

Forum Original Research Communication

Nitroproteomics of Peripheral Blood Mononuclear Cells from Patients and a Rat Model of ALS

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Abstract

Increased levels of 3-nitrotyrosine in the central nervous system have been found in patients and mouse models of familial ALS (fALS), suggesting a possible use of nitrated proteins as biomarkers. We analyzed peripheral blood mononuclear cells (PBMCs), easily accessible samples, from sporadic ALS (sALS) patients and a rat model of fALS (a) to establish whether an increased level of nitrated proteins was present in PBMCs, too, and (b) to identify possible candidate biomarkers. With a proteomic approach, we identified for the first time the major overnitrated proteins in PBMCs from patients and rats at different disease stages. In the rats, their increased levels already were measured at a presymptomatic stage. Among them, actin, ATP synthase, and vinculin overlap between sALS patients and the rat model. Interestingly, in a previous study, actin and ATPase have been found overnitrated in the spinal cord of a mouse model of fALS before disease onset, suggesting their possible involvement in motor neuron degeneration. In conclusion, we observed that an increased level of nitrated proteins was not restricted to the spinal cord but also was present in peripheral cells of patients and an animal model, and that nitrated proteins are promising candidate biomarkers for early diagnosis of ALS. *Antioxid. Redox Signal.* 11, 1559–1567.

Introduction

PEROXYNITRITE FORMATION as a possible consequence of upregulation of nitric oxide synthase (NOS) and superproduction of reactive oxygen species (ROS) by the stimulation of NADPH oxidase (Nox) has been implicated in amyotrophic lateral sclerosis (ALS) (25, 32, 37, 42). Once formed, peroxynitrite may exert its toxic effect through conversion of tyrosine to 3-nitrotyrosine (3-NT), leading to protein nitration and oxidation (33). Increased levels of free 3-NT have been found in the cerebrospinal fluid (CSF) and serum of patients with ALS and in the central nervous system (CNS) of transgenic mice carrying mutations in Cu,Zn superoxide dismutase (SOD1), mouse models of a subset of the familial ALS (fALS) (1, 4, 11, 22, 37, 40). Nevertheless, only recently some 3-NT-modified proteins were properly identified. By

using a proteomic strategy we identified a number of overnitrated proteins in the spinal cord of SOD1^{G93A} mice at a presymptomatic stage of the disease (12). It has been shown that mutant SOD1 has an increased ability to catalyze aberrant oxidative reactions, including protein nitration (5, 21); however, the possible cause of increased 3-NT in non-SOD1-linked cases is not clear.

In ALS, increasing evidence suggests that alterations occurring in the CNS of patients and mouse models (including those related to oxidative stress) also are present in peripheral cells such as lymphocytes and muscles (16–18).

In search for protein biomarkers in sporadic ALS (sALS) patients, we decided to analyze whether tyrosine-nitrated proteins were increased in peripheral blood mononuclear cells (PBMCs) and if they corresponded to those previously found in the spinal cord of mouse models. PBMCs are a

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convenient source for protein biomarker discovery in patients because they can be easily isolated from blood. Moreover, proteins inside the cells are kept under high reducing conditions until analysis. This makes the analysis of nitrated proteins from PBMCs more reliable than analysis of those from serum and CSF, where proteins are exposed to an oxidizing environment that can lead to artifactual nitration (6, 30). We also examined the PBMCs isolated from a transgenic rat model of ALS overexpressing SOD1^{G93A} (2, 27). This model shows phenopathologic features similar to those of the mouse models but has the great advantage of providing more material for examinations, including the blood cells, therefore reducing the number of animals used to the minimum in observance of the ethical requirements for animal experimentation aimed to address the 3R principle.

In this work, we found that protein nitration is substantially increased in sALS patients at two degrees of disease severity as well as in SOD1 mutant rats. Some of these proteins are the same found in the spinal cord of SOD1^{G93A} mice at the presymptomatic stage and during the progression of the disease (12). These nitrated proteins have been detected in PBMCs of the SOD1^{G93A} rats at a presymptomatic stage of the disease, so indications exist for a possible use as protein biomarkers for early diagnosis of ALS.

Materials and Methods

Patients

Patients with sALS according to revised El Escorial criteria (10) were examined after written informed consent. The patients were then divided in two groups according to the Revised ALS Functional Rating Scale score (ALSFERS-R = 0 to 48) (13): patients with an overall functional status of low severity (ALSFERS-R > 24) and patients with high severity (ALSFERS-R ≤ 24). None of the patients had systemic inflammatory conditions as detected by the erythrocyte sedimentation rate test and total blood cell count. Blood samples from healthy donors were provided, after written informed consent, by the Transfusional Medical Centre of Policlinico S. Matteo, Pavia, Italy. For the analysis of total nitrotyrosine level, we examined: 10 ALSFERS-R > 24 sALS patients (five male and five female patients; mean age, 57 ± 9 years), 10 ALSFERS-R ≤ 24 sALS patients (five male and five female patients; mean age, 56 ± 7 years), 11 healthy controls (five male and six female patients; mean age, 53 ± 6); and four multiple sclerosis (MS) patients in remission (two male and two female patients; mean age, 55 ± 5 years). For the nitroproteomic studies, 10 sALS patients (four male and six female patients; mean age, 62 ± 11 years), five with ALSFERS-R ≤ 24 and five with ALSFERS-R > 24, and five healthy donors (all male subjects; mean age, 49 ± 5 years) were examined.

Rat model

Nontransgenic (NTg) and transgenic rats expressing a high copy number of mutant human SOD1 with Gly-93-Ala substitution were bred and maintained at the Mario Negri Institute for Pharmacological Research, Milan, Italy. Animals were housed at the temperature of 21 ± 1°C with relative humidity of 55 ± 10% and 12 h of light. Food (standard pellets) and water were supplied *ad libitum*. Transgenic rats were identified with PCR on DNA from tail biopsies. SOD1^{G93A}

rats were killed at presymptomatic, early-symptomatic, and late-symptomatic stages of disease. NTg rats were used as controls. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. No. 116, 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 Care and use of Laboratory Animals, U.S. National Research Council, 1996).

PBMCs

Samples of peripheral venous blood drawn from patients and controls and of blood sampled directly by intracardiac puncture from rats were collected in EDTA pre-coated vials (Vacuette K3E K3EDTA; Greiner bio-one). PBMCs were isolated from EDTA-blood by Ficoll-Hypaque (Ficoll-Plaque Plus; GE Healthcare) density-gradient centrifugation. Mononuclear cells were harvested from the interface and washed 3 times with phosphate-buffered saline (PBS, pH 7.4) (Invitrogen, Ltd). Platelets were eliminated by an additional wash at 200g, 4°C, for 10 min. Patient and control PBMCs were air-dried for few minutes and stored as pellets at -80°C. Rat PBMCs were resuspended in 250 mM sucrose, Tris-HCl 10 mM, and centrifuged 10 min at 300g, air-dried for few minutes, and stored as pellet at -20°C. PBMC proteins were obtained by cell lysis in 20 mM Tris-HCl, pH 7.5, 0.1% NP40, and 0.1% SDS supplemented with Protease Inhibitors (Sigma). Proteins were quantified by the BCA protein assay (Pierce). Rat PBMC proteins were analyzed in pools (70 µg) from SOD1^{G93A} presymptomatic (PS) (n = 4), symptomatic (S) (n = 4), end-stage (ES) (n = 4) and NTg (n = 4) rats.

Dot-blot analysis

An aliquot (3 µg) of PBMC samples was loaded on nitrocellulose membrane, Trans-Blot Transfer Medium (Bio-Rad), by vacuum deposition on the Bio-Dot SF blotting apparatus (Bio-Rad). Membranes were probed overnight with the anti-3-NT monoclonal antibody (clone HM.11; Hycult Biotechnology) diluted 1:1,000, and then with anti-mouse peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Blots were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) on the Chemi-Doc XRS system (Bio-Rad). Densitometry was determined with Progenesis PG240 v2006 software (Nonlinear Dynamics). Immunoreactivity was normalized to the actual amount of proteins loaded on the membrane, as detected after Red Ponceau staining (Fluka).

Two-dimensional gel electrophoresis (2DE)

Proteins from human and rats PBMCs were prepared for 2DE analysis. Proteins were dissolved in Destreak Solution (GE Healthcare) added with IPG buffer, pH 3-10, NL 0.5% vol/vol (GE Healthcare), and loaded into 7 cm-IPG strip, pI range, 3-10NL (GE Healthcare). Isoelectrofocusing was done in an IPGphor apparatus (GE Healthcare) with the following protocol: 30 V for 270 Vh, 200 V for 200 Vh, 2,000 V for 2,000 Vh, a linear gradient of 3,500 V for 1,375 Vh, 3,500 V for 7,000 Vh, a linear gradient of 8,000 V for 8,625 Vh, 8,000 V for 32,000 Vh, and forever at 30 V. SDS-PAGE was performed by using precast 10% polyacrylamide SDS gel (Invitrogen).

Gels for 2D Western blotting were transferred on a low-fluorescence PVDF membrane (Millipore).

Protein identification

Protein spots were localized and excised from 2D gels with the EXQuest spot cutter (Bio-Rad). Spots were processed and gel-digested with modified trypsin from bovine pancreas (Roche) and identified with mass spectrometry (MS), essentially as previously described (12). Digestion, desalting, and concentration with ZipTip pipette tips with C18 resin (Millipore) and MALDI target deposition were carried out on an automated protein digester DigestPro MS (Intavis AG). Peptide mass fingerprinting and MS/MS analysis were done on a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems). The mass spectra were internally calibrated with trypsin autolysis fragments. The five most abundant precursor ions, of the exclusion mass list (ions from human keratin and trypsin), were selected for MS/MS analysis. The combined MS and MS/MS data were submitted by the GPS Explorer v. 3.6 software (Applied Biosystems) to the MASCOT database search engine (Version 2.1, Matrix Science) and searched with the following parameters: Swissprot 56.5 database over all *Rattus norvegicus* or *Homo sapiens* protein sequences deposited, no fixed modifications, as possible modifications carboamidomethylation of cysteine and oxidation of methionine, one missed trypsin cleavage, a mass tolerance of ± 0.1 Da for the peptide mass values, and of ± 0.3 Da for the MS/MS fragment ion mass values. A protein was regarded as identified if the MASCOT protein score, based on combined MS and MS/MS data, was above the 5% significance threshold for the database (34).

2DE Western blotting (WB) and quantification of the nitration level

Proteins transferred to membrane were visualized with Sypro Ruby Blot Staining (Bio-Rad) and scanned with Molecular Imager FX Laser Scanner (excitation, 532 nm; Bio-Rad). Membranes were probed overnight with the anti-3-NT antibody (clone HM.11; Hycult Biotechnology) diluted 1:1,000. Secondary antibody was goat anti-mouse Cy5 conjugated (GE Healthcare) diluted 1:2,500. The 3-NT signal was captured with the Molecular Imager FX Laser Scanner (excitation, 635 nm; Bio-Rad). The Sypro Ruby blot signal and the 3-NT immunoreactivity signal were captured with the laser scanner with the same parameters for all the membranes of the experiment, controls, and ALS. Densitometry and image analysis were done with Progenesis PG240 v2006 software (Nonlinear Dynamics). Sypro Ruby-stained blot and anti-3-NT blot images were matched by using the specific warping algorithm of the software in the manual mode. The measurement of the 3-NT level of the single protein spot was calculated as the ratio of the pixel volume of the immunoreactive spot on the blot to the pixel volume of the Sypro Ruby-stained spot on the same membrane. The fold increase was calculated as the ratio between the 3-NT level in ALS samples and in controls.

Results

Analysis of total 3-NT level in PBMCs of sALS patients

The analysis of the total 3-NT level was done by dot-blotting with the specific antibody anti-3-NT. Lysates from

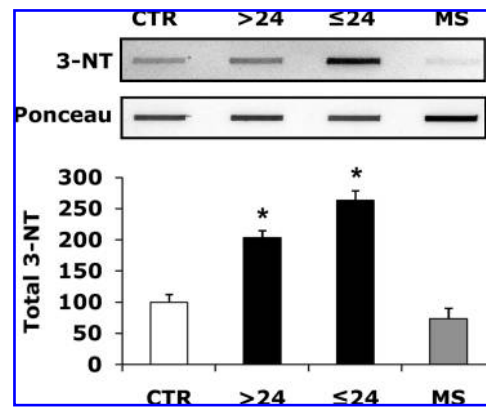


FIG. 1. Analysis of total 3-NT level in PBMCs. Aliquots of 3 μ g of PBMC lysates from sALS patients, ALSFRS-R > 24 ($n = 10$) and ALSFRS-R ≤ 24 ($n = 10$), healthy controls (CTR) ($n = 11$), and MS patients ($n = 4$) were examined with anti-3-NT dot blotting. Representative anti-3-NT blot and relative Red Ponceau-stained membranes are shown. Histogram represents the anti-3-NT immunoreactivity normalized to the actual amount of protein loaded, detected after Red Ponceau staining. Values are expressed as percentages of controls and as the mean \pm SEM. *, Significantly different from controls and MS patients, as assessed with the Mann-Whitney test ($p < 0.05$).

PBMCs of sALS patients with low (ALSFRS-R > 24) and high (ALSFRS-R ≤ 24) degrees of disease severity, healthy controls, and patients with MS were compared. Figure 1 shows a representative dot-blot of the analysis and the relative quantification. Clearly, the sALS patients have a significantly increased level of 3-NT compared with controls and MS patients. This indicates that nitrated proteins in PBMCs are good candidate biomarkers, differentiating ALS at least from MS patients, which may show similar symptoms in the first phase of the disease.

Nitroproteomics of PBMCs from sALS patients

To identify the protein targets of tyrosine nitration in PBMCs, we applied a proteomic approach (12). The nitroproteomic analysis was performed on samples from ALSFRS-R > 24 and ALSFRS-R ≤ 24 sALS patients and controls. The proteins extracted from the cell lysate of each sample were separated by 2DE, blotted, stained with Sypro Ruby blot staining, and probed with the anti-3-NT antibody (Fig. 2A and B). Immunoreactive spots on the membrane were localized on a twin Sypro Ruby-stained 2D gel, and the corresponding proteins identified with mass spectrometry (Fig. 2C and Table 1). All identified proteins, actin, ATP synthase subunit β (ATPase), integrin α -IIb (CD41), α -actinin, vinculin, and filamin-A showed a significantly higher level of nitration in samples from sALS patients if compared with controls (Table 2), except for actin in the ALSFRS-R ≤ 24 patients. In ALSFRS-R ≤ 24 patients, only α -actinin had a significantly higher level of nitration compared with that of ALSFRS-R > 24 patients. The total anti-3-NT immunoreactivity, which comprises the signal from all protein spots on the membrane normalized to the Sypro Ruby blot staining, was higher ($p < 0.01$) in sALS patients than in controls: 4.5 ± 1.7 -fold for patients with ALSFRS-R > 24 and 5.3 ± 1.8 -fold for patients

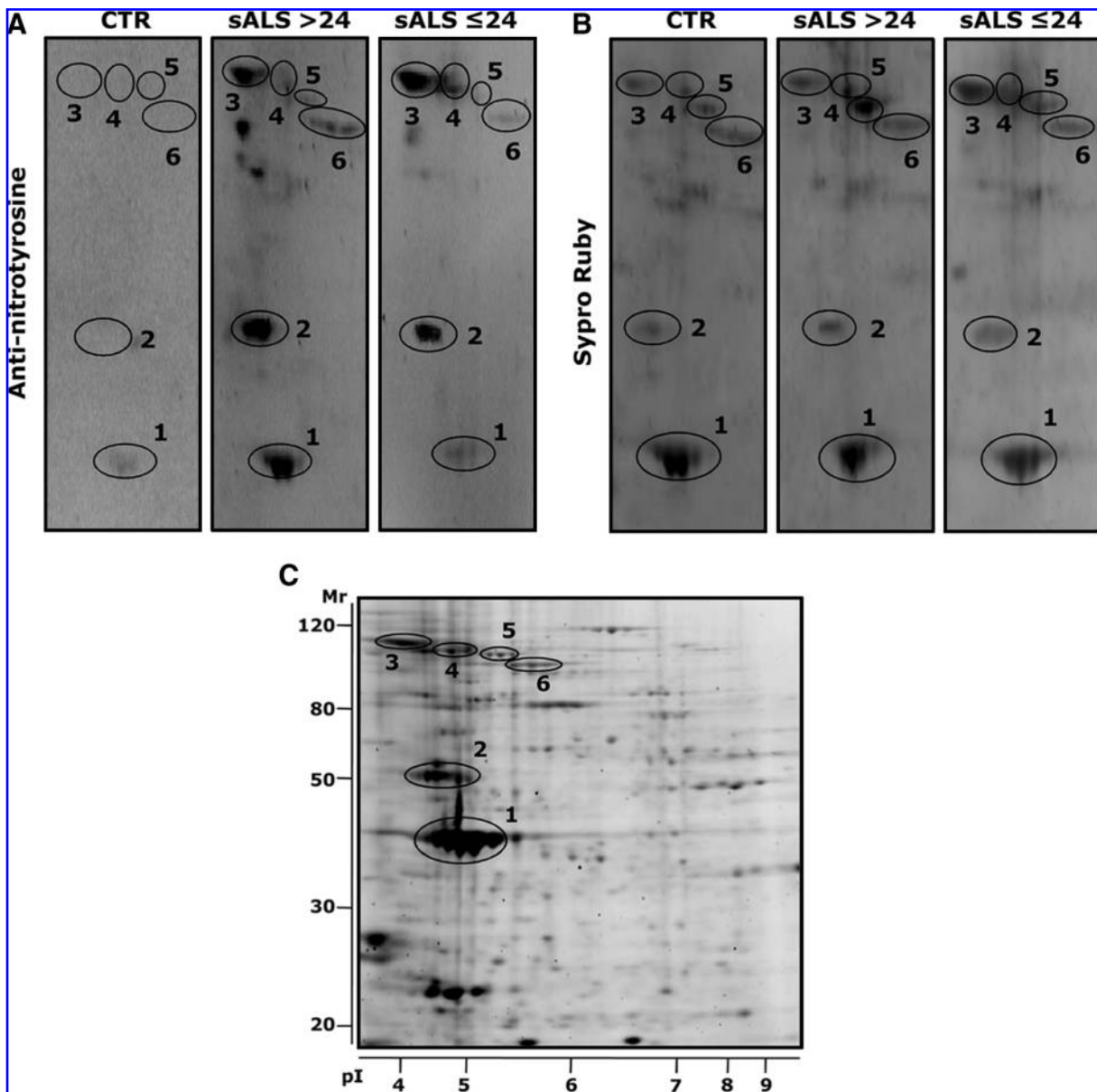


FIG. 2. Identification of nitrated proteins in PBMCs of sALS patients and controls by anti-3-NT 2D WB. (A) Representative anti-3-NT 2D WB with protein samples (80 μ g) from PBMCs of healthy donors (CTR) ($n = 5$), ALSFRS-R > 24 sALS patients ($n = 5$), and ALSFRS-R ≤ 24 sALS patients ($n = 5$). Six major 3-NT immunopositive spots were detected. (B) The anti-3-NT immunoreactivities of the proteins were normalized to the actual protein loaded, as revealed by Sypro Ruby blot staining on the same membrane. (C) Immunopositive protein spots were localized on corresponding Sypro Ruby-stained 2D gel (a representative gel is shown) and identified as actin, ATPase, CD41, α -actinin, filamin A, and vinculin with MALDI TOF/TOF mass spectrometry (see Table 1).

with ALSFRS-R ≤ 24 . Moreover, no differences were found within the group of the patients if stratified by sex and age (younger and older than 70 years) (data not shown).

Nitroproteomics of PBMCs from *SOD1^{G93A}* rats

The analysis of nitrated proteins in *SOD1^{G93A}* rats was performed at different disease stages: presymptomatic (PS), symptomatic (S), and end-stage (ES). For these analyses, we used pooled samples ($n = 4$) for each condition. Figure 3A and B shows representative images of the membranes probed with the anti-3-NT antibody and the corresponding Sypro Ruby-

stained membranes. The proteins were then identified on a corresponding Sypro Ruby-stained 2D gel (Fig. 3C; Table 3). The six major nitrated proteins were actin, ATPase, protein disulfide isomerase (PDI), heat-shock cognate 71-kDa protein (HSC70), 78-kDa glucose-regulated protein (GRP 78), and vinculin. In this study, we also measured the level of the protein nitration at an early stage of the disease, and we found that all of them except PDI were overnitrated before disease onset (Table 4). For all the proteins analyzed, except for vinculin, the level of nitration reached a maximum in correspondence of the symptomatic phase of the disease and then declined at the end stage. This trend was also reflected

TABLE 1. NITRATED PROTEINS IN PBMCS OF sALS PATIENTS AND CONTROLS AS IDENTIFIED BY MALDI TOF/TOF MASS SPECTROMETRY

| Spot | Protein name | AC | Mr ^{calc} | pI ^{calc} | Mr ^{obs} | pI ^{obs} | Cov | Pep | Score |
|------|--------------|--------|--------------------|--------------------|-------------------|-------------------|-----|-----|-------|
| 1 | Actin | P60709 | 42 | 5.3 | 42 | 5.3 | 42 | 13 | 341 |
| 2 | ATPase | P06576 | 56 | 5.2 | 55 | 5.2 | 34 | 12 | 70 |
| 3 | CD41 | P08514 | 113 | 5.2 | 110 | 5.3 | 23 | 19 | 218 |
| 4 | A-Actinin | P12814 | 103 | 5.2 | 108 | 5.3 | 7 | 31 | 69 |
| 5 | Vinculin | P18206 | 124 | 5.5 | 103 | 5.6 | 9 | 8 | 157 |
| 6 | Filamin-A | P21333 | 281 | 5.7 | 107 | 5.7 | 10 | 17 | 97 |

AC, accession numbers from SwissProt database; Mr^{calc} and pI^{calc}, calculated Mr and pI; Mr^{obs} and pI^{obs}, observed Mr and pI; cov, percentage of sequence coverage; pep, matched peptides; score, protein score based on combined MS and MS/MS spectra from MALDI-TOF/TOF analysis with MASCOT search engine (Matrix Science). Spot numbers refer to Fig. 2C. The proteins with a statistically significant ($p < 0.05$) protein score (>54) were considered successfully identified.

by the total anti-3-NT immunoreactivity that was higher in SOD1^{G93A} than in NTg rats at every stage of the disease: 2.3-fold for PS, 4.4-fold for S, and 1.5-fold for ES rats.

Discussion

Increased levels of free and protein-bound 3-NT in the CNS is considered a marker of oxidative stress for different neurodegenerative diseases (33). This study provides the first evidence that specific overnitrated proteins are expressed in the peripheral blood cells of patients and in a rat model of ALS, indicating that they can be identified as potential peripheral biomarkers of the disease. Interestingly, some of these proteins (actin, ATPase, and HSC70) have been found overnitrated in the spinal cords of SOD1 mutant transgenic mice before symptom onset, strengthening the hypothesis of a link between nitrative stress and disease pathogenesis (5, 12, 21).

Analysis of the total 3-NT level in PBMCS, showing a significant remarkable increase in sALS patients in respect to healthy controls and MS patients, revealed that nitrated proteins are good candidates as biomarkers of ALS. The primary pathways leading to production of 3-NT are the upregulation of NOS and the overproduction of ROS, the reaction of which with NO induces the formation of the highly reactive product, peroxynitrite (33). The production of ROS and reactive nitrogen species in ALS may depend on the aberrant gain of toxic function of mutant SOD1 (5), as well as the dysregulation of Nox isoforms (25). Interestingly, the *Nox4* gene was identified as significantly linked to certain sporadic forms of ALS in a whole-genome analysis (19). Both NOS and Nox may be induced by proinflammatory cytokines like TNF- α (3, 26). Levels of this cytokine are elevated in the blood of patients with ALS and in fALS animal models (26, 35), whereas no difference was reported in patients with MS in respect to healthy controls (8, 28). High blood levels of TNF- α might be a key mechanism specifically to induce 3-NT in PBMCS of ALS patients and transgenic SOD1 mutant rodents, linking the mechanisms of inflammation to the oxidative stress.

By using a redox proteomic approach, we compared the changes of the nitrated protein profile of the PBMCS from

TABLE 2. NITRATION LEVEL OF PROTEINS IN PBMCS OF sALS PATIENTS WITH ALSFRS-R > 24 AND ALSFRS-R \leq 24 IN COMPARISON WITH CONTROLS

| Spot | Protein name | Fold, ^a ALSFRS-R > 24 | Fold, ^a ALSFRS-R \leq 24 |
|------|-------------------|-------------------------------------|--|
| 1 | Actin | 3.0 \pm 0.9 | 2.6 \pm 0.9* |
| 2 | ATPase | 6.0 \pm 0.4 | 8.2 \pm 0.9 |
| 3 | CD41 | 6.6 \pm 0.4 | 5.6 \pm 0.4 |
| 4 | α -Actinin | 4.4 \pm 0.1 | 13.9 \pm 1.3** |
| 5 | Vinculin | 4.1 \pm 0.2 | 3.2 \pm 0.1 |
| 6 | Filamin-A | 5.2 \pm 0.9 | 3.6 \pm 0.1 |

^aFold, increase fold of nitration in sALS patients (ALSFRS-R > 24 and ALSFRS-R \leq 24) compared with controls. 3-NT immunoreactivity for the single protein spot (Fig. 2A) was normalized to the actual protein loaded, as revealed by Sypro Ruby blot staining on the same membrane (Fig. 2B). Values are expressed as the mean \pm SEM of the increased fold obtained from each of five experiments. All proteins show an increased fold significantly >1 ($p < 0.05$), by the Bonferroni test, except for actin in ALSFRS-R \leq 24 patients (*). **, Significantly higher fold of nitration compared with ALSFRS-R > 24 patients, as assessed by the Bonferroni test.

sALS patients at two degrees of disease severity with those of healthy controls with the changes observed in the PBMCS from SOD1 mutant rats at different stages of the disease. We identified several major nitrated proteins in PBMCS and found that nearly all of them were abundantly more nitrated in disease than in control conditions, both in the patients and in the animal model.

The advantage in using PBMCS rather than serum or CSF is that proteins are more stable in a cellular milieu than in biofluids, where they can be easily oxidized during sample processing; therefore, their redox state can be more accurately quantified. In our study, a relatively low experimental variability and a clear-cut difference in protein nitration level between sALS and controls were observed for almost all the proteins identified, making easy the discrimination between case and control samples. In addition, these cells and, in particular, lymphocytes from ALS patients have alterations in the intracellular pathway signaling, such as reduction in the antiapoptotic Bcl2 (16, 31) and impairment in the oxidative energy metabolism and calcium homeostasis similar to those described in motor neurons (17), suggesting that they may provide a useful cellular model to study the pathogenetic processes in ALS patients.

Three major overnitrated proteins were identified to overlap between sALS patients and the rat model: actin, ATPase, and vinculin. These proteins were nitrated more than threefold in sALS patients with ALSFRS-R > 24 compared with healthy controls, but were not increasingly nitrated as the disease progressed. In rats, these proteins were substantially overnitrated at a presymptomatic stage of the disease, with even a sixfold increased of nitration for vinculin. At a symptomatic stage for ATPase and actin, the level of nitration reached the maximum, whereas for vinculin, it started to decrease, but still remained 2.6-fold higher than that in controls. At the end stage, the level of nitration of these proteins in the rats did not further increase, but rather decreased, showing a parallel with the sALS patients.

Currently very little is known on the effect of nitration on protein and cellular functions. In most reported studies,

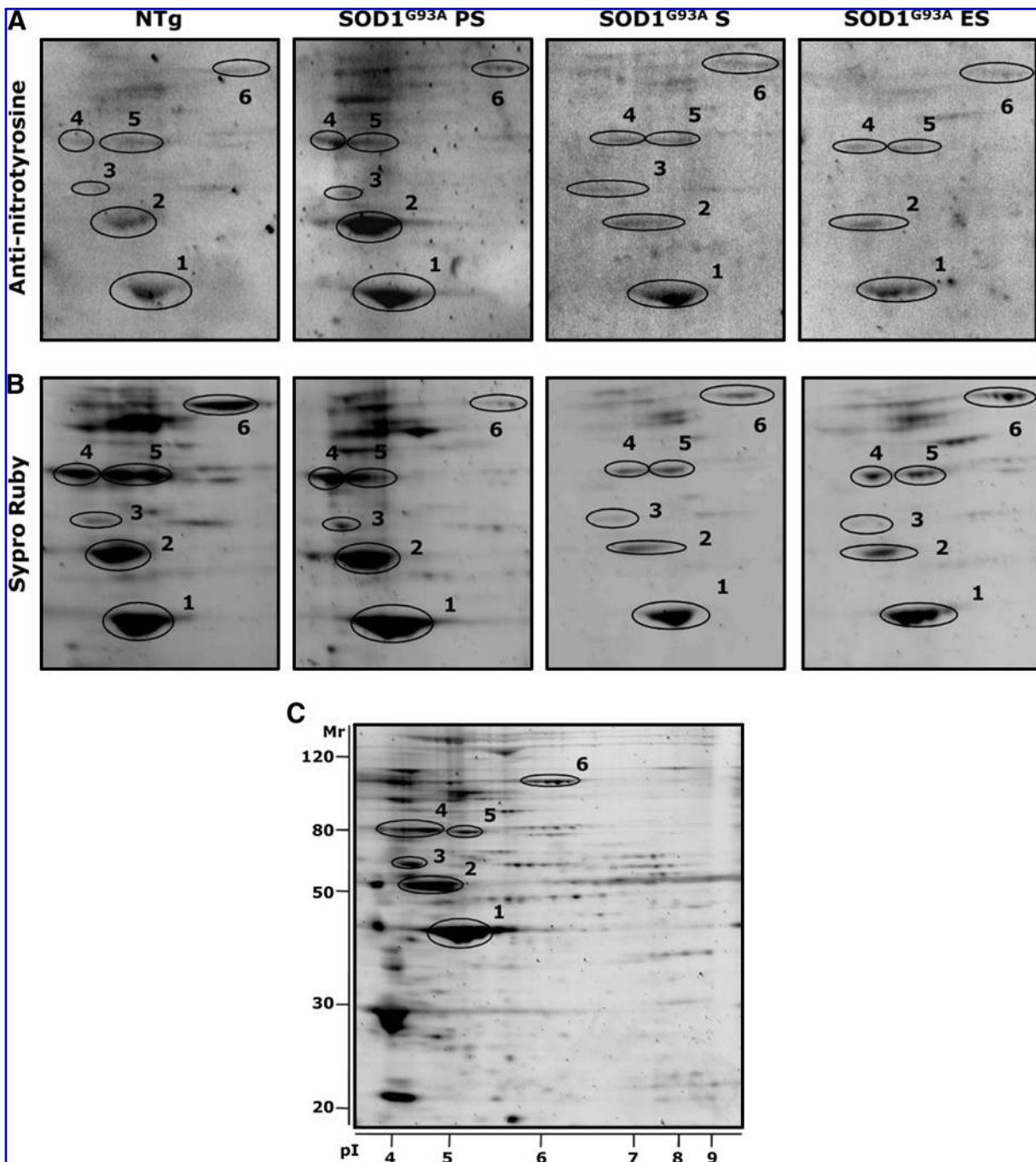


FIG. 3. Analysis of nitrated proteins in PBMCs of transgenic SOD1^{G93A} rats and NTg controls by anti-3-NT 2D WB. (A) Anti-3-NT 2D WB with protein samples (80 μ g) from PBMCs of NTg, presymptomatic (PS), symptomatic (S), and end-stage (ES) SOD1^{G93A} rats. (B) The anti-3-NT immunoreactivities of the proteins were normalized to the actual protein loaded, as revealed by Sypro Ruby blot staining on the same membrane. (C) Immunopositive protein spots were localized on corresponding Sypro Ruby-stained 2D gel (a representative gel is shown) and identified as actin, ATPase, PDI, GRP 78, HSC70, or vinculin with MALDI TOF/TOF mass spectrometry (see Table 3).

nitration of tyrosine has been associated with significant loss of function of the nitrated protein (33). It has been shown that nitration of actin can alter polymerization, leading to a reduced level of actin filaments (14) and, therefore, to an impaired ability to modulate the actin cytoskeleton and the actin-dependent cellular processes, such as chemotaxis, phagocytosis, secretion, and activation of the respiratory

burst (41). Similar effects could be postulated for the other cytoskeleton-associated proteins identified: vinculin, a ubiquitously expressed actin-binding protein that is likely to play a role in cell adhesion, cell shape and motility, and resistance to apoptosis (15, 23, 39); and filamin-A, which belongs to a class of actin-binding proteins with multifunctional properties, such as organization of actin filaments and scaffolds for a wide

TABLE 3. NITRATED PROTEINS IN PBMCS OF SOD1^{G93A} AND NTG RATS AS IDENTIFIED WITH MALDI TOF/TOF MASS SPECTROMETRY

| Spot | Protein name | AC | M _r ^{calc} | pI ^{calc} | M _r ^{obs} | pI ^{obs} | Cov | Pep | Score |
|------|--------------|--------|--------------------------------|--------------------|-------------------------------|-------------------|-----|-----|-------|
| 1 | Actin | P60711 | 42 | 5.3 | 42 | 5.2 | 32 | 14 | 387 |
| 2 | ATPase | P10719 | 56 | 5.2 | 56 | 5.0 | 29 | 16 | 254 |
| 3 | PDI | P04785 | 57 | 4.8 | 57 | 4.5 | 9 | 7 | 206 |
| 4 | GRP 78 | P06761 | 72 | 5.1 | 74 | 4.5 | 30 | 21 | 250 |
| 5 | HSC70 | P63018 | 71 | 5.4 | 74 | 5.5 | 13 | 9 | 100 |
| 6 | Vinculin | P85972 | 123 | 5.5 | 125 | 5.7 | 6 | 7 | 79 |

AC, accession numbers from SwissProt database; M_r^{calc} and pI^{calc}, calculated Mr and pI; M_r^{obs} and pI^{obs}, observed Mr and pI; cov, percentage of sequence coverage; pep, matched peptides; score, protein score based on combined MS and MS/MS spectra from MALDI-TOF/TOF analysis with the MASCOT search engine (Matrix Science). Spot numbers refer to Fig. 3C. The proteins with a statistically significant ($p < 0.05$) protein score (>51) were considered successfully identified.

range of cytoplasmic signaling proteins (24, 38). Because nitrated vinculin and filamin-A were detected in the PBMCS of patients and SOD1^{G93A} rats, but not in the CNS of mice carrying the same SOD1 mutation, we argue that these changes may account for the mechanisms of motor neuron degeneration. These altered proteins may change the cytoskeletal properties of the peripheral cells influencing their motility and their resistance to apoptosis under toxic stimuli, including oxidative stress.

It has been shown that peroxynitrite, possibly through nitration of tyrosine residues, can lead to inhibition of the activation and proliferation of lymphocytes by promoting impairment of tyrosine phosphorylation and apoptotic cell death (9). Recently, studies investigating the systemic immune system of patients with sALS revealed significant changes in peripheral blood T cells (29, 31, 43) and circulating monocytes and their MCP-1 chemokine receptor CCR2 (44), suggesting a major immunologic component in the disease. It has also been shown that, in conditions of nitrative stress, ATPase is nitrated and inactivated, suggesting a possible link between this modification and alterations in the cellular energy metabolism (36). Previous studies from our laboratory found actin and ATPase overnitrated in the spinal cord of SOD1^{G93A} mice at the presymptomatic stage and during disease progression, suggesting that defective ATP synthesis, due to ATPase modifications, and disturbance in the cytoskeleton dynamics, due to oxidation of actin, may contribute to mechanisms of motor neuron degeneration (12).

In conclusion, this work indicates that alterations in the nitrative stress pathways are not restricted to the spinal cord, the tissue specifically affected by the disease, but extend to the periphery and are found in SOD1-linked animal models and sporadic cases. On the basis of these observations, it is possible to speculate that protein nitration could be the effect of converging pathogenetic mechanisms and therefore worthwhile to be studied further from a mechanistic point of view. It would be interesting to know the source of an increased level of tyrosine nitration in peripheral cells. This may be linked to systemic dysfunction of several possible factors, including Nox and mitochondria (17, 25, 42). These elements are thought to play major roles in motor neuron degeneration and ALS pathogenesis (7, 20).

TABLE 4. NITRATION LEVEL OF PROTEINS IN PBMCS OF SOD1^{G93A} RATS AT PRESYMPTOMATIC (PS), SYMPTOMATIC (S), AND END STAGE (ES) OF DISEASE IN COMPARISON WITH NTG RATS

| Spot | Protein name | Fold, SOD1 ^{G93A} PS | Fold, SOD1 ^{G93A} S | Fold, SOD1 ^{G93A} ES |
|------|--------------|-------------------------------|------------------------------|-------------------------------|
| 1 | Actin | 1.3 | 3.0 | 1.7 |
| 2 | ATPase | 3.4 | 4.1 | 2.0 |
| 3 | PDI | 1.0 | 3.4 | — |
| 4 | GRP 78 | 2.0 | 8.8 | 3.8 |
| 5 | HSC70 | 2.0 | 7.1 | 3.0 |
| 6 | Vinculin | 6.0 | 2.6 | 1.4 |

Fold, increased fold of nitration in SOD1^{G93A} rats at three stages of the disease compared with controls. 3-NT immunoreactivity for the single protein spot (Fig. 3A) was normalized to the actual protein loaded, as revealed by Sypro Ruby blot staining on the same membrane (Fig. 3B). The fold increase was calculated as the ratio between the nitration level in SOD1^{G93A} samples and the NTg controls. Samples are pools of four animals for each condition. —, 3-NT immunoreactivity not detected.

Finally, it is important to highlight that nitrated proteins in PBMCS can underlie disease in an early phase of disease progression and therefore are promising candidate biomarkers. Antibodies that specifically recognized these modified proteins may be sensitive and affordable tools with a great potential in biomarker development.

Acknowledgments

This work was supported by grants from the Telethon Foundation (to V.B. and C.B.), the Cariplo Foundation (to V.B. and C.B.), and the Compagnia San Paolo Foundation (to V.B.), and Ministero Salute, Ricerca Finalizzata 2005, h.75. S.P. has a fellowship from the Vialli and Mauro Foundation for research and sport. V.B. is an Assistant Telethon Scientist. We thank Dr. C. Perotti (Immunohaematology and Transfusion Service, Center for Transplant Immunology, IRCCS Policlinico San Matteo, Pavia, Italy) for kindly providing blood samples from healthy controls.

Abbreviations

ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised ALS functional rating scale; ATPase, ATP synthase subunit beta; CD41, integrin α -IIb; CNS, central nervous system; CSF, cerebrospinal fluid; 2DE, two-dimensional gel electrophoresis; ES, end stage; fALS, familial ALS; GRP 78, 78-kDa glucose-regulated protein; HSC70, heat-shock cognate 71-kDa protein; MALDI, matrix-assisted laser desorption ionization; MS, multiple sclerosis; MS, mass spectrometry; MS/MS, tandem MS, NOS, nitric oxide synthase; Nox, NADPH oxidase; 3-NT, 3-nitrotyrosine; NTg, nontransgenic; PBMCS, peripheral blood mononuclear cells; PDI, protein disulfide isomerase; PS, presymptomatic; ROS, reactive oxygen species; S, symptomatic; SOD1, Cu,Zn superoxide dismutase; TOF, time of flight; WB, Western blotting.

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, March 3, 2009; date of acceptance, March 14, 2009.

This article has been cited by:

1. Caterina Bendotti , Maria Teresa Carri . 2009. Amyotrophic Lateral Sclerosis: Mechanisms and CountermeasuresAmyotrophic Lateral Sclerosis: Mechanisms and Countermeasures. *Antioxidants & Redox Signaling* **11**:7, 1519-1522. [[Citation](#)] [[PDF](#)] [[PDF Plus](#)]