Tryptophan 32 Potentiates Aggregation and Cytotoxicity of a Copper/Zinc Superoxide Dismutase Mutant Associated with Familial Amyotrophic Lateral Sclerosis*  

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One familial form of the neurodegenerative disease, amyotrophic lateral sclerosis, is caused by gain-of-function mutations in the gene encoding copper/zinc superoxide dismutase (SOD-1). More than 100 mutations distributed throughout the structure of the protein introduce a toxic gain of function that decreases protein solubility, leading to aggregation and the formation of both detergent-insoluble SOD-1 and inclusions that are a histopathological hallmark of ALS.

The nature of the toxic gain of function imparted by familial ALS (fALS)-causing SOD-1 mutations is not completely understood (reviewed in Ref. 9). These mutations do, however, provide evidence that diverse and relatively minor changes in SOD-1 primary structure can cause fALS. Approximately ninety percent of ALS is sporadic, and it is plausible that post-translational modification of wild-type proteins affects structural changes analogous to those caused by point mutation (10). Indeed, Lewy body-like inclusions in sporadic ALS are immunoreactive with antibodies to SOD-1 (11–13). It has also been demonstrated that oxidative post-translational modification of SOD-1 occurs in vivo with aging (14) and in association with the fALS (7, 15–17), Parkinson (18), and Alzheimer (19) neurodegenerative diseases. There is also considerable evidence that fALS-causing mutations predispose SOD-1 to post-translational modifications (19–21), and that in vitro oxidative modification of SOD-1 induces aggregation (22–24).

An understanding of how modifications that occur in vivo confer or potentiate toxic properties in proteins is lacking. Given the potential importance of post-translational modification of SOD-1, a study was designed to comprehensively characterize modifications that occur in vivo. Modification-prone residues in wild-type SOD-1 isolated from murine and human erythrocytes were identified and changed to residues that were less likely to be modified. Effects upon survival and aggregation were measured to determine whether preventing modification would attenuate the toxicity of SOD-1 with fALS-causing SOD-1 mutations. Tryptophan 32 (Trp-32) was shown to be modified in a significant fraction of as-isolated SOD-1. Herein we report that changing Trp-32 to a residue with a slower rate of oxidative modification, phenylalanine (25), resulted in decreased cytotoxicity of a disease-causing mutant SOD-1 (SOD-1G93A) in a motor neuronal cell culture model and decreased the propensity to form cytoplasmic inclusions.

EXPERIMENTAL PROCEDURES

Blood Samples—B6SJL-TgN(SOD-1wt)2Gur and B6SJL-TgN(SOD-1G93A)1Gur mice, transgenic for human SOD-1wt and SOD-1G93A respectively, were established from breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal facility. Mice hemizygous for the transgene were obtained by breeding hemizygous males with non-transgenic B6SJL females. Human SOD-1 transgenes were identified by PCR as previously described (2). All experiments were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on

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** The abbreviations used are: ALS, amyotrophic lateral sclerosis; CAD, collagen-activated dissociation; fALS, familial amyotrophic lateral sclerosis; LC-MS/MS, liquid chromatography tandem mass spectrometry; PBS, phosphate-buffered saline; SOD-1, copper/zinc superoxide dismutase; wt, wild type.
Animal Care. Blood samples were obtained by cardiac puncture following anesthesia with intraperitoneal injection of ketamine/xylazine. Human blood samples were obtained by finger prick, with 200 μl of blood added to an equal volume of 7.6% sodium citrate in phosphate-buffered saline (PBS), pH 7.2 (citrate PBS). Erythrocytes were separated from plasma by centrifugation at 600 × g for 5 min, washed twice by resuspension in a 20× volume of citrate PBS, and lysed by resuspension in a 5× volume of hypo-osmotic solution of 10 mM ammonium bicarbonate, pH 8.0, using fast aspiration with a 200-μl micropipette tip. The solution was clarified by centrifugation at 15,000 × g for 10 min, and the supernatant was used for subsequent immunopurification.

Antibody to Human SOD-1—Two rabbits were both immunized with four injections (at 3-week intervals) of 1.5 mg of human erythrocyte SOD-1 (hSOD-1) (Sigma-Aldrich) plus TiterMax® Gold Adjuvant (Sigma-Aldrich). Anti-hSOD-1 antibody was affinity-purified using 15 mg of hSOD-1 permanently coupled to a gel affinity column (Pierce) following the manufacturer’s instructions, except for the addition of 2 mM sodium citrate during the coupling step. During coupling optimization, citrate was found to increase coupling efficiency, and 2 mM was found to be the optimal citrate concentration. Because the antibody was purified by affinity to SOD-1, it was likely a mixture of different antibody classes. Further characterization of the antibody revealed that it was able to pull down metallated copper/zinc SOD-1, as well as all post-translational modifications present in the original SOD-1 antigen, including modification of SOD-1 by 1–7 oxygen atoms. The antibody was also able to deplete >90% of SOD-1 from erythrocyte lysates and homogenized spinal cord samples, where depletion was judged by Western blotting using a commercial SOD-1 antibody (SOD-100 Stressgen, Victoria, BC, Canada).

Purification of SOD-1 and Mass Spectrometry—Isolation of hSOD-1 was performed using affinity-purified anti-hSOD-1 polyclonal antibody that was permanently coupled to Ultralink Biosupport medium (Pierce) following the manufacturer’s instructions. Microcolumns of these beads were prepared in pipette tips containing frits (SDR Molecular, Sydney, Australia). For immunopurification, the lysed erythrocyte supernatant was passed and repassed over the column five times. Columns were washed with 40 volumes of 10 mM ammonium bicarbonate, pH 8.0, and SOD-1 was eluted with five volumes of 5% acetic acid directly into two volumes of 10% ammonium hydroxide, making the final pH 8.0. Samples were solvent-exchanged into high pressure liquid chromatography-grade water by centrifugal ultrafiltration (10 kDa) (Centricon, Millipore Corporation, Mississauga, ON, Canada) for direct infusion into the mass spectrometer. Western blot analysis using commercial anti-human (S100) or anti-mouse (S101) antibodies (Stressgen Bioreagents Corporation, Victoria, BC, Canada) confirmed that ≥95% of the immunoreactive SOD-1 was removed from erythrocyte lysates of SOD-1wt transgenic mouse or human blood samples. The entire immunopurification protocol was completed in 20 min, reducing the likelihood of artifactual oxidation. In addition, the presence of modifications in vivo was confirmed by conducting the entire procedure of erythrocyte lysis, immunopurification, and digestion in a sub-parts/million O2 anaerobic glove-box, with samples kept in anaerobic gas-tight syringes until electrosprayed.

Identification of SOD-1 Modifications—Immunopurified intact proteins were infused directly into an MDS-Sciex QSTAR® Pulsar or micromass Q-TOF II high performance liquid chromatography (LC) mass spectrometer. All LC-MS analyses took place using endoproteinase GluC-digested SOD-1, as described in Ref. 26, and injected into an Agilent 1100 nanoflow coupled to a QSTAR® in data-dependent acquisition mode, where the most abundant LC peak at any given time was subject to isolation and collision-activated dissociation (CAD) (27). MS/MS data were correlated to the protein sequence using MASCOT, and data were inspected manually. In this way, endoproteinase-digested fragments for the entire primary sequence of SOD-1 were accounted for. Modifications discovered at the whole-protein level (for example 16 Da, or one oxy-
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FIGURE 2. LC-MS/MS identification of Trp-32 oxidative modification. Typical liquid chromatography-electrospray ionization-mass spectrometry experiment consisting of a total ion chromatogram (A) and a summation of all of the chromatographic peaks between 5 and 21 min (B). B, inset panel, peaks corresponding to the net addition of both a single oxygen and two oxygen atoms to residues 25–40 were identified and localized to Trp-32 by comparing the CAD of molecules at 836 (C), 844 (D), and 852 (E) m/z. Assignments for experimentally observed CAD fragment ions of 844 and 852 m/z-modified residues are shown in respectively labeled tables, with oxygen-modified fragment ions in bold type. Modifications were observed in both the +2H and +3H charge states of residues 25–40 and in the +3H charge state of residues 25–49, which contained one missed trypsin cleavage. The average difference between a given experimental CAD fragment ion and the theoretical fragment ion to which it was assigned was 40 parts/million (0.038 Da), and the standard error for all fragment ions of a modified peptide was 10 parts/million (0.010 Da). Trp-32 is a surface residue; see model in Fig. 3.

gen; see Fig. 1) were used to interrogate the LC-MS data. For example, when oxidative modification by 16 Da was observed in the intact protein, endoproteinase peptide data were searched for peptides that were potentially modified by 16 Da (see Fig. 2B). Putative modified peptides were then subjected to subsequent LC-MS/MS analyses, and the amino acid sequence and sites of modification were derived from CAD data. The crystal structure from the Protein Data Bank (code 1SPD) (28) was used to illustrate modifications, with its surface rendered in gray using PyMOL software.

Spinal Cord-Dorsal Root Ganglion Cultures—Dissociation of spinal cord-dorsal root ganglia from embryonic day 13 CD1 mice (Charles River Laboratories, Wilmington, MA), plating on poly-d-lysine-coated coverslips, culture media, and identification of motor neurons were as previously described (29). Cultures were used 3–6 weeks after plating.

Expression of SOD-1 Variants in Motor Neurons—Subcloning of SOD-1G93A and SOD-1wt cDNAs into the BamHI/HindIII sites of pcDNA3 (Invitrogen) was performed previously (30) using cDNAs provided by Dr. Denise Figlewicz (University of Michigan, Ann Arbor, MI). Trp/Phe (W32F) mutations were created by site-specific mutagenesis of SOD-1G93A and SOD-1wt (TOP Gene Technologies, Quebec, Canada), producing SOD-1W32F/G93A and SOD-1W32F mutants, respectively. Intraneuronal microinjection of plasmids was used to express SOD-1 constructs in motor neurons and to assess their effects on the survival and formation of inclusions as previously described (6). For viability experiments, motor neurons containing the co-injected fluorescent marker were counted daily under epifluorescence microscopy beginning 24 h post-microinjection. The number of motor neurons was expressed as a percentage of those present at 24 h to exclude those dying from injury during microinjection. All experiments involved a minimum of six cultures per condition with 20–80 motor neurons (average of 40) analyzed per culture. All experimental groups were analyzed simultaneously using cultures from the same batch, but each set of conditions was assessed for reproducibility in more than one culture batch. For viability experiments, injectates were coded by an independent researcher, and cell counts for viability were carried out blind to the identity of the experimental condition. Rates of cell death were calculated by a linear curve fit of the data (see Fig. 4) and are expressed as the percentage of death/day. The
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The lowest observed linear regression $r^2$ value was 0.94, and the average value was 0.96.

Immunocytochemistry and Imaging—To visualize protein expression and localization, spinal cord cultures were fixed with 3% paraformaldehyde in PBS, pH 7.3, for 10 min and permeabilized in 0.5% Nonidet P-40/PBS for 1 min followed by additional fixation in 3% paraformaldehyde/PBS for 2 min. Immunocytochemistry with SOD-1 monoclonal antibody (1:300, clone SD-G6, Sigma-Aldrich) was carried out by incubating for 30 min using bovine serum albumin to block nonspecific binding and Alexa-Red 594-conjugated anti-mouse IgG (Molecular Probes Inc., Eugene, OR). Coverslips were mounted onto glass slides using Immumount (Fisher Scientific Company, Ottawa, ON, Canada), and images were captured using a Zeiss LSM 510 confocal microscope and compatible software (Carl Zeiss Canada Ltd., Toronto, ON, Canada).

Statistical Analysis—For the survival assays, statistical significance was determined using analysis of variance between groups (SigmaStat, Systat Software, Inc., Point Richmond, CA). Significance was established at $p \leq 0.05$. Data for percentage of motor neurons with inclusions were tested for normal distribution and then compared using a one-tailed, two-sample $t$ test for equal means under the assumption of unequal variance. A null hypothesis of equal means was rejected when $p \leq 0.05$.

RESULTS

Mass Spectral Evidence for Oxidation of Trp-32 in As-isolated SOD-1—Using immunoaffinity chromatography with antibodies raised against partially oxidized human SOD-1wt, SOD-1 was purified from human blood, and SOD-1wt and SOD-1G93A were purified from transgenic mouse blood. Intact SOD-1 was analyzed by infusion nanoelectrospray mass spectrometry, affording a survey of post-translational modifications. MS data demonstrate that SOD-1 purified from human and mouse erythrocytes was metallated and retained an intact intrasubunit disulfide. Previous studies of SOD-1G93A mettallation in the unattached organs, liver and kidney, also indicated full mettallation (31). The mass-to-charge ratio of SOD-1 was consistent with the oxidation state of the metals being Cu(II) and Zn(II) (Fig. 1) (32, 33). High resolution Fourier transform mass spectrometry (data not shown) in the presence and absence of tri-(2-carboxyethyl) phosphine (21) confirmed the presence of an intrasubunit disulfide bridge. Aside from the expected metallation and disulfide, both SOD-1wt and SOD-1G93A were further post-translationally modified by the addition of between one and six oxygen atoms per monomer (Fig. 1, insets). Oxidatively modified SOD-1 consistently represented 20–30% of total as-isolated SOD-1. Among the most prevalent modifications were SOD-1 modified by one and two oxygen atoms, representing 6 and 5% of total SOD-1, respectively.

To identify sites of oxidative modification, SOD-1 was digested with endoproteinase GluC, creating peptides that are amenable to MS/MS analysis. Peptides were separated by reverse phase liquid chromatography (Fig. 2A), their masses were measured using MS, and peptides were isolated and subjected to CAD (Fig. 2, C and D). The peptide mass and fragment masses were used to “sequence” and identify peptides using the MASCOT search engine followed by manual validation, and the full amino acid sequence of SOD-1 was accounted for. Individual peptides of SOD-1 were surveyed for oxidative modification (Fig. 2B, inset), and potentially modified peptides were sequenced in subsequent LC-MS/MS experiments. Following this procedure, Trp-32 was determined to be modified by one and two oxygen atom(s) (Fig. 2, D and E). Post-translational modification of both intact protein and Trp-32 were present in preparations from both aerobic and anaerobic conditions, indicating that oxidative modification occurred in vivo, not as an artifact from exposure to air during purification or digestion (Fig. 3).

Survival and Aggregation of SOD-1W32F/G93A—In previous studies, recombinant SOD-1 was oxidized at Trp-32 and aggregated following exposure to peroxide or superoxide in vitro (22, 25). Rates of oxidation-induced aggregation of SOD-1wt were decreased by replacing Trp-32 with Phe (25). This finding, combined with the identification, in this study, of oxidized Trp-32 in as-isolated SOD-1, provided the impetus to test whether Trp-32 plays a role in the toxicity or aggregation of mutant SOD-1 in living motor neurons. Trp-32 was replaced with Phe in SOD-1G93A and in the SOD-1wt control. The G93A mutant was chosen because it retains normal dismutase activity (34) and has been relatively well characterized. Plasmids encoding SOD-1wt, SOD-1W32F, SOD-1G93A, and SOD-1W32F/G93A were expressed in the motor neurons of long term dissociated spinal cord-dorsal root ganglia cultures (6, 29).

As previously reported (6), motor neurons expressing SOD-1G93A died at a considerably faster rate over the 10 days of observation relative to motor neurons expressing SOD-1wt (Fig. 4B). The presence of the W32F mutation significantly reduced the death of motor neurons conferred by the G93A mutation from 6.8 to 3.6%/day (Fig. 4A). In fact, viability of motor neurons expressing SOD-1 with the double mutation (W32F and G93A) did not differ significantly from the controls; SOD-1wt (3.0%/day), SOD-1W32F (4.3%/day), and SOD-1W32F/G93A (3.6%/day) survived as a group (Fig. 4C).

SOD-1wt distributed diffusely throughout the cell (6) (data not shown), whereas a proportion of motor neurons expressing SOD-1G93A contained SOD-1 immunoreactive inclusions (Fig. 5A). Not only did introduction of the W32F muta-
tion into SOD-1G93A prolong the survival of motor neurons, it also delayed aggregation of the protein into inclusions (Fig. 5, B and C).

DISCUSSION

The results presented herein indicate that oxidation of Trp-32 occurs in vivo as a natural modification to SOD-1 and that the Trp-32 residue is an important mediator of the toxicity of an SOD-1G93A mutant associated with fALS. Exchanging Trp-32 with the less oxidation-prone Phe decreased the toxicity of SOD-1G93A to motor neurons, reducing both lethality and the formation of cytoplasmic, SOD-1-containing inclusions. This study does not rule out the possibility that a property of Trp-32 other than its propensity for oxidative modification promotes toxicity of mutant SOD-1. For instance, tryptophan is among the most critical residues in the formation of protein-protein and protein-ion interactions (35–37). There are, however, a number of circumstantial arguments supporting the involvement of oxidative modification of Trp-32 in the promotion of G93A toxicity. 1) Trp-32 was found to be oxidized in vivo. 2) Trp is the most oxidizable of all of the amino acids, and Trp oxidation is associated with in vitro aggregation (23, 38). 3) Mutating Trp to Phe also slows the rate of aggregation of recombinant SOD-1 wt following oxidative modification in vitro (25). 4) Oxidative modification of Trp results in the formation of free carbonyl moieties that go on to form advanced glycation end products; carbonyl-containing as well as anionic detergent- and dithiothreitol/β-mercaptoethanol-resistant high molecular weight species of mutant SOD-1 have been detected in cultured cells (2, 3) and in both tissues and filter-purified aggregates of transgenic mice expressing mutant SOD-1 (39, 40).

Direct measurement of the relationship between Trp-32 oxidation and protein conformation in vivo would require physical separation of oxidized and unmodified SOD-1, which would be difficult to achieve. Although there is controversy over the relationship between the aggregation of mutant SOD-1 and disease-related toxicity (reviewed in Ref. 41), only treatments that reduce the formation of cytoplasmic mutant SOD-1 inclusions also prolong survival in the primary motor neuron culture model used in the present study (29, 30, 42–45). This culture system has the distinct advantage of utilizing primary motor neurons, cells with particular vulnerability to mutant SOD-1 toxicity and ALS generally (46, 47).

In previous in vitro oxidation studies, the major modifications identified by MS or amino acid analysis were oxidation of active site His residues (24, 26) or Trp-32 oxidation (22, 25). In all in vitro studies, activity and presumably copper were lost upon oxidation. In the present study, no more than 2% of SOD-1 isolated from erythrocytes was modified at His residues, and oxidatively modified SOD-1 retained its metals and presumably its activity (48).

In vitro oxidation is metal-catalyzed Fenton-type oxidation by hydroxyl radicals and may not mimic in vivo oxidation where SOD-1 is surrounded by diverse oxidants. SOD-1W32F/G93A and SOD-1W32F exhibit similar toxicity to SOD1 wt (*, p < 0.001, day 4; **, p < 0.01, day 7; ***, p < 0.0001 at days 8–10 using analysis of variance and the Holm-Sidak method of pairwise group comparison). Bonferroni and Tukey tests confirmed significance at each time point.
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A

B

C

FIGURE 5. Mutation of Trp-32 to Phe delays aggregation of SOD1G93A into inclusions in cultured motor neurons. A, a motor neuron after microinjection with a SOD-1G93A expression vector, stained with anti-SOD-1 antibodies, revealed punctate, SOD-1-positive cytoplasmic inclusions. B, the percentage of motor neurons microinjected with plasmid encoding SOD-1G93A and SOD-1W32F/G93A that contained SOD-1-positive cytoplasmic inclusions. C, the W32F mutation significantly reduced the percentage of motor neurons with mutant SOD-1 inclusions at post-microinjection day 3. There was no observable difference in the size or distribution of the aggregates observed in a given SOD-1G93A or SOD-1W32F/G93A inclusion-containing cell, although a significantly lower proportion of SOD-1W32F/G93A-injected cells contained aggregates at day 3. Normal distribution of the data allowed for weighted test comparison of data (p < 0.005, day 3). No significant difference was observed at days 5 and 7.

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