OXIDATIVE MODIFICATIONS AND AGGREGATION OF Cu/Zn SUPEROXIDE DISMUTASE ASSOCIATED WITH ALZHEIMER'S AND PARKINSON'S DISEASES*  
Joungil Choi‡, Howard D. Rees‡||, Susan T. Weintraub¶, Allan I. Levey‡||, Lih-Shen Chin‡, and Lian Li‡  
From the ‡Department of Pharmacology and of ||Neurology, Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA, 30322, and ¶Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229  
Running Title: SOD1 oxidation in neurodegenerative diseases  
Address correspondence to: Lian Li, Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322-3090, Tel: 404-727-5987; Fax: 404-727-0365; E-Mail: lianli@pharm.emory.edu

Although oxidative stress has been strongly implicated in the pathogenesis of Alzheimer's disease (AD) and Parkinson's disease (PD), the identities of specific protein targets of oxidative damage remain largely unknown. Here, we report that copper-zinc superoxide dismutase (SOD1), a key antioxidant enzyme whose mutations have been linked to autosomal dominant neurodegenerative disorder familial amyotrophic lateral sclerosis (ALS), is a major target of oxidative damage in AD and PD brains. By using a combination of two-dimensional gel electrophoresis, immunoblot analysis, and mass spectrometry, we have identified four human brain SOD1 isoforms with isoelectric point (pI) of 6.3, 6.0, 5.7, and 5.0, respectively. Of these, the SOD1 pI 6.0 isoform is oxidatively modified by carbonylation and the pI 5.0 isoform is selectively accumulated in AD and PD. Moreover, Cys-146, a cysteine residue of SOD1 which is mutated in familial ALS, is oxidized to cysteic acid in AD and PD brains. Quantitative Western blot analyses demonstrate that the total level of SOD1 isoforms is significantly increased in both AD and PD. Furthermore, immunohistochemical and double fluorescence labeling studies reveal that SOD1 forms proteinaceous aggregates that are associated with amyloid senile plaques and neurofibrillary tangles in AD brains. These findings implicate, for the first time, the involvement of oxidative damage to SOD1 in the pathogenesis of sporadic AD and PD. This work suggests that AD, PD, and ALS may share a common or overlapping pathogenic mechanism(s) which could potentially be targeted by similar therapeutic strategies.

Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease) are chronic neurodegenerative disorders characterized by selective neuronal death and the accumulation of insoluble proteinaceous deposits, such as senile plaques and neurofibrillar tangles in AD, Lewy bodies in PD, and hyaline- and skein-like inclusion bodies in ALS (1, 2). Increasing evidence indicate that oxidative stress plays a critical role in the pathogenesis of these slowly progressive neurodegenerative diseases (3-5). For example, both AD and PD have been associated with increased production of reactive oxygen species (ROS), which could result from genetic predisposition and/or environmental factors, such as exposure to pesticides (4). Postmortem analyses reveal that the overall levels of oxidative damage to proteins, lipids and DNA are elevated in AD and PD brains (6, 7). The most widely used marker for oxidative damage to proteins is the presence of carbonyl groups, which can be introduced into proteins by direct oxidation of Pro, Arg, Lys, and Thr side chains, or by Michael addition reactions with products of lipid peroxidation or glycooxidation (3). Elevation in the total level of protein carbonyls has been documented in both AD and PD (6, 7). However, the identities of the oxidized proteins modified by carbonylation or other types of oxidation remain largely unknown. Furthermore, it remains to be determined whether the protein targets of oxidative damage to proteins are identical to those identified in AD and PD.
oxidative damage are the same or are different in AD and PD.

As a first step towards a molecular understanding of the pathogenic mechanism of oxidative stress in neurodegenerative diseases, we performed a search for specific protein targets of oxidative damage in AD and PD brains by using a proteomic approach that combined two-dimensional gel electrophoresis, immunological detection of protein oxidation, and mass spectrometry (8). Here we report that copper-zinc superoxide dismutase (SOD1; E.C.1.15.1.1) is a major target of oxidative damage in AD and PD brains. SOD1 is a key antioxidant enzyme that catalyses the disproportionation of superoxide radicals into molecular oxygen and hydrogen peroxide, which is then decomposed by catalase and glutathione peroxidase (9). Mutations in SOD1 have been identified as the cause for autosomal dominant familial form of ALS, a neurodegenerative disorder characterized by the loss of motor neurons in the spinal cord and brain (10-12). However, little is currently known about the role of SOD1 in the more common, sporadic form of ALS, such as AD and PD. In the present study, we investigated the changes in the expression, localization, and oxidative modifications of SOD1 in the brains of patients with idiopathic AD or PD. Our results suggest that oxidative damage to and aggregation of SOD1 plays a crucial role in the neurodegeneration associated with AD and PD.

Materials and Methods

Human Brain Samples—Frontal cortex tissues from five clinically diagnosed PD, five AD, and five healthy non-demented control subjects (Table 1) were obtained from the Emory Alzheimer’s Disease Center brain bank. The neuropathological diagnosis of PD was based on the presence of nigral degeneration and Lewy bodies. The diagnosis of AD was established using CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) criteria (13). ApoE genotypes (Table 1) were determined for all subjects as previously described (14).

Sample Preparation, DNP-derivatization and Two-dimensional Gel Electrophoresis—Brain tissues were homogenized in a buffer containing 50 mM Tris-HCl and protease inhibitors (1 mM PMSF, 0.5 mg/ml Benzamidine, 1 µM Aprotinin, 10 µM Leupeptin, 1 µM Pepstatin A, and 1 µM Bestatin), followed by centrifugation at 13,000 X g. Protein samples (350 µg) were applied to 17-cm immobilized pH gradient (IPG) strips (pH 4-7) in an IPG re-swelling tray, and the strips were then isoelectrically focused on a Protein IEF Cell (Bio Rad, Hercules, CA) for 24,000 V-hr (15). Following isoelectric focusing, the strips were immediately subjected to in-strip DNP-derivatization as described previously (16) by 15-min incubation in 2 N HCl/10 mM DNPH at 25 °C. After being washed with 2 M Tris-base/30 % (v/v) glyceral for 15 min, the strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glyceral, and 1.0 % (w/v) dithiothreitol. The strips were then re-equilibrated for 15 min in the same buffer containing 2.5 % (w/v) iodoacetaamide instead of dithiothreitol. Second-dimension separation was performed on SDS-polyacrylamide gradient gels (10-20% porosity polyacrylamide) using the Ettan-DALT slab gel SDS-PAGE system (Amersham Biosciences, Piscataway, NJ).

SYPRO Ruby Staining—Duplicate samples of brain proteins were subjected to two-dimensional gel electrophoresis as described above. Proteins in one gel were stained with SYPRO Ruby protein gel stain (Bio Rad, Hercules, CA), while proteins in the other gel were electroblotted to polyvinylidene difluoride (PVDF) membranes using the Ettan-DALT system. For SYPRO Ruby staining, proteins were first fixed in the gel using 40% methanol/10% acetic acid (v/v) for 30 min. The gel was then incubated in SYPRO Ruby protein gel stain (Bio Rad, Hercules, CA), while proteins in the other gel were electroblotted to polyvinylidene difluoride (PVDF) membranes using the Ettan-DALT system. For SYPRO Ruby staining, proteins were first fixed in the gel using 40% methanol/10% acetic acid (v/v) for 30 min. The SYPRO Ruby stained gel was placed in a light-tight cabinet directly on a transilluminator, and the fluorescence generated by excitation with UV light at 365 nm was recorded using a cooled, computerized CCD camera-based imaging system (Alpha Innotech, San Leandro, CA).

Immunoblotting and Image Analyses—The PVDF membranes were removed from the Ettan-DALT electrophoretic apparatus and incubated for 1 hr with phosphate-buffered saline containing 3% (v/v) Tween and 5% (w/v) non-fat dried skim milk (PBS-TM). Membranes were incubated overnight.
at 4 °C with anti-DNP primary antibody (Molecular Probes, Eugene, OR) at 1:16,000 dilution or with anti-SOD1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:2000 dilution as described previously (17). After extensive washing with PBS-TM, the membranes were then incubated for 1 hr at 4 °C with a 1:16,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma, St. Louis, MO). Immunostained proteins were detected by using a SuperSignal chemiluminescence kit (Pierce, Rockford, IL) and an Alpha Innotech imaging system. Digitized images from SYPRO Ruby-stained gels and immunoblots were analyzed using the 2-DE gel analysis program PD Quest (Bio Rad, Hercules, CA). The data were analyzed statistically by ANOVA.

Mass Spectrometry—Spots of interest were excised from the gels and digested in situ with trypsin (modified; Promega, Madison, WI). The resulting digests were subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF /MS) and capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI/MS/MS). MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager-DE STR. The peptide mass maps produced by MALDI-TOF /MS were searched against the published databases by means of Mascot (Matrix Science) and the MS Fit component of Protein Prospector (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Electrospray ionization mass spectra were acquired on a Finnigan LCQ ion trap mass spectrometer adapted for microspray ionization sample introduction. On-line HPLC separation of the tryptic peptides was accomplished with a Michrom MAGIC 2002 micro HPLC: column, PicoFrit™ (New Objective; 75 µm i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MSB5; 5 µm, 300 Å); mobile phase A, 0.5% acetic acid/0.005% TFA; mobile phase B, 90% acetonitrile/0.5% acetic acid/0.005% trifluoroacetic acid; linear gradient of 2 to 72% B in 30 min; flow rate, 0.4 µl/min. As a part of the data-dependent acquisition protocol, the four most intense ions in each survey scan were sequentially fragmented in the ion trap by collision-induced dissociation using an isolation width of 2.5 and a relative collision energy of 35%. Uninterpreted MS/MS spectra were analyzed by the SEQUEST component of the LCQ software and by Mascot (Matrix Science). Assignment of the MS/MS fragments was verified by comparison with the predicted ions generated in silico by GPMAW (Lighthouse Data).

Immunohistochemistry—Brains were fixed in 4% paraformaldehyde, cryoprotected in sucrose, and serial sections (50 µm) of cingulate cortex were cut on a cryostat. Sections were treated with 3% H2O2 to eliminate endogenous peroxidase activity and blocked with a solution containing 8% goat serum, 0.1% Triton X-100, 0.9% NaCl, and 50 mM Tris-HCl (pH 7.2). Sections were then incubated overnight at 4 °C with mouse monoclonal anti-SOD1 antibody (0.5 µg/ml; BD Biosciences) or monoclonal anti-Aβ antibody (4G8; 1:5000; Signet) followed by incubation with biotinylated secondary antibody (1:200). Bound antibodies were detected using a standard avidin-biotinylated peroxidase complex (ABC) method (Vectastain Elite ABC kit, Vector Laboratories). Sections were observed using a Leica DC500 microscope, and images were captured with a C4742-95 Hamamatsu digital camera.

Double Fluorescence Labeling Confocal Microscopy—Brain tissues from patients with Alzheimer’s disease were fixed in 4% paraformaldehyde for 2-3 days. Blocks of cingulate cortex were cryoprotected in 30% sucrose and frozen sections were cut on a sliding microtome at 50 µm. Sections were rinsed in phosphate-buffered saline, treated with 3% H2O2, and blocked with normal horse serum (8%), avidin (10 µg/ml), and Triton X-100 (0.1%) in Tris-buffered saline (TBS) for 1 hr before incubation with mouse monoclonal antibody to SOD1 (1 µg/ml; BD Biosciences) in TBS containing 50 µg/ml biotin for 2 days at 4°C. Following TBS rinses, sections were incubated with biotinylated goat anti-mouse secondary antibody (Vector Laboratories, 1:200), followed by detection using the ABC method. Signal amplification was achieved using tyramide-Cy3 reagent (Perkin-Elmer). Sections were counterstained with 1% thioflavin-S (Sigma-Aldrich) for 20 min, rinsed in 70% ethanol, and coverslipped. Images were acquired using a Zeiss LSM510 Meta confocal microscope.
RESULTS

Molecular Characterization of Altered Protein Oxidative Modification in AD and PD Brains—To investigate alterations in protein oxidation associated with AD and PD, we performed comparative high-resolution, two-dimensional gel electrophoresis experiments on protein samples obtained from AD and PD brains and age-matched controls (Table 1). Brain protein extracts were resolved by isoelectric focusing on an immobilized-pH-gradient strip followed by in-strip DNP derivatization (reacting with protein carbonyls) and second-dimensional separation by SDS-PAGE. Total proteins in the 2-D gels were visualized by staining with SYPRO Ruby, and oxidatively modified proteins were detected by immunoblotting with anti-DNP antibody. Comparison of anti-DNP immunoblots with SYPRO Ruby-stained 2-D gels revealed that a protein spot which electrophoresed on the 2-D gel with apparent molecular mass (Mr)/isoelectric point (pI) values of 16 kDa/6.0 exhibited enhanced oxidation in AD and PD brains (Fig. 1). Quantification results showed that the specific oxidation level of this spot was increased approximately 7.5-fold in AD and 9-fold in PD compared to age-matched control brains (Table 2).

Identification of SOD1 as a Major Target of Oxidative Damage in AD and PD Brains by Mass Spectrometry—For determination of the identity of the oxidized protein spot indicated in Fig. 1, the spot was excised from the 2-D gels and its identity was determined by mass spectrometric analysis. The spot was unambiguously identified as human Cu/Zn superoxide dismutase (SOD1, Accession Number NP_000445; Swiss-Prot P00441) by using HPLC-electrospray ionization tandem mass spectrometry (Fig. 2). This assignment is further strengthened by the agreement between the apparent Mr/pI values of 16 kDa/6.0 exhibited enhanced oxidation in AD and PD brains (Fig. 1). Quantification results showed that the specific oxidation level of this spot was increased approximately 7.5-fold in AD and 9-fold in PD compared to age-matched control brains (Table 2).

Immunoblot Analysis Reveals the Presence of Four Brain SOD1 Isoforms and Confirms the Identified Oxidized Protein as the SOD1 pI 6.0 Isoform—To confirm the protein identification result obtained by mass spectrometry, we performed 2-D gel electrophoresis of human brain samples followed by immunoblotting with a rabbit polyclonal anti-SOD1 antibody. As shown in Fig. 3, anti-SOD1 antibody specifically recognized four distinct protein spots with the same molecular mass (16 kDa) and different isoelectric points (pI = 6.3, 6.0, 5.7, and 5.0, respectively), demonstrating the presence of four SOD1 isoforms in human brain. Of these four SOD1 spots, only one spot (pI = 6.0) was immunoreactive to anti-DNP antibody, indicating that the pI 6.0 isoform of SOD1 was oxidatively modified by carbonyl formation (Fig. 3A and B). The specific oxidation of the 16 kDa/pI 6.0 SOD1 spot is consistent with the results described in Fig. 1.

The SOD1 pI 5.0 Isoform Is Selectively Accumulated in AD and PD Brains—We next examined whether or not the four SOD1 isoforms were differentially expressed in AD, PD, and control brains by two-dimensional immunoblot analysis (Fig. 4, A-C). Quantification results (Fig. 4D) showed a more than 6-fold increase in the relative level of the pI 5.0 isoform of SOD1 in AD and PD compared with age-matched controls. The different isoelectric points of the SOD1 isoforms (Fig. 4) suggest the presence of some types of post-translational modification that alter the charge of SOD1 protein. To test this possibility, the four different pI spots of SOD1 were individually excised from the 2-D gels and their tryptic digests were analyzed by MALDI-TOF/MS/MS and HPLC-ESI/MS/MS. Despite repeated attempts, efforts at identifying the post-translational modification that causes the pI shifts of SOD1 have so far been unsuccessful.

The Total Level of SOD1 Protein Is Increased in AD and PD Brains—Our two-dimensional immunoblotting data (Fig. 4) suggest that the total level of all four SOD1 isoforms is increased in AD and PD brains. To further investigate this possibility, we performed quantitative Western blot analysis using protein samples obtained from AD and PD brains and age-matched controls (Table 1). The fact that all four SOD1 isoforms have the same molecular mass (Fig. 3) allowed us to use one-dimensional gel electrophoresis for direct comparison of the total SOD1 levels of various brain samples on the same
gel. After electrophoresis, the gels were subjected to immunoblotting with antibodies against SOD1 and actin. The relative level of SOD1 in each sample was measured by quantification of the intensity of the SOD1 band and normalized to the actin level in the corresponding sample (Fig. 5). The results indicated that the total level of SOD1 was increased 2.4-fold in PD and 2.5-fold in AD brains compared to controls.

**Accumulation of SOD1 protein aggregates in AD Brains**—Oxidative modification may convert proteins into forms that are more prone to aggregation and/or more resistant to proteolytic degradation, resulting in the formation of protein aggregates (18). Because our biochemical data revealed that SOD1 was significantly oxidized and accumulated in AD and PD brains, we used immunohistochemistry to investigate whether SOD1 aggregates in these diseases. In control human brains, intense SOD1 immunoreactivity was observed in pyramidal neurons in addition to moderately stained neurons and neuropilis throughout the neocortex (Fig. 6A). Strong SOD1 immunostaining was also found in astrocytes in white matter (Fig. 6B). PD brains exhibited similar SOD1 immunostaining patterns compared to the controls (data not shown). However, aggregation of SOD1 protein was occasionally seen in the cytoplasm of some neurons (Fig. 6C), although we did not detect SOD1 immunoreactivity in Lewy bodies of PD brains (data not shown). In AD brains, prominent SOD1 immunoreactivity was observed in aggregates with granular or globular appearances (Fig. 6D and E). Many of these SOD1-positive deposits had diameters similar to amyloid plaques labeled by anti-Aβ antibody in adjacent brain sections (Fig. 6F).

**SOD1 Aggregates Are Associated with Senile Plaques and Neurofibrillary Tangles in AD Brains**—To further characterize SOD1 aggregates, we performed double fluorescence labeling experiments using anti-SOD1 antibody and thioflavin S, a widely used dye that binds to fibrillar, β-pleated sheet structures. As shown in Fig. 7 and 8, there was very little overlap between SOD1 immunostaining and thioflavin S labeling, suggesting that SOD1 aggregates *per se* are amorphous protein deposits with little fibrillar, β-pleated sheet content. However, SOD1 aggregates were closely associated with thioflavin S-positive amyloid plaques (Fig. 7) and neurofibrillary tangles and neuropil threads (Fig. 8).

**DISCUSSION**

SOD1 is a ubiquitously expressed antioxidant enzyme that plays a key role in the cellular defense against harmful superoxide radicals (9, 19). The importance of SOD1 in neurodegeneration was first discovered by the identification of more than 100 different point mutations in SOD1 as causative genetic defects for familial ALS (20). In this study, we found that SOD1 is one of the major targets of oxidative damage in AD and PD brains and provided evidence supporting the involvement of oxidative damage to and aggregation of SOD1 in the two common neurodegenerative disorders AD and PD.

By using a combination of two-dimensional gel electrophoresis, immunoblot analysis, and mass spectrometry, we have identified four human brain SOD1 isoforms with isoelectric point (pI) of 6.3, 6.0, 5.7, and 5.0, respectively. The chemical nature of the modifications responsible for the differences in the pI value of the SOD1 isoforms remains to be determined. It is possible that some of the SOD1 pI isoforms represents differentially metallated forms of SOD1. SOD1 is a homodimeric metalloprotein containing one Cu (II) and one Zn (II) ion per subunit (19). SOD1 protein is known to first fold into a dimeric apo form and then acquires Cu (II) from the copper chaperone for SOD1 (21). Although the mechanism of the Zn (II) acquisition is yet unknown, Zn-deficient wild type and mutant SOD1 have been implicated in ALS (19, 22). The predicted pI of SOD1 in its metallated holo form, partially metallated form (Cu-free or Zn-free state), and metal-free apo form is 6.4, 6.0, and 5.7, respectively. We observed that in the control human brains, SOD1 exists mainly in three isoforms (pI = 6.3, 6.0, and 5.7), of which the pI 6.0 and pI 5.7 isoform are most abundant, accounting for about 55% and 35% of total SOD1 protein, respectively. The observed pI 6.0 isoform is likely to be the metallated holo form of SOD1, whereas the pI 5.7 isoform may represent the partially metallated form. Consistent with this assumption, copper incorporation experiments revealed that the Cu-free form of SOD1...
constitutes ~35% of the total SOD1 in human lymphoblasts (23).

Our data showed no significant change in the relative levels of pI 6.3, pI 6.0, and pI 5.7 isoforms of SOD1 in AD and PD brains compared with age-matched controls. In contrast, the level of the more acidic SOD1 isoform (pI = 5.0) is increased more than 6 folds in AD and PD brains. The selective accumulation of the acidic SOD1 pI isoform is analogous to the acidic pI shift of familial PD-associated DJ-1 protein in response to oxidative stress (24, 25). In the case of DJ-1, the acid pI shift is thought to result from direct oxidation of DJ-1 by H₂O₂, leading to the formation of cysteine sulfenic or sulfonic acid (24, 25). A similar oxidative modification of SOD1 might be responsible for the accumulation of the acidic SOD1 pI 5.0 isoform in AD and PD brains.

Our proteomic results indicated that the SOD1 pI 6.0 isoform, but not the other three SOD1 isoforms (pI = 6.3, 5.7, and 5.0), is heavily oxidized in AD and PD brains by carbonylation, an irreversible oxidative modification that is widely used as a marker of oxidative damage (3). A 7.5 to 9-fold increase in the specific oxidation level of the SOD1 pI 6.0 isoform was observed in AD and PD compared to age-matched control brains, providing the first direct evidence for occurrence of oxidative damage to SOD1 in sporadic AD and PD. In addition, we found that Cys-146 of the SOD1 pI 6.0 isoform is oxidized irreversibly to cysteine sulfonic acid in AD and PD brains. Cysteine contains a thiol group (Cys-SH) that can be reversibly oxidized to a sulfenic acid (Cys-SOH) or sulfonic acid (Cys-SO₂H) by low concentrations of H₂O₂ and irreversibly oxidized to a sulfonic acid (Cys-SO₃H) by high concentrations of H₂O₂ (26-28). The identified irreversible cysteine oxidation of SOD1 is consistent with previous in vitro studies showing that SOD1 undergoes oxidative damage by its own reaction product H₂O₂ (29, 30). The identified irreversible cysteine oxidation of SOD1 is consistent with previous in vitro studies showing that SOD1 undergoes oxidative damage by its own reaction product H₂O₂ (29, 30). The vulnerability of SOD1 to H₂O₂-mediated oxidative damage underscores the importance of H₂O₂ clearance by catalase and peroxidases. Elevated levels of H₂O₂ in the brain have been implicated in AD and PD (6). It has been shown that β-amyloid (Aβ) peptide can directly catalyze the production of H₂O₂ in vitro, suggesting that Aβ deposits may be a source of H₂O₂ in AD brain (31, 32). The increased H₂O₂ levels in AD and PD may overwhelm the cellular H₂O₂ defense systems, leading to various oxidative products, including oxidized SOD1.

Our findings raise a possibility that the identified oxidative modifications of SOD1 might contribute to the pathogenesis of sporadic AD and PD in a manner similar to the genetic mutations of SOD1 in causing neurodegeneration in familial ALS. In agreement with this possibility, Cys-146, a cysteine residue which we found to be irreversibly oxidized in AD and PD brains, is mutated to an arginine (Cys146Arg) in familial ALS (33). Ample evidence indicates that SOD1 mutations cause neurodegeneration by a gain of cytotoxic function, rather than a loss of enzymatic activity (19, 20). Transgenic mice expressing familial ALS-associated mutant SOD1 develop severe motor neuron degeneration (34), whereas neither transgenic mice expressing the human wild-type SOD1 (35) nor SOD1 knockout mice (36) exhibit such ALS-like phenotype. The mechanism by which SOD1 mutants cause neurodegeneration is not understood, but it has been suggested that the toxic gain of function is a consequence of mutation-induced misfolding of SOD1, which leads to the formation of cytotoxic SOD1 aggregates (11, 19). The oxidative modifications of SOD1 identified in AD and PD brains may induce misfolding and/or protease resistance of SOD1, thereby promoting the accumulation and aggregation of SOD1. In support of this possibility, we found that the total level of SOD1 protein is significantly increased in both AD and PD. Moreover, we observed the presence of prominent SOD1-immunoreactive protein aggregates in AD brains. These aggregates are unlikely to be non-specific staining artifacts of the anti-SOD1 antibody because similar SOD1 aggregates have been observed in AD brains using different anti-SOD1 antibodies (37). Although we were unable to detect SOD1 immunostaining in Lewy bodies in our PD samples, the previously reported association of SOD1 with Lewy bodies (38) is consistent with our hypothesis.

We have further characterized SOD1 aggregates in AD brains by double fluorescence labeling confocal microscopy and found that these aggregates are not stained with thioflavin S, indicating that SOD1 aggregates are not fibrillar amyloid deposits. The granular morphology and non-fibrillar nature of SOD1 aggregates in AD
brains resemble the SOD1-positive inclusion bodies found in both familial and sporadic forms of ALS (12, 39, 40). In AD brains, we found that SOD1 aggregates are closely associated with amyloid senile plaques and neurofibrillary tangles. The co-occurrence of SOD1 aggregates with the hallmark lesions of AD and PD suggests a common or overlapping mechanism(s) underlying the formation of pathological inclusions in AD, PD, and ALS.

Although the genetic defects underlying some of the rare familial forms of AD, PD, and ALS have recently been identified, the causes of the common, sporadic forms of these diseases remain unclear. Our work suggests that oxidative damage to and subsequent aggregation of SOD1 may contribute to the neurodegeneration associated with AD and PD. It would be worthwhile to determine if the identified oxidative modifications of SOD1 also take place in sporadic ALS. The overlapping pathogenic mechanisms of these neurodegenerative diseases raise an exciting possibility that similar potential therapeutic strategies might be utilized for treating these disorders.

REFERENCES

FOOTNOTES

* We thank Christopher A. Carroll of the Institutional Mass Spectrometry Laboratory at the University of Texas Health Science Center at San Antonio (supported in part by National Institutes of Health Grant CA54174) for mass spectrometric analyses. This work was supported by grants from National Institutes of Health (AG021489 and NS047575 to L.L.), the Emory University Research Committee and the Emory Collaborative Center (ES012068) for Environmental Research on Parkinson's Disease (to L.-S.C.), and by the Emory Center for Neurodegenerative Disease-Merck Scholar Award (to L.L.).

1 The abbreviations used are: AD, Alzheimer's disease; PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenyl; PVDF, polyvinylidene difluoride; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; ROS, reactive oxygen species; 2-DE, two-dimensional electrophoresis; IPG, immobilized pH gradient; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ESI/MS/MS, electrospray ionization tandem mass spectrometry.
Fig. 1. Oxidation of specific proteins in AD and PD brains. Protein samples of control (A), PD (B), and AD (C) brains (350 µg) were subjected to 2-D gel electrophoresis, followed by SYPRO Ruby staining for total proteins (left panel) or by immunostaining with anti-DNP antibody for oxidized proteins (right panel). Arrow indicates a protein spot with apparent molecular mass/pl values of 16 kDa/6.0 that exhibits elevated oxidation in both AD and PD.

Fig. 2. Identification of the oxidized protein spot in Fig. 1 as SOD1 by mass spectrometry. The protein spot indicated in Fig. 1 was excised from the 2-D gels and analyzed by HPLC-ESI tandem mass spectrometry. Collision-induced dissociation mass spectrum of the ion at m/z 992.0 (1+) in AD and PD brains indicates the identity of this ion as SOD1 peptide 144-153. Peptide fragments are indicated using the nomenclature of Roepstorff and Fohlman (41). C*, cysteic acid.

Fig. 3. Identification of four SOD1 isoforms and confirmation of the identified oxidized protein as the SOD1 pI 6.0 isoform. Protein extracts (350 µg) from a PD brain were subjected to 2-D gel electrophoresis, followed by immunoblotting with anti-DNP antibody (A) or with anti-SOD1 antibody (B). The result shows four SOD1 isoforms with different pI values. The oxidized protein spot identified by mass spectrometry as SOD1 represents the pI 6.0 isoform of SOD1.

Fig. 4. Accumulation of SOD1 pI 5.0 isoform in AD and PD brains. Protein samples of control (A), PD (B), and AD (C) brains were subjected to 2-D gel electrophoresis, followed by immunoblotting with anti-SOD1 antibody. The relative level of each SOD1 isoform was measured by quantification of the intensity of the individual SOD1 isoform using the 2-D gel analysis program PD Quest, and expressed as a percentage of the total level of all four SOD1 isoforms. Values represent mean ± SEM for 5 PD, 5 AD, and 5 control individuals. The asterisk indicates statistically significant (p < 0.05) increase in the level of SOD1 pI 5.0 isoform in PD or AD versus control.

Fig. 5. The total level of SOD1 protein is increased in AD and PD brains. A. Protein extracts of control, PD, and AD brains were subjected to one-dimensional gel electrophoresis, followed by immunoblotting with anti-SOD1 antibody. Each lane represents a different individual from the control, PD, and AD groups. B. The relative SOD1 level was measured by quantification of the intensity of the 16-kDa SOD1 band and normalized to the actin level in the corresponding brain extract. Bar graph shows the results (mean ± SEM) from 5 PD, 5 AD, and 5 control individuals. The asterisk indicates statistically significant (p < 0.05) increase in the total level of SOD1 protein in PD or AD versus control.

Fig. 6. Immunohistochemical analysis of SOD1 expression in PD, AD, and control brains. Sections of cingulate cortical regions from control (A and B), PD (C) or AD (D-F) brains were immunostained with antibodies against SOD1 (A-E) or Aβ (F). Scale bar = 20 µm in (A), (B), and (D); 10 µm in (C), (E), and (F).

Fig. 7. Association of SOD1 aggregates with senile plaques in AD brains. Sections of cingulate cortical regions from AD brains were stained with anti-SOD1 antibody (A and D) and thioflavin-S (B and E). Images were obtained by confocal microscopy. Superimposed images (C and F) revealed the association of SOD1 aggregates with thioflavin S-positive amyloid plaques.

Fig. 8. Association of SOD1 aggregates with neurofibrillary tangles in AD brains. Sections of cingulate cortical regions from AD brains were stained with anti-SOD1 antibody (A and D) and thioflavin-S (B and
Images were obtained by confocal microscopy. Superimposed images (C and F) revealed the association of SOD1 aggregates with thioflavin S-positive amyloid plaques. Scale bar = 10 μm.
### Table 1. Demographic data of human subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Sex</th>
<th>ApoE genotype</th>
<th>PMI (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>76.0 ± 11.0</td>
<td>1</td>
<td>4</td>
<td>2/3 (2), 3/3 (3)</td>
</tr>
<tr>
<td>PD</td>
<td>72.5 ± 6.5</td>
<td>5</td>
<td>0</td>
<td>2/3 (2), 3/3 (3)</td>
</tr>
<tr>
<td>AD</td>
<td>70.5 ± 20.5</td>
<td>1</td>
<td>4</td>
<td>3/3 (2), 3/4 (2), 4/4 (1)</td>
</tr>
</tbody>
</table>

1 Values represent mean ± SD.
2 The number of subjects of each genotype is given in parenthesis.
3 Postmortem interval (PMI) values represent mean ± SD.

**TABLE 1**
Table 2. Increased oxidation of the 16 kDa / pI 6.0 protein spot in PD and AD brains compared to controls

<table>
<thead>
<tr>
<th>Samples (n = 5)</th>
<th>Specific oxidation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>PD</td>
<td>44.2 ± 8.9*</td>
</tr>
<tr>
<td>AD</td>
<td>36.5 ± 6.0*</td>
</tr>
</tbody>
</table>

The specific oxidation index of spot in Fig. 1 was obtained by the normalization of the intensity of the anti-DNP immunostain to the intensity of the protein stain. Values represent mean ± SEM for 5 individuals of each of the PD, AD, or control group. *p < 0.05

TABLE 2
FIGURE 1
FIGURE 2
Figure 4
FIGURE 5
Oxidative modifications and aggregation of Cu/Zn superoxide dismutase associated with Alzheimer’s and Parkinson’s diseases

Joungil Choi, Howard D. Rees, Susan T. Weinraub, Allan I. Levey, Lih-Shen Chin and Lian Li

J. Biol. Chem. published online January 19, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M414327200

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

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/01/19/jbc.M414327200.citation.full.html#ref-list-1