and 2% of non-ionic detergent Triton X-100 and shaken for 5 hours at 4 °C. Samples were centrifuged at 10000 g at 4 °C for 15 minutes to obtain Triton-resistant pellets.

Dot blot—This analysis was done using a dot-blot apparatus, Minifold II Slot-Blot System (Schleicher & Schuell BioScience). A polyvinylidene difluoride (PVDF) membrane (Millipore) was soaked in methanol for a few seconds and then in ultrapure water for 2 minutes and conditioned in 20 mM Tris-HCl, pH 7.4, prior to assembly in the apparatus. Soluble proteins were directly loaded onto the membrane, while Triton-insoluble proteins were first resuspended in 0.5% sodium dodecyl sulfate (SDS) in 20 mM Tris-HCl, pH 7.4, then diluted 1:10 with the same Tris-HCl buffer before application. Deposition of each sample was made on the membrane by vacuum filtration. Aliquots (2 µg) of samples from Tg SOD1 G93A or Tg SOD1 WT mice of different ages were loaded on the membrane. The membrane was probed with the anti-NT antibody as described in the Western
sections at the L3-5 level (30°C 
Transverse lumbar spinal cord they sank, and finally frozen in 2-methylbutane overnight, then to 30% sucrose solution until they sank, and finally frozen in 2-methylbutane at –45°C. Transverse lumbar spinal cord sections at the L3-5 level (30 µm thick) were cut on a cryostat and processed free-floating in multiwell plates. Cryosections were permeabilized with 70% methanol in PBS for 30 min at room temperature (RT) and blocked with 5% normal goat serum, 0.1% Triton X-100 in PBS for 30 min at room temperature. Then sections were incubated overnight, with constant agitation, with primary antibody (monoclonal anti-NT, clone HM.11, HyCult Biotechnology) diluted 1:100 in PBS containing 0.1% Tween and 10% normal goat serum. Immune reactions were revealed by 60 min incubation in goat anti-mouse biotinylated IgG diluted 1:200 (Vector Laboratories), followed by 60 min incubation in avidin-biotin-peroxidase complex (ABC, Vector), using 3'-3-diaminobenzidine as chromogen. The specificity of the NT-immunostaining was evaluated in sections from control mice processed with omission of the primary antiserum or with primary antiserum that was preabsorbed with 3-NT, and developed under the same conditions.

**Two-dimensional electrophoresis**—Soluble proteins extracted from the spinal cord of G93A or WT mice were precipitated overnight with 4 volumes of methanol at –20°C, and resuspended in 8 M urea. Two thirds of the sample from a spinal cord were used for preparative two-dimensional electrophoresis (2-DE); one third after 2-DE was examined by Western blotting (WB). 2-DE was done essentially as previously described (24). Briefly, home-made 8-cm long immobilized pH gradient (IPG) gel covering, with an exponential course, the pH range 4–10, prepared according to a recipe (25), were used. Aliquots of sample solution were loaded on the IPG gels near the anode on a stack of Paratex pads and run in a Multiphor II apparatus (Amersham Biosciences). Pairs, with a WT and a G93A sample, were mounted tail-to-end on a gradient, of 4-20% polyacrylamide, SDS gel cast in a Protean II apparatus (Bio-Rad) with the discontinuous buffer system of Laemmli. After completion of the run, the preparative 2-DE gel was stained with Coomassie blue and the other 2-DE gel was transferred onto a PVDF membrane.

**Western blotting**—Each WB experiment was done on a single PVDF membrane, containing WT and G93A samples run in parallel. The membranes were incubated for 1 hour at RT with a blocking buffer (5% milk in Tris buffered saline containing 0.1% Tween) and probed overnight at 4°C with monoclonal antibody against-NT diluted 1:1000 (clone HM.11, HyCult Biotechnology) in blocking buffer. The membrane was then washed and incubated for 1 hour at RT with goat anti-mouse peroxidase-conjugated secondary antibody diluted 1:5000 (Santa Cruz Biotechnology). The immunopositive spots were visualized using a sensitive chemiluminescent protein detection system, ECL Plus (Amersham Biosciences). To reveal false immunopositive spots, membranes were first stripped with Restore™ Western Blot Stripping Buffer (Pierce) and then chemically reduced to convert NT to aminotyrosine, with 10 mM sodium dithionite in 50 mM pyridine-acetate buffer, pH 5.0, for one hour at RT, as previously described (26). After the reaction the membranes were extensively washed with distilled water and probed again with the anti-NT antibody. The immunopositive spots detected after the reduction were not considered for further analysis.

**Gel/blot image analysis and quantification**—The 2-DE gels and the 2-D WB images were captured by a high-resolution scanner, Expression 1680 Pro (Epson). Densitometry and image analysis were done by Progenesis software, Workstation version 2003.03 (Nonlinear Dynamics). Gel/blot comparison was done by using the specific warping algorithm of the software in the manual mode, placing seeding points on recognizable, intense immunopositive spots. The relative immunoreactivity for each protein spot was calculated as the ratio of the pixel volume of the immunoreactive spot on the blot to the pixel volume of the matched spot on the 2-DE gel. NT-immunoreactive spot pixel volumes were normalized to the total immunoreactivity of the blot, and gel spot pixel volumes were normalized to the total spot volume of the gel. The fold increase or decrease in NT-immunoreactivity
was calculated as the ratio between the relative immunoreactivity in Tg SOD1 G93A and in Tg SOD1 WT mice. Fold changes of relative NT-immunoreactivity in Tg SOD1 G93A compared to Tg SOD1 WT mice in the four experiments were expressed as the mean ± SEM. Statistical analysis was done by one-sample Student’s t test, with p<0.05 indicating fold changes significantly greater than 1.

**Protein identification**—Protein spots were excised from 2-DE gels, de-stained for a few hours in 25 mM ammonium bicarbonate/40% ethanol and washed with sequentially rising percentages of acetonitrile. Proteins were in gel-digested overnight at 30 °C with trypsin (Promega) at a concentration of 12 ng/µL in a 25 mM ammonium bicarbonate/10% acetonitrile solution. When necessary tryptic digests were concentrated and desalted using ZipTip® pipette tips with C18 resin and 0.2 µL bed volume (Millipore). Peptide mass fingerprinting (PMF) was done on a ReflexIII™ MALDI mass spectrometer (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid as matrix, as previously described (27). The mass spectra were internally calibrated with trypsin autolysis fragments, routinely obtaining accuracy better than 50 ppm. Database (NCBI) searches were done using the Mascot program (http://www.matrixscience.com), allowing up to one missed trypsin cleavage and a mass tolerance of ±0.1 Da. In the Mascot searching *Mus musculus* sequences deposited in NCBI, probability-based MOWSE scores (Pappin, 1993) greater than 61 were considered significant (p<0.05). In case of a low score, the identification was confirmed by PMF analysis of endoprotease V8 (Sigma) protein digest. The enzyme was used at a concentration of 12 ng/µL in a 25 mM ammonium bicarbonate/5% acetonitrile solution. Spectra originating from parallel protein digestions were compared pairwise to discard common peaks from endoprotease V8 autodigestion. Database searches were done with the Mascot program, allowing up to two missed cleavages.

**RESULTS**

NT-immunoreactivity is higher in spinal cord of Tg SOD1 G93A mice than Tg SOD1 WT mice at all stages of the disease—We measured the total NT-immunoreactivity, which comprises free and protein-bound NT, in spinal cord and hippocampus soluble protein extracts from Tg SOD1 G93A mice at presymptomatic (9 weeks), symptomatic (14 weeks) and late (20 weeks) stages of the disease and from age-matched control SOD1 WT mice. We found dot-blot analysis particularly suitable for this type of experiment since it involves limited manipulation of the sample and NT modification is maximally preserved, while in classical SDS polyacrylamide gel electrophoresis/WB analysis boiling the sample with reducing agents may cause the conversion of NT to aminotyrosine (18). In addition, only a small amount of sample is required and all the replicates can be analyzed at the same time, allowing accurate comparison.

At all ages examined, the level of NT-immunoreactivity in G93A spinal cord soluble protein extracts was higher than for age-matched controls (Fig.1A), with a peak of increase of about 2 folds at a presymptomatic stage. We performed the same experiment with soluble protein extracts from hippocampus, which is not affected by the disease. In hippocampus (Fig. 1B) the levels of NT-immunoreactivity were comparable for G93A and WT samples at 14 and 20 weeks. The NT level in G93A samples was higher, but not significantly, only at 9 weeks. On the other hand, the NT level in hippocampus extracts was about 2.5 folds less than in spinal cord extracts (data not shown).

Nitrated proteins do not selectively accumulate in protein aggregates—We tested whether nitrated proteins participate in the formation of protein aggregates, and accumulate in cell inclusions. The most widely seen inclusions in ALS immunostain for ubiquitin (both sporadic and FALS) and for SOD1 (SOD1-linked FALS cases) (28). Triton-insoluble fractions of protein extracts are rich in ubiquitinated proteins (29) and in SOD1 (30). The Triton-resistant pellet was our protein aggregate preparation. Proteins were extracted from spinal cord of six Tg SOD1 G93A or Tg SOD1 WT mice at 9, 14 and 20 weeks of age and NT-immunoreactivity was measured in the Triton-insoluble fraction by dot-blot analysis. The levels of NT-immunoreactivity were not significantly different between G93A and WT samples at all ages examined (data not shown). As shown in Fig. 2, NT-immunoreactivity was substantially lower, about 10 folds, in the aggregates from G93A and WT samples at all ages compared to soluble extracts from 9-week-old Tg SOD1 WT mice.

NT-immunolabeling appears as a punctuate staining in the motor neuron perikarya of presymptomatic Tg SOD1 G93A mice—The overall intensity of immunostaining was comparable in non-Tg, SOD1 WT and...
presymptomatic SOD1 G93A mice (Fig. 3A-C). However, in four out of six presymptomatic SOD1 G93A mice it was noticed a peculiar punctuate staining in the motor neurons with spots intensely immunolabeled (Fig. 3C). This phenomenon was observed in highly vacuolated, degenerating motor neurons as well as in apparently healthy ones. At the symptomatic stage there was an overall increase of NT-immunoreactivity throughout the grey and white matter of the whole lumbar spinal cord sections, but a punctuate staining was not observed (Fig. 3D). The increase in immunoreactivity was diffuse in the neuropil and was particularly concentrated in small cells and small tissue elements. Some of them appeared as markedly shrunken neurons while others showed morphology typical of hypertrophic astrocytes and reactive microglia cells. 

Identification of nitrated proteins in spinal cords of presymptomatic Tg SOD1 G93A and age-matched Tg SOD1 WT mice—To investigate the impact of nitrated proteins in ALS pathogenesis we concentrated our attention on mice at a presymptomatic stage of the disease in comparison with age-matched healthy control Tg mice. Nitrated proteins in spinal cords of Tg SOD1 G93A or Tg SOD1 WT mice were analyzed using a proteomic approach. Soluble proteins were extracted from spinal cord of 9-week-old mutated Tg and age-matched healthy control Tg mice. Each sample was separated in duplicate by isoelectrofocusing. The second dimension for the G93A and WT samples was run in the same gel, in duplicate. One of the 2-DE gels was then transferred to a single membrane for WB analysis using anti-NT antibody, and the other was stained with Coomassie blue. The images of blot and corresponding Coomassie-stained 2-DE gel were overlaid by the Progenesis software, using the warping/matching algorithm in the manual mode. The 2-DE gel spots corresponding to the immunoreactive spots were excised and subjected to PMF analysis for identification. Figure 4A-B shows a representative gel and its corresponding anti-NT WB. Several immunoreactive spots are present in both WT and G93A samples. Few were considered false-positive because they were still present in the blot after reducing treatment with sodium dithionite or because they could not be matched to visible Coomassie-stained gel spots. The remaining 32 were identified as listed in Table 1.

The nitrination patterns for WT and G93A mice are very similar, whereas the level of nitrination for some proteins differs. For example, as can be seen in the magnification of a portion of the blot in Fig. 4C-D, the levels of nitrination of creatine kinase, B chain (CKB) and actin are respectively 2.7 and 2.0 folds higher in G93A than in WT mice (Fig. 4E-F) (see also Table 2). The relative immunoreactivity toward the anti-NT antibody, as a quantification of the level of specific protein nitratin, could be measured (Table 2), since WT and G93A samples were blotted to the same membrane for consistent experimental conditions. The increased nitratin (>1.5 fold) in G93A compared to WT for each protein in this WB experiment is reported in Table 2.

Over-nitrated proteins in presymptomatic Tg SOD1 G93A mice—In the 2-D WB/gel experiments, we found a mean value of 1.7 ± 0.4 (p=0.07) fold increase of total NT-immunoreactivity in G93A compared to WT samples. We used a controlled proteomic-based experimental setting to compare WT and G93A samples, but we found several differences for the individual proteins among the various WB experiments. In combination with the intrinsic variability of the immunoblotting technique, it is likely that the extension and dynamics of protein nitratin vary widely for the individual protein in different animals. However, we observed a number of proteins, listed in Table 3 and shown in Figure 5, which were nitrated in all the experiments and had a higher level of nitratin in Tg SOD1 G93A compared to Tg SOD1 WT mice in at least three different WB. We define these proteins “over-nitrated”. Among them, we reported a highly significant increase in NT level for heat shock cognate 71 kDa protein, ATP synthase beta chain and alpha enolase. Finally, a special pattern was observed for alpha synuclein, which was significantly “under-nitrated” in mutated mice in all four experiments by 2 ± 0.4 folds. This could depend on the high propensity of alpha synuclein, exposed to nitrating agents, to form peculiar nitrated cross-linked oligomers, through covalent o,o′-dityrosine bonds (31), which may result in a decrease of the specific level of nitratin in the spot of the monomeric form.

Identification of the sites of nitratin by MALDI mass spectrometry—Some of the NT-immunopositive proteins, particularly the ones with higher relative immunoreactivity, were also confirmed as being nitrated by MALDI mass spectrometry (MS). During ionization/desorption in MALDI MS, nitrated fragments undergo a series of phodecomposition reactions involving
the loss of one or two oxygens that can also be accompanied by further reductive reactions (32,33). Though this could contribute to the dispersion of the signal of the nitrated fragments, in our hands MALDI MS appears rather sensitive for the analysis of in vivo nitrated proteins. In fact, we detected Tyr-containing tryptic fragments together with possibly related modified fragments with mass shifts of 45, 31, 29, 15, 13 Da compatible with NO2-, NHOH-, NO-, NH2- and N-Tyr modifications respectively. Table 4 reports the mass ions of the unmodified tryptic fragments, the potentially nitrated tryptic fragments and their phodecomposition products. Figure 6A-D gives examples of MS analysis of the nitrated fragments for actin, alpha enolase, phosphoglycerate mutase and CKB. Further MS/MS analysis of the possibly nitrated peptides was not possible because of low recovery of the modified peptides, as already observed by others when analyzing 2-DE separated proteins (34).

DISCUSSION

Markers of oxidative damage and increased levels of free NT have been found in Tg mice and in patients with ALS (6,8,9), but no comprehensive study on the protein targets has yet been reported. Our investigation was set out to clarify the role of oxidative stress, in particular protein nitration, in ALS pathogenesis by a specific proteomic approach.

We used one of the most widely studied ALS animal models, the Tg SOD1 G93A mouse, which exhibit many of the hallmarks of the human disease and enabled us to analyze nitrated proteins in the different stages of disease progression (20). For comparison we used age-matched healthy Tg SOD1 WT mice. Tg SOD1 WT and G93A mice express transgenic SOD1 at the same level, as measured by densitometric analysis (data not shown), allowing accurate proteome map comparisons. Technical problems exist in the detection of nitrated proteins because: (i) the modification is rare in tissues and in biological fluids, even in pathological conditions (10); (ii) the modification is lost in strongly reducing conditions and possibly also under the action of not yet identified denitrase enzymes (17,18); (iii) common chemical and immunological methods may detect false-positive nitrated proteins (35,36). Therefore, special care was needed in each experiment to avoid under- or over-estimation, as described in the Materials and Methods section. Using these methods NT-bound proteins could be analyzed with high sensitivity in spinal cords of Tg SOD1 G93A/WT mice.

The soluble protein fraction from mice at presymptomatic, symptomatic and end stages showed considerably higher levels of NT-immunoreactivity, indicating the presence of free and protein-bound NT, compared to controls. This increase was disease-specific since it occurred basically in spinal cord of mutated Tg mice and not (except for a tendency at 9 weeks of age) in extracts from hippocampus, tissue not affected by the disease. Furthermore, the increase appeared to be an early event. In fact, at 9 weeks our line of Tg mice had no evident sign of neuromuscular deficits, even though alterations of the mitochondria are observed at a morphological level (37). Nitrated proteins did not specifically accumulate in the aggregate fraction. In fact, only a small percentage of the proteins in the aggregates were NT-immunopositive. One explanation is that the modification renders the protein more polar and water-soluble, and only in selected cases, the protein may become poorly soluble and form aggregates, e.g. alpha synuclein whose nitro-group extensively affects the protein structure causing cross-linking and oligomerization (31). It cannot be excluded, however, that even a small amount of insoluble nitrated proteins may serve as a seed for aggregation for other proteins. Interesting and original observations in this study were the spots of intense NT-immunoreactivity in the perikarya of motor neurons at the early stage of degeneration, in presymptomatic SOD1 G93A mice. We have not identified the nitrated protein(s) present in these spots, but this calls for further investigation. However, whatever the composition of these nitrated microaggregates, they do not seem to cause massive accumulation and aggregation of nitrated proteins in the motor neurons at a later phase of degeneration. In fact, although there is a steep rise in NT-immunoreactivity in the spinal cord sections at the advanced stages of the disease, the signal appears homogeneously distributed in the reactive astrocytes and in shrunken motor neurons, except for some scattered, intensely labeled, round formations reminiscent of axonal spheroids (38). In addition, the pattern of distribution of NT-immunoreactivity is different from that of ubiquitinated protein aggregates, suggesting that nitrated proteins do not accumulate in those aggregates (30).

We therefore made a detailed proteomic analysis of the soluble fraction of spinal cord...
homogenates of 9-week-old SOD1 G93A and SOD1 WT mice. We identified 32 proteins nitrated in vivo, under physiological or pathological conditions. These can be grouped in classes based on their recognized functions: chaperone, energy metabolism, GDP/GTP exchange regulator, cytoskeletal, antioxidant, and others (Table 1). With the proteomic approach a relative quantification of the level of specific nitration for individual proteins was made in ALS-mice in comparison with healthy mice. Behavior was not similar for all the proteins in different experiments. This cannot be explained simply as individual variability or experimental artifact. Other authors have observed a similar pattern and suggested that protein nitration is a dynamic process, where hypothetical denitrase removes the modification under certain conditions (15,16). In any case, a small group of proteins, possibly the most susceptible to oxidation, were substantially over-nitrated: alpha and gamma enolase, ATP synthase beta chain, heat shock cognate 71 kDa protein and actin. Persistent alterations of the function of these proteins may have important implications for cell metabolism. Inhibition of function is the most widely reported consequence of protein Tyr nitration, but there are a few examples of a gain of function as well as no effect on function (10,39). On this basis, we could consider a potential role of nitration in ALS pathogenesis, possibly interfering with multiple pathways, as summarized in the scheme in Figure 7.

Enolase is a dimeric enzyme of the glycolytic pathway responsible for catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate. In neuronal tissues the enzyme is present as a homodimer or heterodimer of alpha and gamma subunits. The gamma subunit is mainly located in neurons, and the alpha subunits in glial cells. Increasing evidence links enolase-dependent pathways to several pathologies, especially autoimmune and neurodegenerative disorders (40). Abnormally oxidized forms of alpha enolase have been suggested as a cause of the impaired glucose metabolism observed in brains of Alzheimer’s disease patients (41,42).

The ATP synthase (ATPase) beta-chain subunit belongs to the F1 catalytic core of the mitochondrial ATPase complex, which has a key role in energy production. ATPase, by complex rotational movements of its subunits, couples the proton gradient generated by the respiratory chain to the synthesis of ATP from ADP and inorganic phosphate. The nitro-group may strongly influence the interactions between the subunits and impair the complex machinery of ATP production. This is compatible with reduced ATP synthesis in the spinal cord mitochondria of symptomatic SOD1 G93A mice (43).

Heat shock cognate 71 kDa protein (HSC71) is a constitutively expressed member of the heat shock protein (HSP) 70 family. HSC71 is a molecular chaperone and facilitates the degradation by the proteasome of several proteins, including mutant SOD1, in an ubiquitination-dependent manner (44). Proteinaceous inclusions containing mutant SOD1 have been detected in ALS-transgenic mice and patients with sporadic and familial ALS. Neurons may be relatively deficient in their ability to induce certain HSPs upon stress and up-regulation of protein chaperones protects cells from mutant SOD1 toxicity (45). Very recently it has been shown that treatment with arimoclomol, a coinducer of HSPs, raised the levels of HSP70 and 90 in the spinal motor neurons of SOD1 G93A mice and significantly delayed disease progression (46).

Actin is a major cytoskeletal protein in neurons, involved in many aspects of cell motility, vesicle transport and membrane turnover. Its organization in filaments is fundamental for its function. Oxidation and in particular nitration of actin can alter actin polymerization (47). The motor neuron, with its extensive network of cell processes, needs a particularly efficient transport system to transfer cellular components synthesized in the cell body to their correct destination. In ALS mice cytoskeletal abnormalities and slowing axonal transport have been observed before disease onset (48,49).

A definite explanation of the association of nitration of specific proteins with the expression of a pathological phenotype calls for detailed functional studies. This remains a challenge because conditions that favor protein nitration can readily induce the concomitant oxidation of other amino acids that might be crucial in protein function.

As recently reviewed, protein nitration may function as a cell signaling mechanism (14), however, its significance and, in particular, its relationship of competition or cooperation with protein Tyr phosphorylation, is still an open question (50). To address these problems properly we must identify the sites of nitration and phosphorylation of proteins in vivo. However, only a limited number of proteins have
been rigorously analyzed. We identified 16 sites of nitration in proteins in vivo by MALDI MS. Among the sites identified, it is interesting to note that Tyr43 in alpha enolase, reported to be susceptible to phosphorylation (51), was found to be nitrated. It would be worthwhile investigating further how this influences alpha enolase function. It is important to note that alpha enolase, even though abundantly expressed in most cells, is not a housekeeping gene (40). Alpha enolase also has other, apparently unrelated functions, linked to its expression on the surface of a variety of eukaryotic cells (40).

Mildly oxidized proteins are readily degraded by the proteasomal system, while severely oxidized proteins are poor substrates for proteases and may accumulate. Tyr nitration makes proteins more prone to proteasomal degradation, i.e. the rate of proteolytic cleavage mediated by the 20S proteasome subunit is higher for nitrated proteins (52-54). Protein nitration might indeed play a signaling role targeting the proteins for degradation and determine an overload of the proteasome system.

Moreover, we and another group found a reduction of 20S proteasome levels in the motor neurons of Tg SOD1 G93A mice (30,55) at the presymptomatic stage. This may contribute to increase the levels of nitrated proteins, which do not specifically accumulate in the aggregates, except perhaps for the spots intensely NT-immunolabeled showed in the motor neurons.

In conclusion, several factors may contribute to the increasing oxidative and nitrative stress in ALS: mutated SOD1, excitotoxicity, mitochondrial dysfunction, etc. All these may, on one hand, exacerbate existing mitochondrial alterations and, on the other, lead to protein tyrosine nitration, which in turn may cause motor neuron degeneration by multiple pathways: directly, affecting protein functions important for cell metabolism and catabolism, and/or indirectly, through signaling mechanisms (Fig. 7). Protein nitration may therefore be an important physiological regulator and ALS pathology may occur when the system is excessively perturbed.

REFERENCES

1. Rakhit, R., Cunningham, P., Furtos-Matei, A., Dahan, S., Qi, X. F., Crow, J. P., Cashman, N. R., Kondejewski, L. H., and Chakrabarty, A. (2002) J Biol Chem 277, 47551-47556
2. Simpson, E. P., Yen, A. A., and Appel, S. H. (2003) Curr Opin Rheumatol 15, 730-736
3. Singh, P., Mann, K. A., Mangat, H. K., and Kaur, G. (2003) Mol Cell Biochem 243, 139-145
4. Bowling, A. C., Schulz, J. B., Brown, R. H., Jr., and Beal, M. F. (1993) J Neurochem 61, 2322-2325
5. Ferrante, R. J., Browne, S. E., Shinobu, L. A., Bowling, A. C., Baik, M. J., MacGarvey, U., Kowall, N. W., Brown, R. H., Jr., and Beal, M. F. (1997) J Neurochem 69, 2064-2074
6. Beal, M. F., Ferrante, R. J., Browne, S. E., Matthews, R. T., Kowall, N. W., and Brown, R. H., Jr. (1997) Ann Neurol 42, 644-654
7. Beckman, J. S., Carson, M., Smith, C. D., and Koppenol, W. H. (1993) Nature 364, 584
8. Bruijn, L. I., Beal, M. F., Becher, M. W., Schulz, J. B., Wong, P. C., Price, D. L., and Cleveland, D. W. (1997) Proc Natl Acad Sci USA 94, 7606-7611
9. Klivenyi, P., Ferrante, R. J., Matthews, R. T., Bogdanov, M. B., Klein, A. M., Andreassen, O. A., Mueller, G., Werner, M., Kaddurah-Daouk, R., and Beal, M. F. (1999) Nat Med 5, 347-350
10. Greenacre, S. A., and Ischiropoulos, H. (2001) Free Radic Res 34, 541-581
11. Yamakura, F., Taka, H., Fujimura, T., and Murayama, K. (1998) J Biol Chem 273, 14085-14089
12. Duda, J. E., Giasson, B. I., Chen, Q., Gur, T. L., Hurtig, H. I., Stern, M. B., Gollomp, S. M., Ischiropoulos, H., Lee, V. M., and Trojanowski, J. Q. (2000) Am J Pathol 157, 1439-1445
13. Giasson, B. I., Duda, J. E., Murray, I. V., Chen, Q., Souza, J. M., Hurtig, H. I., Ischiropoulos, H., Trojanowski, J. Q., and Lee, V. M. (2000) *Science* **290**, 985-989
14. Schopfer, F. J., Baker, P. R., and Freeman, B. A. (2003) *Trends Biochem Sci* **28**, 646-654
15. Aulak, K. S., Koeck, T., Crabb, J. W., and Stuehr, D. J. (2004) *Am J Physiol Heart Circ Physiol* **286**, H30-38
16. Koeck, T., Fu, X., Hazen, S. L., Crabb, J. W., Stuehr, D. J., and Aulak, K. S. (2004) *J Biol Chem* **279**, 27257-27262
17. Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998) *Proc Natl Acad Sci U S A* **95**, 11584-11589.
18. Balabanli, B., Kamisaki, Y., Martin, E., and Murad, F. (1999) *Proc Natl Acad Sci U S A* **96**, 13136-13141
19. Ghezzi, P., and Bonetto, V. (2003) *Proteomics* **3**, 1145-1153
20. Bendotti, C., and Carri, M. T. (2004) *Trends Mol Med* **10**, 393-400
21. Rosen, D. R. (1993) *Nature* **364**, 362
22. Bendotti, C., Tortarolo, M., Suchak, S. K., Calvaresi, N., Carvelli, L., Bastone, A., Rizzi, M., Rattray, M., and Mennini, T. (2001) *J Neurochem* **79**, 737-746
23. Ouyang, Y. B., and Hu, B. R. (2001) *Neurosci Lett* **298**, 159-162
24. Gianazza, E., Eberini, I., Villa, P., Fratelli, M., Pinna, C., Wait, R., Gemeiner, M., and Miller, I. (2002) *J Chromatogr B Analyt Technol Biomed Life Sci* **771**, 107-130
25. Gianazza, E. (1998) in *2-D Proteome Analysis Protocols* (LinK, A. J., ed), pp. 175-188, Humana Press, Totowa
26. Miyagi, M., Sakaguchi, H., Darrow, R. M., Yan, L., West, K. A., Aulak, K. S., Stuehr, D. J., Hollyfield, J. G., Organisciak, D. T., and Crabb, J. W. (2002) *Mol Cell Proteomics* **1**, 293-303
27. Laragione, T., Bonetto, V., Casoni, F., Massignan, T., Bianchi, G., Gianazza, E., and Ghezzi, P. (2003) *Proc Natl Acad Sci U S A* **100**, 14737-14741
28. Wood, J. D., Beaujeux, T. P., and Shaw, P. J. (2003) *Neuropathol Appl Neurobiol* **29**, 529-545
29. Hayashi, T., Takada, K., and Matsuda, M. (1992) *J Neurosci Res* **31**, 561-564
30. Cheroni, C., Peviani, M., Cascio, P., De Biasi, S., Monti, C., and Bendotti, C. (2004) *Neurobiol Dis*, in press
31. Souza, J. M., Giasson, B. I., Chen, Q., Lee, V. M., and Ischiropoulos, H. (2000) *J Biol Chem* **275**, 18344-18349
32. Petersson, A. S., Steen, H., Kalume, D. E., Cairdahl, K., and Roepstorff, P. (2001) *J Mass Spectrom* **36**, 616-625
33. Sarver, A., Scheffler, N. K., Shetlar, M. D., and Gibson, B. W. (2001) *J Am Soc Mass Spectrom* **12**, 439-448
34. Kanski, J., Alterman, M. A., and Schoneich, C. (2003) *Free Radic Biol Med* **35**, 1229-1239
35. Viera, L., Ye, Y. Z., Estevez, A. G., and Beckman, J. S. (1999) *Methods Enzymol* **301**, 373-381
36. Yi, D., Ingelse, B. A., Duncan, M. W., and Smythe, G. A. (2000) *J Am Soc Mass Spectrom* **11**, 578-586.
37. Bendotti, C., Calvaresi, N., Chiveri, L., Prellle, A., Moggio, M., Braga, M., Silani, V., and De Biasi, S. (2001) *J Neurol Sci* **191**, 25-33
38. Bendotti, C., Atzori, C., Piva, R., Tortarolo, M., Strong, M. J., DeBiasi, S., and Migheli, A. (2004) *J Neuropathol Exp Neurol* **63**, 113-119
39. Ischiropoulos, H. (1998) *Arch Biochem Biophys* **356**, 1-11
40. Pancholi, V. (2001) *Cell Mol Life Sci* **58**, 902-920
41. Castegna, A., Aksenov, M., Thongboonkerd, V., Klein, J. B., Pierce, W. M., Booze, R., Markesbery, W. R., and Butterfield, D. A. (2002) *J Neurochem* **82**, 1524-1532
42. Castegna, A., Thongboonkerd, V., Klein, J. B., Lynn, B., Markesbery, W. R., and Butterfield, D. A. (2003) *J Neurochem* **85**, 1394-1401
43. Mattiazz, M., D'Aurelio, M., Gajewski, C. D., Martushova, K., Kiaei, M., Beal, M. F., and Manfredi, G. (2002) *J Biol Chem* **277**, 29626-29633
44. Uruhitani, M., Kurisu, J., Tateno, M., Hatakeyama, S., Nakayama, K., Kato, S., and Takahashi, R. (2004) *J Neurochem* **90**, 231-244
45. Bruening, W., Roy, J., Giasson, B., Figlewicz, D. A., Mushynski, W. E., and Durham, H. D. (1999) *J Neurochem* **72**, 693-699
46. Kieran, D., Kalmar, B., Dick, J. R., Riddoch-Contreras, J., Burnstock, G., and Greensmith, L. (2004) *Nat Med* **10**, 402-405
47. Clements, M. K., Siemsen, D. W., Swain, S. D., Hanson, A. J., Nelson-Overton, L. K., Rohn, T. T., and Quinn, M. T. (2003) *J Leukoc Biol* **73**, 344-355
48. Williamson, T. L., and Cleveland, D. W. (1999) *Nat Neurosci* **2**, 50-56
49. Farah, C. A., Nguyen, M. D., Julien, J. P., and Leclerc, N. (2003) *J Neurochem* **84**, 77-86
50. Monteiro, H. P. (2002) *Free Radic Biol Med* **33**, 765-773.
51. Songyang, Z., and Cantley, L. C. (1995) *Trends Biochem Sci* **20**, 470-475
52. Gow, A. J., Duran, D., Malcolm, S., and Ischiropoulos, H. (1996) *FEBS Lett* **385**, 63-66
53. Grune, T., Blasig, I. E., Sitte, N., Roloff, B., Haseloff, R., and Davies, K. J. (1998) *J Biol Chem* **273**, 10857-10862
54. Souza, J. M., Choi, I., Chen, Q., Weisse, M., Daikhin, E., Yudkoff, M., Obin, M., Ara, J., Horwitz, J., and Ischiropoulos, H. (2000) *Arch Biochem Biophys* **380**, 360-366
55. Kabashi, E., Agar, J. N., Taylor, D. M., Minotti, S., and Durham, H. D. (2004) *J Neurochem* **89**, 1325-1335

**FOOTNOTES**

*We wish to thank Giuliano Grignaschi for help in collecting the animal tissues, and Pietro Ghezzi and Roberto Chiesa for critical review of the manuscript. This work was supported by grants from the Telethon Foundation (TCP0010 to V.B.), the Cariplo Foundation (S01010FCRA to V.B) and the Italian Ministry of the University and Research (FIRB Negoziali, Protocol RBNEO1B5WW_008). V.B. is an Assistant Telethon Scientist.

*The abbreviations used are: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismutase; NT, nitrotyrosine; FALS, familial form of ALS; Tg, transgenic; WT, wild-type; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; PBS, phosphate buffered saline; RT, room temperature; 2-DE, two-dimensional electrophoresis; WB, Western blotting; matrix-assisted laser desorption/ionization, MALDI; PMF, peptide mass fingerprinting; MS, mass spectrometry; IPG immobilized pH gradient; CKB, creatine kinase, B chain; ATPase, ATP synthase; HSC71, heat shock cognate 71 kDa protein; HSP, heat shock protein.

**FIGURE LEGENDS**

Fig. 1. NT-immunoreactivity in soluble protein extracts of spinal cord and hippocampus of Tg SOD1 G93A mice throughout disease progression in comparison with healthy mice. NT-immunoreactivity was measured by dot-blot analysis of protein extracts from spinal cord (A) and hippocampus (B) of Tg SOD1 G93A mice at 9, 14 and 20 weeks of age, corresponding to an early, symptomatic and late stage of the disease. Age-matched Tg SOD1 WT mice were used as controls. Six mice per genotype and age were used for the analysis. NT-immunoreactivity was normalized to the total protein loaded, as measured by densitometry of the dot-blot membrane after Coomassie staining. Values are the means ± SEM of NT-immunoreactivity of the samples calculated as a percentage of the 9-week-old WT sample.
mean. Asterisk indicates G93A sample mean significantly higher (p < 0.05) than age-matched WT sample mean, as assessed by Student’s t test.

Fig. 2. Comparison of the level of NT-immunoreactivity in soluble protein extracts and aggregates. NT-immunoreactivity was measured by dot-blot analysis of soluble protein extracts from spinal cord of 9-week-old Tg SOD1 WT mice and of Triton-insoluble proteins extracts from spinal cord of Tg SOD1 WT and Tg SOD1 G93A mice at 9, 14 and 20 weeks of age. Dot-blot analysis was done on a pool of three G93A and WT mice, loading on the membrane a 2 µg-aliquot of each pooled sample. NT-immunoreactivity was normalized to the total protein loaded as measured by densitometry of the dot-blot membrane after Coomassie staining. Values are expressed in arbitrary units.

Fig. 3. NT-immunoreactivity in ventral horn of transverse sections of lumbar spinal cord from non-Tg (A), SOD1 WT (B) and SOD1 G93A mice at presymptomatic (C) and symptomatic (D) stages. In non-Tg and SOD1 WT mice low NT-immunostaining is distributed in the grey matter except for the motor neurons that show a higher immunolabeling (arrows; see also insets at higher magnification). In presymptomatic SOD1 G93A mice (C) some motor neurons show a remarkable, punctuated staining of perikarya. This is evident in both, massively vacuolated, (lower inset in C) and apparently normal (upper inset in C) motor neurons. In symptomatic SOD1 G93A mice (D) a higher, diffuse NT-immunoreactivity is observed in both grey and white matter. Small intensely immunolabeled cells, apparently hypertrophic astrocytes, upper inset in (D), are distributed in grey and white matter. Intensely labeled shrunken motor neurons (lower inset in D) are observed. Scale bars: 100 mm lower power; 20 mm insets.

Fig. 4. Analysis of nitrated proteins in Tg SOD1 WT/G93A mice using a proteomic-based strategy. A representative 2-DE gel (A) and the corresponding anti-NT 2-D WB (B) are shown, with the WT and G93A samples displayed respectively on the left and right sides of the panels A and B. Coomassie-stained gel spots and matching immunoreactive spots are circled and numbered. The corresponding proteins were identified as reported in Table 1. Magnifications of portions of the WB of the WT sample (C) and of the G93A sample (D) are shown. The two immunoreactive spots in C and D were creatine kinase, B chain and actin and are over-nitrated in the G93A sample. The 3-D visualization of the blot image analysis is shown for creatine kinase, B chain and actin from WT (E) and G93A (F) samples.

Fig. 5. Over-nitrated proteins. Representative portions of the anti-NT 2-D WB where the immunoreactive spots corresponding to the over-nitrated proteins actin, alpha and gamma enolase, ATP synthase beta chain, and heat shock cognate 71 kDa protein are indicated by arrows in WT and G93A samples.

Fig. 6. Identification of the nitration sites by MALDI MS. Spectra of nitrated fragments of actin (A), alpha enolase (B), phosphoglycerate mutase 1 (C), and creatine kinase, B chain (D). The mass ion of the unmodified tryptic fragment (*) and of the NT-modified fragment (Y-NO2) or of its photodecomposition products (Y-NHOH, Y-NH2) are indicated.

Fig. 7. Possible multifunctional role of protein nitration in ALS pathogenesis. Schematic view of the biochemical pathways, in which protein nitration is potentially involved, leading to neurodegeneration in ALS, either directly via function inhibition of specific proteins, such as actin, ATPase and HSC71, and/or indirectly through signaling mechanisms, interfering with protein degradation pathways and phosphorylation cascades.
| spot | protein                                      | AC  | Mr$_{calc}$ | pI$_{calc}$ | Mr$_{obs}$ | pI$_{obs}$ | score | cov | pep | fun |
|------|---------------------------------------------|-----|-------------|-------------|------------|------------|-------|-----|-----|-----|
| 1    | heat shock protein 4                         | Q99L75 | 94.0        | 5.1         | 110        | 4.9        | 222   | 50  | 44  | C   |
| 2    | aconitase 2, mitochondrial                   | Q99K10 | 85.5        | 8.1         | 86         | 7.5        | 225   | 37  | 25  | E   |
| 3    | heat shock cognate 71 kDa protein            | P63017 | 70.8        | 5.4         | 70         | 5.2        | 98    | 35  | 21  | C   |
| 4    | dihydropyrimidinase related-2                | O08553 | 62.2        | 6.0         | 70         | 5.6        | 116   | 33  | 18  | -   |
| 5    | stress-induced phosphoprotein 1              | Q8BPH3 | 62.4        | 6.0         | 65         | 6.3        | 128   | 39  | 25  | -   |
| 6    | Rab GDP dissociation inhibitor alpha          | P50396 | 50.5        | 5.0         | 63         | 4.7        | 107   | 49  | 18  | G   |
| 7    | protein disulfide isomerase A3               | P27773 | 54.2        | 5.8         | 56         | 5.6        | 166   | 42  | 22  | C   |
| 8    | dihydropyrimidinase related-3                | Q62188 | 61.9        | 6.0         | 65         | 5.8        | 104   | 59  | 39  | -   |
| 9    | dihydropyrimidinase related-2*               | O08553 | 62.2        | 6.0         | 65         | 5.9        | 78    | 31  | 13  | -   |
| 10   | ATP synthase beta chain                      | P56480 | 51.7        | 5.0         | 55         | 4.7        | 155   | 48  | 22  | E   |
| 11   | alpha enolase                                | P17182 | 47.0        | 6.4         | 47         | 5.8        | 167   | 67  | 28  | E   |
| 12   | alpha enolase                                | P17182 | 47.0        | 6.4         | 47         | 5.8        | 156   | 67  | 25  | E   |
| 13   | alpha enolase                                | P17182 | 47.0        | 6.4         | 47         | 5.8        | 168   | 54  | 24  | E   |
| 14   | gamma enolase                                | P17183 | 47.1        | 5.0         | 48         | 4.7        | 212   | 61  | 27  | E   |
| 15   | actin                                        | P60710 | 41.7        | 5.2         | 42         | 5.0        | 150   | 69  | 30  | T   |
| 16   | creatine kinase, B chain                     | Q04447 | 42.6        | 5.4         | 42         | 5.3        | 183   | 75  | 31  | E   |
| 17   | fructose-bisphosphat aldolase C              | P05063 | 39.2        | 6.8         | 40         | 6.7        | 164   | 49  | 23  | E   |
| 18   | fructose-bisphosphat aldolase C              | P05063 | 39.2        | 6.8         | 40         | 6.8        | 191   | 56  | 27  | E   |
| 19   | L-lactate dehydrogenase B chain              | P16125 | 36.4        | 5.7         | 36         | 5.5        | 77    | 47  | 19  | E   |
| 20   | glyceroldehydes-3-phosphate DH               | P16858 | 35.7        | 8.4         | 36         | 7.0        | 64    | 33  | 9   | E   |
| 21   | glyceroldehydes-3-phosphate DH               | P16858 | 35.7        | 8.4         | 36         | 7.8        | 80    | 44  | 19  | E   |
| 22   | phosphoglycerate mutase 1                    | Q9DBJ1 | 28.7        | 6.7         | 28         | 6.0        | 137   | 64  | 20  | E   |
| 23   | carbonic anhydrase II                        | P00920 | 29.0        | 6.5         | 29         | 6.4        | 93    | 66  | 15  | O   |
| 24   | Rho GDP-dissociation inhibitor 1             | Q99PT1 | 23.3        | 5.1         | 25.5       | 4.8        | 113   | 68  | 16  | G   |
| 25   | adenylyl kinase isoenzyme 1*                 | Q9ROY5 | 21.5        | 5.6         | 22         | 5.0        | 33    | 35  | 7   | O   |
| 26   | superoxide dismutase [Mn]                    | P09671 | 22.2        | 7.3         | 22         | 6.8        | 68    | 39  | 8   | A   |
| 27   | superoxide dismutase [Mn]                    | P09671 | 22.2        | 7.3         | 22         | 7.2        | 89    | 53  | 13  | A   |
| 28   | coflin, muscle isofrom                      | P45591 | 18.7        | 7.6         | 18         | 7.0        | 64    | 60  | 10  | T   |
| 29   | coflin, non-muscle isoform*                  | P18760 | 18.5        | 8.2         | 18         | 8.0        | 76    | 68  | 12  | T   |
| 30   | beta synuclein b*                           | Q91ZZ3 | 14.0        | 4.4         | 14         | 4.1        | 38    | 31  | 4   | -   |
| 31   | alpha synuclein b*                          | O55042 | 14.0        | 4.7         | 14         | 4.5        | 78    | 45  | 8   | -   |
| 32   | neurofilament triplet L protein*             | P08551 | 61.5        | 4.6         | 70         | 4.6        | 82    | 30  | 18  | T   |

*AC, accession numbers are from SwissProt or TrEMBL databases; Mr$_{calc}$ and pI$_{calc}$, calculated Mr and pI; Mr$_{obs}$ and pI$_{obs}$, observed Mr and pI; score, probability score in Mascot program; cov, percentage of sequence coverage; pep, matched peptides; fun, protein function: C, chaperone, E, energy metabolism, G, GDP/GTP exchange regulator, T, cytoskeletal, A, antioxidant, O, other.

*The identification was confirmed by PMF analysis after V8 endoproteinase digestion.

Protein spots 30 and 32 are NT-immunopositive in other blots (data not shown).

DH, dehydrogenase.
Table 2. Increase in relative NT-immunoreactivity in Tg SOD1 G93A compared to WT mice

| spot | protein                              | WB^a | gel^b | RF | WB | gel | RI | RIG93A/RIWT^d |
|------|--------------------------------------|------|-------|----|----|-----|----|----------------|
| 1    | heat shock protein 4                 | 0.02 | 0.24  | 0.09 | 0.04 | 0.22 | 0.18 | +2.1          |
| 2    | aconitase 2, mitochondrial           | 0.10 | 0.92  | 0.10 | 0.42 | 0.80 | 0.53 | +5.1          |
| 3    | heat shock cognate 71 kDa            | 2.37 | 0.34  | 7.02 | 2.79 | 0.18 | 15.48 | +2.2          |
| 4    | dihydropyrimidinase related-2        | -    | 0.35  | -   | 0.18 | 0.21 | 0.85 | +             |
| 5    | stress-induced phosphoprotein 1      | 0.03 | 0.54  | 0.05 | 0.14 | 0.41 | 0.34 | +6.6          |
| 6    | protein disulfide isomerase A3       | 0.02 | 0.21  | 0.08 | 0.04 | 0.25 | 0.18 | +2.2          |
| 7    | dihydropyrimidinase related-3        | 0.02 | 0.21  | 0.09 | 0.15 | 0.16 | 0.96 | +10.2         |
| 8    | dihydropyrimidinase related-2        | 0.08 | 0.99  | 0.08 | 0.29 | 0.57 | 0.51 | +6.2          |
| 9    | ATP synthase beta chain              | 0.19 | 0.45  | 0.42 | 0.29 | 0.31 | 0.91 | +2.2          |
| 10   | alpha enolase                        | 5.22 | 0.75  | 6.92 | 10.94 | 1.01 | 10.79 | +1.6          |
| 11   | actin                                | 3.42 | 1.11  | 3.07 | 5.94 | 0.95 | 6.27 | +2.0          |
| 12   | creatine kinase, B chain             | 1.77 | 1.11  | 1.59 | 4.68 | 1.10 | 4.27 | +2.7          |
| 13   | L-lactate dehydrogenase B chain      | 0.13 | 0.66  | 0.20 | 0.62 | 0.60 | 1.03 | +5.1          |
| 14   | phosphoglycerate mutase 1            | 0.40 | 0.30  | 1.32 | 0.87 | 0.39 | 2.25 | +1.7          |
| 15   | carbonic anhydrase II                | 1.41 | 0.67  | 2.09 | 2.09 | 0.56 | 3.75 | +1.8          |
| 16   | Rho GDP-dissociation inhibitor 1     | 0.14 | 1.47  | 0.09 | 0.35 | 1.47 | 0.24 | +2.6          |
| 17   | adenylate kinase isoenzyme 1         | 0.02 | 0.46  | 0.04 | 0.04 | 0.38 | 0.11 | +2.5          |
| 18   | superoxide dismutase [Mn]            | 3.43 | 0.54  | 6.42 | 1.83 | 0.49 | 3.72 | +1.7          |
| 19   | superoxide dismutase [Mn]            | 0.09 | 0.18  | 0.50 | 0.04 | 0.15 | 0.26 | +2.0          |

^aWB, NT-immunoreactive spot pixel volume normalized to the total immunoreactivity of the blot, as measured by Progenesis.
^bGel, gel spot pixel volume normalized to the total spot volume of the gel, as measured by Progenesis.
^cRI, relative immunoreactivity calculated for each spot as the ratio between pixel volume of the immunoreactive spot on the blot and pixel volume of the matched spot on the 2-DE gel. ^dRIG93A/RIWT, fold increase (+) in NT-immunoreactivity calculated as the ratio between the RI in Tg SOD1 G93A and Tg SOD1 WT mice.
Table 3. Over-nitrated proteins in presymptomatic Tg SOD1 G93A compared to Tg SOD1 WT mice

| spot | protein name                           | fold±  | p-value |
|------|----------------------------------------|--------|---------|
| 3    | heat shock cognate 71 kDa protein      | 1.6±0.3| < 0.05  |
| 10   | ATP synthase beta chain                | 2.1±0.6| < 0.05  |
| 12   | alpha enolase                          | 1.4±0.2| < 0.05  |
| 14   | gamma enolase                          | 3.1±2.3| > 0.05  |
| 15   | actin                                  | 3.7±2.5| > 0.05  |

*Increase fold of relative NT-immunoreactivity in Tg SOD1 G93A compared to Tg SOD1 WT mice. Values are the means ± SEM of the increase fold obtained from each of four experiments, p < 0.05 indicates increase fold significantly greater than 1, by one-sample Student’s t test.*
| Protein Name, Spot No. | Tryptic Fragment Sequence | m/z<sub>calc</sub><sup>a</sup> | m/z<sub>exp</sub><sup>b</sup> | -NO<sub>2</sub><sup>b</sup> | -NHOH<sup>b</sup> | -NO<sup>b</sup> | -NH<sub>2</sub><sup>b</sup> | -N<sup>b</sup> |
|----------------------|--------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Rab GDP dissociation inhibitor alpha, 6 | TDDYLDQPCLETINR | 1852.84<sup>c</sup> | 1852.81 | 1867.86 |
| Dihydropyrimidinase-related-3, 8 | GNVVFGEPIASLGIDGTHYWSK | 2448.21 | 2448.14 | 2493.12 | 2479.08 | 2463.04 |
| ATP synthase beta chain, 10 | AHHGYSVFAGVGER | 1406.68 | 1406.68 | 1473.74<sup>*</sup> | 1457.72<sup>*</sup> |
| Alpha enolase, 12 | AAVPSGASTGIYEALELR | 1804.94 | 1804.94 | 1820.00 |
| Gamma enolase, 14 | LGAEVYHTLK | 1130.62 | 1130.69 | 1143.71 |
| Actin, 15 | QEYDEGPSIVHR | 1516.70 | 1516.66 | 1547.66 | 1531.67 |
| Creatine kinase, B chain, 16 | VLTPELYAELR | 1303.72 | 1303.73 | 1348.70 |
| Fructose-bisphosphate aldolase C, 18 | PSYPALSAEQK | 1327.66 | 1327.66 | 1356.68 | 1364.67<sup>*</sup> | 1340.67 |
| Glyceraldehyde-3-phosphate dehydrogenase, 21 | LISWYDNEYGYSNR | 1799.80 | 1799.78 | 1824.67 |
| Phosphoglycerate mutase 1, 22 | DAGYEDICFTSVQK | 1979.78 | 1979.67 | 2024.77 |
| Carbonic anhydrase II, 23 | QSPVDDTATAQHDPALQPLLISYDK | 2836.43 | 2836.50 | 2851.57 |
| Rho GDP-dissociation inhibitor 1, 24 | AEEYEFITMEAPK | 1783.80 | 1783.77 | 1798.88 |
| Superoxide dismutase [Mn], 26 | HHAAYVNNLNATEEK | 1710.82 | 1710.79 | 1741.87 |
| Cofilin, muscle isoform, 28 | QILVGDGDTVEDPYTSFK | 2196.10 | 2196.11 | 2225.12 | 2211.10 |
| Alpha synuclein, 31 | EGVLYVGSKTK | 1180.66 | 1180.65 | 1247.68<sup>*</sup> |

<sup>a</sup>m/z<sub>calc</sub>, calculated m/z of the unmodified tryptic fragment.

<sup>b</sup>m/z<sub>exp</sub>, experimentally measured m/z of the unmodified and Y-modified tryptic fragment ion. The fragment ions carrying Y-NO<sub>2</sub>, Y-NHOH, Y-NO, Y-NH<sub>2</sub> and Y-N have a mass shift of +45, +31, +29, +15 and +13 Da, respectively).

<sup>c</sup>m/z of the fragment with carboxamidomethylated cysteine.

<sup>d</sup>DH, dehydrogenase.

* Sodium-adduct mass ion (+22 Da).
Figure 1
Figure 2
Figure 4
Figure 5

| Protein                        | WT          | G93A        |
|--------------------------------|-------------|-------------|
| actin                          |             |             |
| alpha enolase                  |             |             |
| gamma enolase                  |             |             |
| ATP synthase beta chain        |             |             |
| heat shock cognate 71 kDa     |             |             |
Figure 6
Figure 7
Protein nitration in a mouse model of familial amyotrophic lateral sclerosis: Possible multifunctional role in the pathogenesis
Filippo Casoni, Manuela Basso, Tania Massignan, Elisabetta Gianazza, Cristina Cheroni, Mario Salmona, Caterina Bendotti and Valentina Bonetto

J. Biol. Chem. published online February 7, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413111200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts