

Neurobiology

β -Amyloid Mediated Nitration of Manganese Superoxide Dismutase

Implication for Oxidative Stress in a APP^{NLh/NLh} X PS-1^{P264L/P264L} Double Knock-In Mouse Model of Alzheimer's Disease

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Alzheimer's disease is a multifactorial, progressive, age-related neurodegenerative disease. In familial Alzheimer's disease, A β is excessively produced and deposited because of mutations in the amyloid precursor protein, presenilin-1, and presenilin-2 genes. Here, we generated a double homozygous knock-in mouse model that incorporates the Swedish familial Alzheimer's disease mutations and converts mouse A β to the human sequence in amyloid precursor protein and had the P264L familial Alzheimer's disease mutation in presenilin-1. We observed A β deposition in double knock-in mice beginning at 6 months as well as an increase in the levels of insoluble A β 1-40/1-42. Brain homogenates from 3-, 6-, 9-, 12-, and 14-month-old mice showed that protein levels of manganese superoxide dismutase (MnSOD) were unchanged in the double knock-in mice compared to controls. Genotype-associated increases in nitrotyrosine levels were observed. Protein immunoprecipitation revealed MnSOD as a target of this nitration. Although the levels of MnSOD protein did not change, MnSOD activity and mitochondrial respiration decreased in knock-in mice, suggesting compromised mitochondrial function. The compromised activity of

MnSOD, a primary antioxidant enzyme protecting mitochondria, may explain mitochondrial dysfunction and provide the missing link between A β -induced oxidative stress and Alzheimer's disease. (*Am J Pathol* 2006, 168:1608–1618; DOI: 10.2353/ajpath.2006.051223)

Alzheimer's disease (AD) is a multifactorial, progressive age-related neurodegenerative disease that affects more than four million persons in the United States.¹ The pathological hallmarks of AD are extracellular A β deposits, neurofibrillary tangles, synaptic loss, and neuronal degeneration. A β plaques are composed of 40- and 42-mer peptides (A β 1-40 and A β 1-42) that are proteolytically produced from amyloid precursor protein (APP).² Three genes, presenilin 1 (PS-1), PS-2, and APP are causatively linked with the pathogenesis of early onset familial AD (FAD). The findings that A β is central to the pathogenesis of AD³ and that the AD brain is under significant oxidative stress were united into a comprehensive model for neurodegeneration in the brain in AD based on A β -associated free radical generation.⁴ The brain in AD has marked oxidative damage as manifested by increased protein oxidation, lipid peroxidation, free radical formation,⁴ 3-nitrotyrosine (3-NT) levels,^{5,6} advanced glycation end products,⁷ and DNA/RNA oxidation products.^{8,9}

Manganese superoxide dismutase (MnSOD) is a homotetramer consisting of identical 24-kd subunits. The translated precursor in cytosol contains an N-terminal 24-amino acid sequence signaling mitochondrial com-

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partmentalization. The mature protein protects the cells against cytotoxic O_2^- . The importance of this enzyme is evident from MnSOD knockout mice that suffer from a defect of mitochondrial iron-sulfur centers, a modification proving lethal to newborns.^{10,11} Activity of this antioxidant enzyme declines in the aging process.¹² Because the AD brain is under intense oxidative stress, any dysfunction of MnSOD may lead to progression of the disease.

Peroxynitrite anion ($ONOO^-$) is a potent biological oxidant that has been implicated in diverse forms of free radical-induced tissue injury.¹³ Peroxynitrite is produced by the reaction of O_2^- and $\cdot NO$, and this peroxynitrite can compromise the functional and/or structural integrity of target proteins.¹⁴ Increased levels of nitrotyrosine⁵ and 4-hydroxynonenal¹⁵ are associated with degenerating neurons in AD, suggesting pathogenic roles of peroxynitrite and membrane lipid peroxidation in this disease.

Mutations in the APP gene and PS-1 gene lead to increased levels of $A\beta$, which appear to contribute to the disease process. One study demonstrated altered levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ distinguish AD from normal aging.¹⁶ Recently, several transgenic animals have been shown to have increased $A\beta$ peptide deposition and some of the pathological characteristics similar to AD patients.^{17,18} Crossing APP-Tg mutant mice and PS-1-Tg mutant mice resulted in increased $A\beta$ production and accelerated amyloid deposition in the brains of these animals.^{19,20} However, increased $A\beta$ levels in these models may, in part, be attributable to an increase in copy number of the transgene.

The aim of this study was to use a mouse model that resembles the natural progression of $A\beta$ pathology similar to that observed in AD patients and to gain insight into potential causes of the mitochondrial alterations that occur during the progression of AD. The model that we used is a homozygous knock-in APP^{NLh/NLh} \times PS-1^{P264L/P264L} (APP/PS-1).^{21,22} The results presented here demonstrate that these mice have an age-dependent accumulation of $A\beta$ in the brain, consistent with an increase in both $A\beta_{1-40}$ and $A\beta_{1-42}$ and an accelerated decline in mitochondrial function associated with a decrease in activity of MnSOD because of nitration. These results suggest a novel $A\beta$ -mediated nitrative inactivation of MnSOD and inhibition of mitochondrial function in AD.

Materials and Methods

Mutant Mouse Lines

APP^{NLh/NLh} \times PS-1^{P264L/P264L} mutant mice were generated using the Cre-lox knock-in technology.^{21,22} The APP strategy introduced the Swedish FADK670N/M671L mutations and changed the mouse sequence for $A\beta$ to be identical to the human sequence (NLh). These mice demonstrate proper cleavage of the APP protein to generate the $A\beta$ peptide. The PS-1 mutation targeted codons 264 and 265 of the mouse PS-1 gene to introduce the proline to leucine (P264L) mutation. When PS-1 mutant mice (P264L) are crossed with the mutant APP mice (NLh), the mutations are driven by the endogenous promoters of

the APP and PS-1 genes, and expression is limited to the replacement of these two endogenous genes and not by the expression of multiple transgenes.

Genotyping of Mice

APP/PS-1 mice were maintained on a CD-1/129 background. Wild-type (WT) mice were obtained from heterozygous APP/PS-1 matings and maintained as a separate line for use as controls. The APP/PS-1 mutant mice were monitored for maintenance of the knock-in gene by PCR analysis of tail snip DNA.²¹ The APP NLh mutation was screened with primers spanning the loxp sequence in intron 15 of the targeted locus (5'-CACACCAAGAAGTACAATAGAGGG-3' and 5'-CCTGGGTTGTAGGGACTGTACTTG-3') (Invitrogen, Carlsbad, CA). WT mice showed a single band at 214 bp, whereas the homozygous mutant mice had a single band at 298 bp. The PS-1 P264L mutation was identified using primers spanning exon 8 (5'-CCCGTGGAGGTCAGAAGTCAG-3' and 5'-TTACGGGTTGAGCCATGAATG-3')²² (Invitrogen). WT mice showed a single band at 142 bp, whereas the homozygous PS-1 knock-in mutant mice showed a single band at 219 bp. The mice used in all experiments were either WT or homozygous for APP/PS-1 mutation.

Immunocytochemistry for $A\beta$

To determine the deposition of $A\beta$ in APP/PS-1 mice, one brain hemisphere from 3-, 6-, 9-, 12-, and 14-month-old mice was fixed in 4% formaldehyde, processed in the standard manner, embedded in paraffin, and sectioned at 10- μ m thickness. The sections were deparaffinized, hydrated, and immersed in 88% formic acid for 3 minutes and washed in distilled water. After blocking with 15% filtered horse serum in automation buffer (Biomedica Corp., Foster City, CA) for 1 hour at room temperature, the sections were immunostained with 10D-5 monoclonal antibody (1:100) (NCL-B-amyloid) and a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA). The sections were developed using an ABC reagent kit (Vector Laboratories) and counterstained with hematoxylin.

Tissue Preparation for $A\beta$ Enzyme-Linked Immunosorbent Assay (ELISA)

Cerebral cortices were serially extracted with Tris-buffered saline, pH 7.4, followed by RIPA buffer [0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 5 mmol/L ethylenediaminetetraacetic acid in Tris-buffered saline] and finally in 70% formic acid. Protease inhibitor cocktail (pepstatin A, leupeptin, TPCK, TLCK, soybean trypsin inhibitor, 1 μ g/ml of each in 5 mmol/L ethylenediaminetetraacetic acid) was added during Tris-buffered saline and RIPA extraction. To measure the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, the super-

natant obtained from the SDS extraction step was analyzed by ELISA.

Sandwich ELISA to Determine the Levels of Insoluble Brain A β 1-40 and A β 1-42

The sandwich ELISA used here was performed as described previously,²³⁻²⁵ and the absorbencies falling within the standard curve for each assay were converted to pmol. The sandwich ELISA consisted of a capture monoclonal antibody (mAb, BAN50) that was specific for the first 10 amino acids in A β and was used in conjunction with one of two different detection antibodies, BA27 mAb specific for A β species ending at amino acid 40 or BC05 mAb specific for A β species ending at position 42(43). This ELISA had a detection limit of 3 to 6 fmol/well for A β 1-40 and 1-42/43. The mAbs BAN50, BA27, and BC05 were prepared as described previously, and each ELISA result was normalized for the dilution and tissue weight (pmol/ μ g wet tissue).

Mitochondrial Isolation

Mice were euthanized; brains (excluding cerebellum) of three mice from each age group were pooled, homogenized in 5 ml of ice-cold mitochondrial isolation buffer containing 0.225 mol/L D-mannitol, 0.075 mol/L sucrose, 20 mmol/L HEPES, 1 mmol/L EGTA, and 1% bovine serum albumin, pH 7.2, in a Dounce homogenizer with a glass pestle. The homogenized brains were then diluted with isolation buffer to a final volume of 10 ml, centrifuged at 1500 \times *g* for 5 minutes. The supernatant was kept on ice, and the pellet resuspended in 3 ml of isolation buffer, homogenized, and centrifuged at 1500 \times *g* for 5 minutes. The supernatants were combined and centrifuged at 13,500 \times *g* for 10 minutes. The brown mitochondrial pellets were resuspended in 100 μ l of isolation buffer and kept on ice, and the protein concentration was determined by Bradford assay.

Measurement of Mitochondrial Respiration

Mitochondrial proteins were resuspended in buffer containing 0.25 mol/L sucrose, 50 mmol/L HEPES, 2 mmol/L MgCl₂, 1 mmol/L EGTA, 10 mmol/L KH₂PO₄, and 0.5% bovine serum albumin, pH 7.4. Oxygen consumption was measured using a Clark-type electrode oxygraph (Hansatech Inc., Norfolk, UK) with 10 mmol/L pyruvate and 5 mmol/L malate as substrate in the absence of exogenous ADP (state 2 respiration) and after addition of 300 mmol/L ADP (state 3 respiration). The ATPase inhibitor oligomycin (100 μ g/ml) was then added to inhibit mitochondrial respiration. In normally coupled mitochondria, the addition of oligomycin slows respiration to a rate similar to that of state 2, whereas in uncoupled mitochondria oligomycin inhibition is reduced. Respiratory control ratio (RCR) was calculated as the ratios between state 3 and state 2 respirations.

Immunoprecipitation

Isolated mitochondrial protein (200 μ g) was resuspended in 200 μ l of RIPA buffer (9.1 mmol/L Na₂HPO₄, 1.7 mmol/L NaH₂PO₄, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% v/v Nonidet P-40, and 0.1% SDS, pH 7.2). Polyclonal nitrotyrosine antibody (anti-rabbit, 2 μ g/ml; Cayman Chemical, Ann Arbor, MI) was added and incubated overnight at 4°C. Protein A/G agarose (20 μ l) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the mixture and incubated overnight. Immunocomplexes were collected by centrifugation at 1000 \times *g* for 5 minutes at 4°C, followed by washing with RIPA buffer, four times. Immunoprecipitated samples were recovered by resuspending in 2 \times sample loading buffer, immediately fractionated by reducing SDS/polyacrylamide gel electrophoresis (PAGE), and analyzed by Western blot.

Western Blot Analysis

Equal amounts of brain homogenated proteins were resuspended in 2 \times sample loading buffer and separated on 12.5% SDS-PAGE. After separation by SDS/PAGE proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and blocked with 5% nonfat dried milk in 50 mmol/L Tris, pH 7.9, 150 mmol/L NaCl, and 0.05% (v/v) Tween-20. After blocking, the blots were incubated overnight at 4°C with appropriate primary antibody (rabbit, anti-MnSOD IgG, dilution 1:10,000; Upstate Technology, Lake Placid, NY), followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Probed membranes were washed three times, and immunoreactive proteins were detected using enhanced chemiluminescence (Amersham Corp., Piscataway, NJ).

MnSOD Activity Assay

SOD activity in the homogenized brain sample was measured by the nitro blue tetrazolium (NBT)-bathocuproine sulfonate reduction inhibition method.²⁶ This is an assay based on the competition reaction between SOD and the indicator molecule NBT. When increasing amounts of protein (containing SOD activity) were added to the system, the rate of NBT reduction was progressively inhibited. Potassium cyanide at 5 mmol/L was used to inhibit Cu/ZnSOD and thus measured only MnSOD activity. The assay mixture also contained catalase to remove H₂O₂ and diethylenetriaminepentaacetic acid to chelate metal ions capable of redox cycling and interfering with the assay system. One unit of SOD activity was defined as the amount of SOD protein that caused a 50% reduction in the background rate of NBT reduction.

Statistical Analysis

Statistical significance was analyzed by two-way analysis of variance, followed by Newman-Keuls multiple comparison test when applicable. The experiments were re-

peated at least three times, and the graphs were drawn using Graph Pad Prism, version 3.02.

Results

A β Deposition in APP/PS-1 Mice

To determine the deposition of A β , we examined brain sections that were immunostained with 10D-5 antibody (Figure 1). The brains from WT mice contained no plaques at any age. The APP/PS-1 animals showed no deposition of A β at the age of 3 months (Figure 1A). At the age of 6 months (Figure 1B), a few microscopic, scattered, small A β plaques were found in the frontal cortex. By 9 months (Figure 1C) there were a few larger A β plaques and more scattered smaller A β deposits. By 12 months (Figure 1D), A β deposition was more prominent in the neocortex and spread of plaques to the hippocampus occurred, with many larger confluent A β plaques and many small and moderate size plaques. By 14 months, there were larger numbers of A β deposits and A β in small blood vessel walls in brain and leptomeninges. These results suggest that there was an age-related, regional dependence to A β deposition in APP/PS-1 mice.

Increased Levels of A β 1-40 and 1-42 Fractions in APP/PS-1 Mice

The accumulation of diverse species of A β peptides in amyloid plaques is a multistep process including the conversion of soluble A β into insoluble derivatives that assemble into amyloid fibrils and aggregate in extracellular deposits.¹⁶ We examined whether the APP/PS-1 mice model showed an increase in levels of SDS-soluble fractions of A β 1-40 and 1-42 in cerebral cortical tissue. APP/PS-1 mice exhibited a trend of age-related increase in the load of both species of A β (Figure 2).

Unchanged MnSOD Protein Levels in WT and APP/PS-1 Mice

Expression of MnSOD is highly inducible by oxidative stress-inducing agents. To determine whether increases in A β produced increases in MnSOD enzyme levels, Western blot analyses of brain homogenate from animals of different ages of both WT and APP/PS-1 mutant mice were performed. The protein levels of MnSOD did not change when compared between genotypes or between different ages within a genotype (Figure 3).

Increased Nitration of MnSOD in APP/PS-1 Mice

MnSOD is susceptible to peroxynitrite-induced inactivation.²⁷ To determine whether A β -induced oxidative stress was associated with nitration and inactivation of MnSOD, nitration of MnSOD was detected by Western blot analysis of SDS/PAGE-fractionated mitochondrial proteins,

which had been immunoprecipitated using a polyclonal anti-nitrotyrosine antibody. Immunodetection with polyclonal anti-MnSOD demonstrated detectable levels of nitrotyrosine in WT mice at all ages tested. In contrast, the APP/PS-1 mice showed an increasing trend in the level of immunoreactive-nitrated MnSOD (Figure 4A), but the increase was not statistically significant at $P < 0.05$. The increase was significant when compared between the two genotypes ($P < 0.01$).

Decreased SOD Activity in APP/PS-1 Mice

MnSOD catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, and this conversion was used to estimate the activity of MnSOD. The activity of MnSOD in APP/PS-1 mice was significantly decreased ($P < 0.0001$) when compared to the WT mice. WT mice showed a significant (** $P < 0.05$) decrease in MnSOD activity at 12 and 14 months when compared to 3-month-old mice of their own genotype (Figure 4C). There was also a significant decrease (* $P < 0.05$) in activity of MnSOD in APP/PS-1 mice when compared to their age-matched WT controls (Figure 4C).

Decreased Mitochondrial Respiration in APP/PS-1 Mice

MnSOD is a primary antioxidant enzyme protecting mitochondria from oxidative injury. To determine whether a reduction of MnSOD activity would affect mitochondrial respiratory function, oxygen consumption by isolated mitochondria was measured as an indicator of the mitochondrial respiration activity. Pyruvate and malate were used as substrates to determine the function of brain mitochondria from WT and APP/PS-1 mice via complex I respiration. The results showed that the RCR of mitochondria was significantly (* $P < 0.01$) decreased in 9- and 12-month-old APP/PS-1 mice when compared to age-matched WT mice and also when compared to 3-month-old mice of both genotypes (** $P < 0.001$) (Figure 5). The results suggest that in APP/PS-1 mice there is inhibition of NAD-linked state 3 respiration rate, which is mediated through complex 1 of the mitochondrial electron transport chain. WT mice showed a small decrease in mitochondrial respiration with age; however, it was not statistically significant.

Discussion

Numerous reports have described attempts to recapitulate the hallmark pathologies of AD in the rodent brain by overexpression of human APP or APP fragments in transgenic models;^{16,28,29} examples include, Tg2576 mice that overexpress human APP695³⁰ with Swedish FAD mutations, mice obtained by crossing Tg2576 mice and transgenic PS1-P264L mice,^{20,31} and the crosses of other Swedish APP transgenic mice with transgenic FAD mutant PS-1 mice.^{19,32} In this study, we used a double gene-targeted APP^{NLh/NLh}/PS-1^{P264L/P264L} mouse model

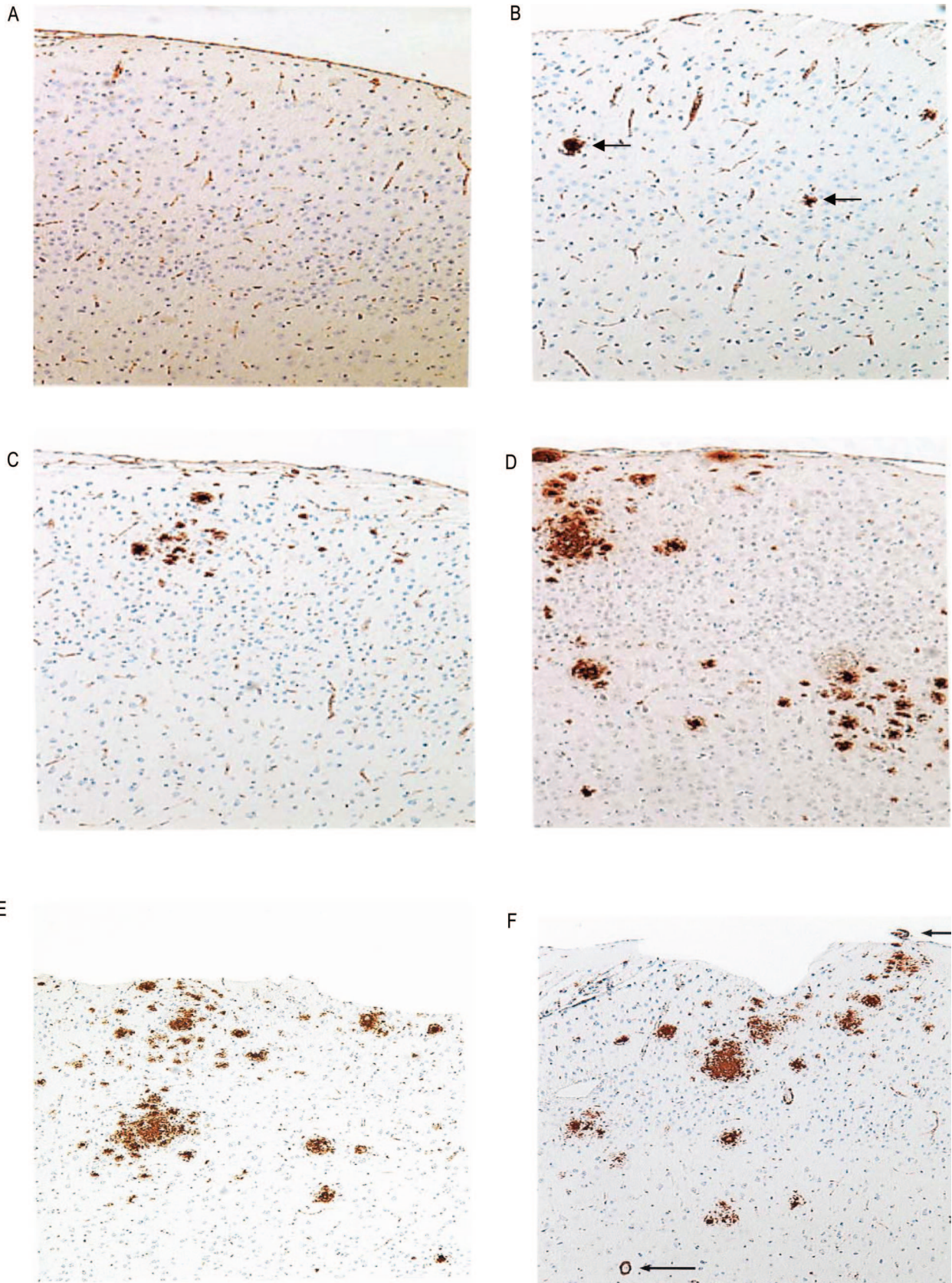


Figure 1. Sections of frontal cortex from APP/PS-1 mice immunostained with 10D-5 antibody for A β . **A:** Three-month-old animal showing no amyloid immunostaining in cortex. **B:** Six-month-old mouse showing rare small deposits of A β (arrows). **C:** Nine-month-old mouse showing increased A β deposits. **D:** Twelve-month-old mouse demonstrating numerous variable size deposits of A β . **E:** Fourteen-month-old mouse showing numerous diffuse A β deposits in cortex. **F:** Fourteen-month-old mouse demonstrating many A β deposits in cortex in parenchymal and leptomeningeal vessels (arrows). Original magnifications, $\times 100$.

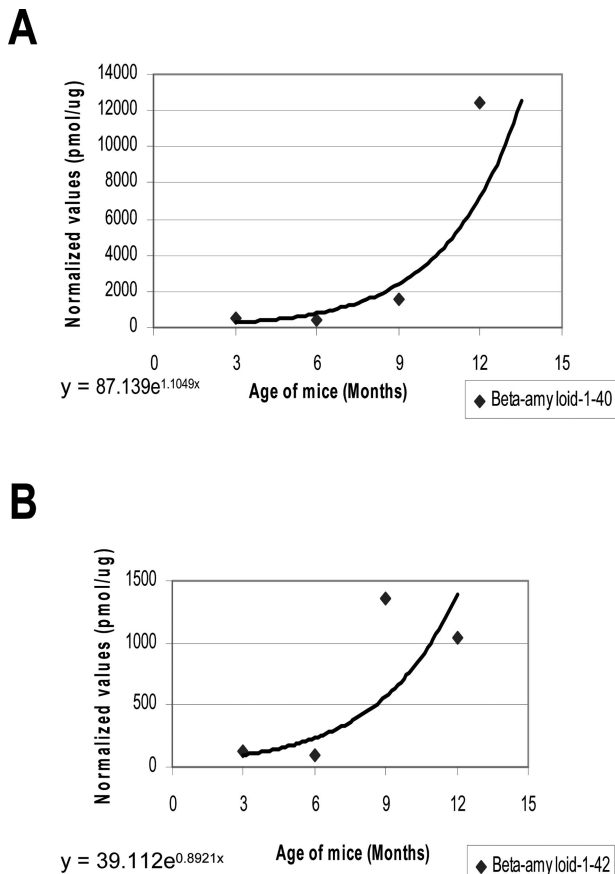


Figure 2. Levels of A β 1-40 and 1-42 in APP/PS-1 mice. A β 1-40 and A β 1-42 levels were measured by ELISA from different groups ($n = 5$ per group) as described in the Materials and Methods section. Both species of A β showed an increasing trend in the levels associated with age ($R^2 = 0.8072$ for A β 1-40 and $R^2 = 0.702$ for A β 1-42).

for amyloid deposition without the overexpression of APP.³³

Humanization of the mouse A β gene sequence results in an approximate threefold increase in amyloidogenic processing of recombinant APP in rat hippocampal neurons infected with Semliki Forest virus expression constructs,³⁴ and single mutant PS-1 allele was sufficient to elevate the concentration of A β 42 in brain and speed the onset of amyloid deposition and reactive astrogliosis.²² Our results using mice homozygous for APP/PS-1, which completely lack WT APP or PS-1 and expressed both mutant FAD APP and PS-1 at natural levels, showed age-dependent increases in amyloid pathology (Figure 2) in accordance with the results obtained from APP 695_{SWE} transgenic mice having PS-1 P264L knock-in mutation.^{22,33} The observed accelerated A β deposition in APP/PS-1 mice also seems to have region dependence in relation to age, with deposition seen first in frontal cortex and later encompassing other cortical regions and hippocampus. Thus, this humanized mouse model should serve as a useful model to study the A β -induced pathology in human AD.

Measurement of A β 1-40 and 1-42 showed that there was an increasing trend in both species in APP/PS-1 mice. Our findings are in concordance with that of Wang

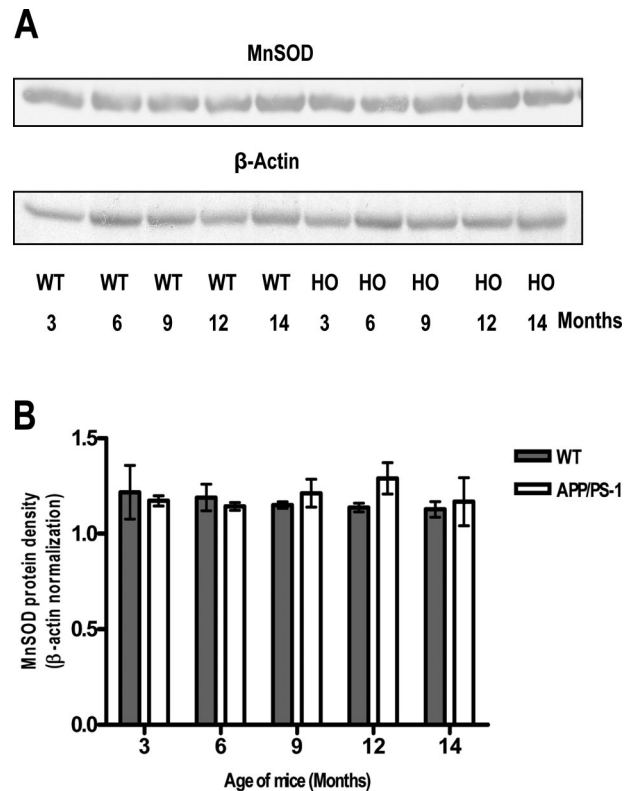


Figure 3. Representative (out of three) immunoblot (A) and densitometry analysis (B) showing the levels of MnSOD. Protein (30 μ g) extracted from brain specimens of APP/PS-1 knock-in and WT mice were run on 12.5% SDS-polyacrylamide gel electrophoresis. Protein levels of MnSOD did not show significant age- and genotype-associated alterations. β -Actin was used to normalize protein loading. HO, APP/PS-1.

and colleagues¹⁷ who demonstrated an increase in average levels of A β 1-40 and A β 1-42 in AD. Our finding of increased quantity of A β 1-40 and A β 1-42, is consistent with the possibility that A β 1-42 serves as the initial seeding event for plaque formation and that increased levels of A β 1-40 play a role in growing plaques and may be mechanistically linked to the onset and progression of AD.¹⁷

The AD brain is under pronounced oxidative stress, as manifested by protein oxidation, lipid peroxidation, DNA and RNA oxidation, widespread peroxynitrite-induced damage, advanced glycation end products, and altered antioxidant enzyme expression. Via mechanisms that are inhibited by antioxidants such as vitamin E, A β causes brain cell protein oxidation, lipid peroxidation, and ROS formation, among other oxidative stress responses, suggesting that this peptide is a source of oxidative stress in the brain.³⁵ Other sources of oxidative stress in AD are likely, ranging from altered mitochondrial function, trace metal ion imbalances to binding of altered metal ion to biomolecules.³⁶ Our results indicating increased nitration of MnSOD protein signify an increase in oxidative stress in the brain of the APP/PS-1 mice and suggest a compromise in mitochondrial function of APP/PS-1 mice attributable to increased oxidative stress.

The mitochondrion, a major subcellular source of ROS^{37,38} plays a pivotal role in apoptosis.³⁹ However, the mitochondrion is also a site of cellular protection against

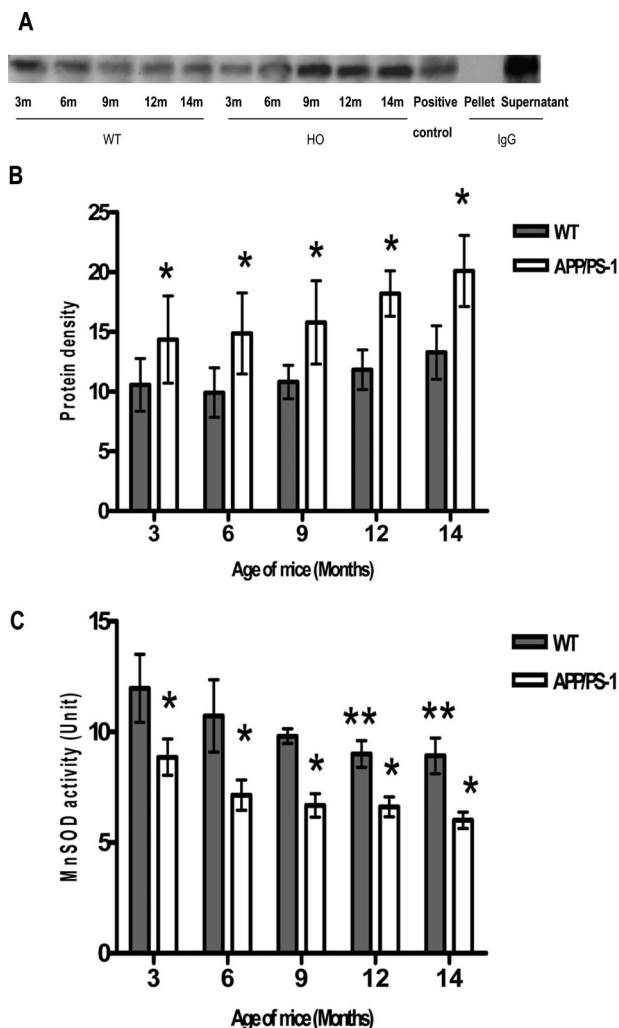


Figure 4. Immunoprecipitation of nitrotyrosine with MnSOD. Isolated mitochondrial protein (200 μ g) was precipitated with polyclonal nitrotyrosine antibody and analyzed by Western blotting using MnSOD antibody. **A:** Nitrotyrosine co-immunoprecipitated with MnSOD showed that MnSOD is nitrated. HO, APP/PS-1; positive control, brain homogenate + peroxyntirite (2 μ mol/L), provided by Dr. Timothy R. Miller, Graduate Center for Toxicology, University of Kentucky, Lexington, KY; pellet, pellet of the isolated mitochondrial protein (200 μ g) precipitated with preimmune IgG, analyzed by Western blotting using MnSOD antibody; supernatant of the isolated mitochondrial protein (200 μ g) precipitated with preimmune serum, analyzed by Western blotting using MnSOD antibody. **B:** Densitometric analysis and subsequent statistical analysis by two-way analysis of variance showed significant differences ($*P < 0.01$) when compared between genotypes. **C:** MnSOD activity in the brain specimen was measured by the NBT-bathocuproine sulfonate reduction inhibition method.²⁶ Statistical analysis by two-way analysis of variance showed significant age- and genotype-dependent decreases ($P < 0.0001$) in activity of MnSOD. APP/PS-1 mice, at all ages, showed significant decreases ($*P < 0.05$) in MnSOD activity when compared with age-matched WT mice. WT mice at 12 and 14 months showed significant decrease ($**P < 0.05$) in MnSOD activity when compared with 3-month-old WT mice. Immunoprecipitation and MnSOD activity was performed in three sets of animals.

ROS that involves an elaborate antioxidant defense system, especially MnSOD. Our studies indicate that the expression pattern of MnSOD in APP/PS-1 mice remained unaltered in all age groups, but SOD activity in APP/PS-1 mice was significantly reduced when compared to age-matched WT mice, suggesting that the protein was inactivated. Our finding is in accordance with that reported by Macmillan-Crow and colleagues²⁷ in a

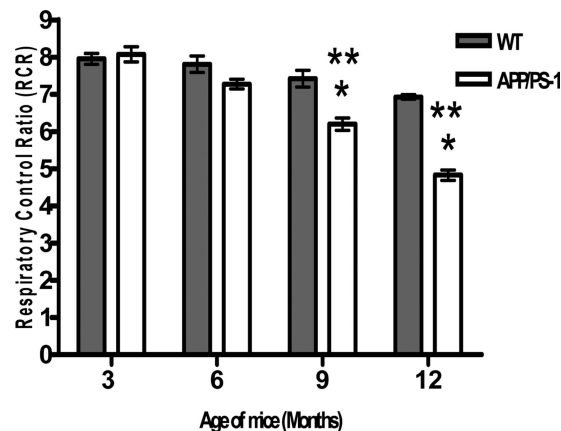


Figure 5. Decline in mitochondrial respiration via complex I in APP/PS-1 mice. Oxygen consumption was measured using a Clark-type electrode oxygraph. RCR was calculated as the ratios between state 3 and state 2 respirations. APP/PS-1 mice at 9 and 12 months of age showed a significant decrease ($*P < 0.01$) in mitochondrial respiration when compared to its age-matched WT mice and a significant decrease ($**P < 0.001$) compared to 3-month-old mice of both genotypes. Statistical analysis: two-way analysis of variance followed by Newman-Keuls multiple comparisons test.

chronic rejecting renal model. The decreased activity of MnSOD is attributable to nitration of tyrosine residues^{5,40} that occurs on the MnSOD protein. Tyrosine nitration (3-nitrotyrosine, 3-NT) is an *in vivo* posttranslational protein modification with potentially significant biological implications^{14,41} and has been detected in a number of human and animal models of disease.³⁹ 3-NT is increased in the hippocampus and cerebral cortex of aged rats,⁴² the cerebrospinal fluid of aged humans,⁴³ and the subcortical white matter of aged monkeys.⁴⁴ 3-NT formation is the hallmark of reactive peroxynitrite (ONOO⁻), and this modification can compromise the functional and/or structural integrity of target proteins,⁴⁰ especially MnSOD.⁴⁵ After diffusing into mitochondria, NO can inhibit oxygen consumption by complex IV. Inhibition by NO is reversible; however, this process decreases electron transport and could potentially increase the concentration of O₂^{-•}. MnSOD has a function to eliminate O₂^{-•} from the mitochondrial matrix space. But, the concentration of NO required to inhibit complex IV is sufficient to compete effectively with MnSOD for O₂^{-•} by a rapid reaction generating peroxynitrite, which can nitrate tyrosine residues and inactivate MnSOD to increase further the intramitochondrial level of O₂^{-•}.²⁷ O₂^{-•} reacts with NO faster than with MnSOD⁴⁶; therefore, if mitochondria contain inactive MnSOD, this peroxynitrite can further nitrate MnSOD and inactivate MnSOD to increase the intramitochondrial level of superoxide. Consequently this peroxynitrite-mediated amplification cycle would result in a progressive increase of mitochondrial levels of peroxynitrite, which can induce additional cytotoxic effects²⁷ including inactivation of complexes I and II in the mitochondrial respiratory chain.⁴⁷

Myeloperoxidase (MPO), one of the principle hemoproteins stored in the azurophilic granules of neutrophils and monocytes, is also a catalyst of nitrotyrosine formation via nitrite oxidation to the potent nitrating species nitrogen dioxide (:NO₂).⁴⁸ It has been demonstrated in

vascular tissues that MPO significantly contributes to nitrotyrosine formation *in vivo*.^{48,49} Further, it has been shown that MPO^{-/-} animals have reduced nitrotyrosine immunoreactivity than WT mice.⁴⁹ MPO is not only present in neutrophils and monocytes, but also in microglia. Microglia are quiescent in normal brain but can become activated in response to neuronal damage or various other stimuli, including aggregated A β .^{50,51} In AD, MPO has been shown to be co-localized around the A β plaques and also in microglia-macrophages that are present around the plaques. It has also been shown that A β treatment of the mouse microglia cell line BV-2 resulted in strong induction of MPO mRNA expression.⁵² From these observations and the fact that senile plaques are surrounded by activated microglia, it can be cautiously speculated that MPO may also contribute to the nitration of MnSOD.

Despite major advances in the study of nitration of proteins, the effect of nitration on protein turnover and the pathways that degrade the nitrated proteins have not been fully elucidated. There are reports about faster degradation of proteins treated with peroxyxynitrite or a generator of nitric oxide and superoxide by 20S proteasome.⁵³ Consistent with this, Souza and colleagues⁵⁴ reported that a single nitrating event is sufficient to target proteins for degradation by the proteasome. Although protein nitration is generally viewed as an irreversible event, activities that appear to specifically repair nitrated proteins have been reported in human and rat tissues.^{55,56} Thus it tempting to speculate that nitration of MnSOD may be reversible. In this regard, our APP/PS-1 mouse model is ideal for testing pharmacological intervention by mitochondrially targeted antioxidants such Mito-Q or Mito-Vit E.

Mito-Q [mixture of mitoquinol-10-(6'-ubiquinoyl)decyltriphenylphosphonium and mitoquinone-10-(6'-ubiquinonyl)decyltriphenylphosphonium]⁵⁷ and MitoVit E [2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol bromide]⁵⁸ have been found to destroy superoxide in the mitochondrial matrix.⁵⁸ Mito-Q (Mito-Q₁₀) has been shown to be an effective antioxidant against lipid peroxidation, peroxyxynitrite, and superoxide.⁵⁹ Pretreatment with Mito-Q and Mito Vit-E have been shown to 1) significantly abrogate the lipid peroxide-induced 2'-7'-dichlorofluorescein fluorescence and protein oxidation in bovine aortic endothelial cells; 2) inhibit cytochrome c release, caspase-3 activation, and DNA fragmentation; 3) inhibit H₂O₂ and lipid peroxide-induced inactivation of complex I and aconitase, thus preventing the production of superoxide; 4) inhibit TfR overexpression and mitochondrial uptake of ⁵⁵Fe, thereby inhibiting apoptosis; and 5) restore the mitochondrial membrane potential and proteasomal activity.⁶⁰ Further, the pro-oxidant effect or superoxide production by Mito-Q₁₀ was found to be insufficient to cause any damage but led to hydrogen peroxide production and nitric oxide consumption.⁵⁹ Despite these results, the effect of these compounds on the reversal of damage that has already been caused by oxidative stress has not been investigated.

The antioxidant efficacy of Mito-Q₁₀ is due to its conversion to ubiquinol by complex II of the mitochondrial

respiratory chain, but its reoxidation back to ubiquinone by complex III is ineffective. In ubiquinol form, Mito-Q₁₀ quenches ONOO⁻ and becomes oxidized, which can be reduced by complex II to ubiquinol, making it available to quench more ONOO⁻.⁵⁹ Mito-Q₁₀ may be effective in preventing the nitration of MnSOD and may serve as an effective therapeutic intervention to slow the progression of AD.

Mitochondrial respiratory dysfunction and oxidative stress have been associated with many neurodegenerative diseases.⁶¹ Results obtained in this study on mitochondrial respiration using pyruvate plus malate as substrate demonstrate that in APP/PS-1 mice there is inhibition of NAD-linked state 3 respiration rate, which is mediated through complex I of the mitochondrial electron transport chain. RCR represents functional integrity of isolated mitochondria.⁶² A high value of RCR indicates the utilization of substrates is tightly coupled to the production of ATP.⁶³ The respiration injury would predict a low coupling efficiency of the mitochondrial electron transport and an increased likelihood of electron leakage during respiration, leading to O₂^{-•} radical formation and increased oxidative stress. Our finding is consistent with that of Kokoszka and colleagues⁶⁴ who reported a decrease in state III respiratory states and the RCR of liver mitochondria from both homozygous and heterozygous knock-out mice for the gene encoding the MnSOD protein, *Sod2*. Cardiac mitochondria from *Sod2*^{+/-} mice, which have 50% reduction in MnSOD activity, showed altered mitochondrial function as exemplified by decreased respiration by complex I and an increase in the sensitivity of the permeability transition pore induction.⁶⁵ Thus, mitochondria from the heart (*Sod2*^{+/-}) and liver (*Sod2*^{-/-} and *Sod2*^{+/-}) show evidence of increased oxidative damage compared with mitochondria isolated from *Sod2*^{+/+} mice. Yen and colleagues⁶² showed that MnSOD selectively protected state 3 respiration activity through complex I substrates and prevented complex I inactivation in heart mitochondria treated with the anthracyclin antibiotic, adriamycin. These results indicate that MnSOD plays a critical role in oxidative stress responses and in maintenance of mitochondrial respiration and the lack or reduced activity that leads to increased sensitivity of the animals to oxidative stress-induced mitochondrial damage. The decreased activity of MnSOD in APP/PS-1 mice can be implicated as a cause of decreased state 3 respiration rate seen in these mice. Our study, which shows a decline in mitochondrial respiration in aged (9 and 12 months) WT mice, also implicates the effects of mitochondrial ROS production in the process of aging. Our findings are consistent with studies that demonstrate a decline in respiratory function of mitochondria with age⁶⁶⁻⁶⁸ and decreased mitochondrial respiratory function in aged or aging mice, due to deficiencies in the MnSOD.⁶⁴ Our results also suggest that nitrative inactivation of MnSOD may be, at least in part, responsible for the decline in the mitochondrial function of aging AD mice. Biochemical analyses of brain specimens from patients with AD have shown abnormalities in the components of electron transport chain, particularly in the activity of cytochrome oxidase (COX).^{69,70} Inhibition of COX

could cause depressed ATP synthesis and bioenergetic impairment in AD. In addition, the decreased COX function could cause diversion of electrons from their normal pathway into reaction with molecular oxygen in the neocortex and hippocampus in AD, resulting in increased O_2^- .³⁶ Superoxide can contribute to mitochondrial impairment by generating additional reactive species, particularly hydroxyl radical, and peroxynitrite that can inactivate mitochondrial proteins leading to further decline in mitochondrial respiration, decreased ATP production, and ultimately neuronal cell death.⁷¹

Although there is no consensus about the activity level of MnSOD in AD, there have been reports of reduction of SOD activity in AD frontal cortex, hippocampus, and cerebellum⁷² and elevation of SOD activity in the caudate nucleus of AD.⁷³ However, there are reports of increased nitrotyrosine immunoreactivity in neurons of AD.^{74,75} To our knowledge the identity of the nitrated proteins in the AD brain is not known. Our results showing decreased activity of MnSOD in relation to age and nitration represents the first study directed to the identification of nitrated proteins in AD brain.

The mouse model used in our study has the sequence of A β identical to the human sequence. Further, the mutations introduced are driven by endogenous promoters of APP and PS-1 genes, and expression is limited to the replacement of these two endogenous genes and not by the expression of multiple transgenes. The amyloid pathology observed in this model is similar to that found in AD. Thus, the results obtained from this model may prove useful in unraveling the pathogenesis of AD-induced oxidative stress.

In conclusion, in this study we demonstrate an increased and accelerated deposition of A β in APP/PS-1 mice, increased levels of A β 1-40/1-42, increased nitrotyrosine and subsequent inactivation of MnSOD, and impaired mitochondrial respiration in APP/PS-1 mice in association with age. The increased levels of A β 1-40/1-42, essential for aggregation and subsequent neurotoxic properties, are observed from 6 months on. It is tempting to speculate that the increased A β may be the cause of alterations seen in MnSOD and the associated decrease in mitochondrial function. These changes may lead to altered function of mitochondrial permeability transition pore,⁶⁶ which increases the propensity to undergo apoptosis. This is important because findings indicate that neuronal cell death associated with A β peptide are apoptotic in nature.⁷⁶ Thus, our studies which indicate mitochondrial dysfunction, an age-associated increase in nitration of MnSOD, and its concomitant decrease in antioxidant activity, may provide the missing link between A β -induced oxidative stress and progression of AD.

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References

1. Evans DA, Funkenstein HH, Albert MS, Scherr PA, Cook NR, Chown MJ, Hebert LE, Hennekens CH, Taylor JO: Prevalence of Alzheimer's disease in a community population of older people. *JAMA* 1989, 262:2551-2556
2. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y: Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron* 1994, 13:45-53
3. Selkoe DJ: Clearing the brain's amyloid cobwebs. *Neuron* 2001, 25:177-180
4. Butterfield DA, Lauderback CM: Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid β -peptide-associated free radical oxidative stress. *Free Radic Biol Med* 2002, 32:1050-1060
5. Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G: Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* 1997, 17:2653-2657
6. Castegna A, Thongboonkerd V, Klein JB, Lynn B, Markesbery WR, Butterfield DA: Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 2003, 85:1394-1401
7. Butterfield DA, Griffin S, Munch G, Pasinetti GM: Amyloid β -peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. *J Alzheimer's Dis* 2001, 4:193-201
8. Mecocci P, MacGarvey U, Beal MF: Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 1994, 36:747-751
9. Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S, Smith MA: RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci* 1999, 19:1959-1964
10. Lebovitz RM, Zhang H, Vogel H, Cartwright Jr J, Dionne L, Lu N, Huang S, Matzuk MM: Neurodegeneration, myocardial injury and perinatal death in mitochondrial superoxide dismutase deficient mice. *Proc Natl Acad Sci USA* 1996, 93:9782-9787
11. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH: Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995, 11:376-381
12. Wei YH, Lee HC: Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Exp Biol Med* 2002, 227:671-682
13. Wiseman H, Halliwell B: Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J* 1996, 313:17-29
14. Beckman JS: Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 1996, 9:836-844
15. Montine KS, Olson SJ, Amarnath V, Whetsell WO, Graham DG, Montine TJ: Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of ApoE4. *Am J Pathol* 1997, 150:437-443
16. Games D, Adams D, Alessandrini R, Barbour R, Borthellette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, Guido T, Hagan S, Johnson-Wood K, Khan K, Lee M, Leibowitz P, Lieberburg I, Little S, Masliah E, McConlogue L, Montoya-Zavala M, Mucke L, Paganini L, Penniman E, Power M, Schenk D, Seubert P, Snyder B, Soriano F, Tan H, Vitale J, Wadsworth S, Wolozin B, Zhao J: Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* 1995, 373:523-527
17. Wang J, Dickson DW, Trojanowski JQ, Lee VM: The levels of soluble versus insoluble brain A β distinguish Alzheimer's disease from normal and pathologic aging. *Exp Neurol* 1999, 158:328-337
18. Hsia AY, Masliah E, McConlogue L, Yu GQ, Tatsuno G, Hu K, Kholodenko D, Malenka RC, Nicoll RA, Mucke L: Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc Natl Acad Sci USA* 1999, 96:3228-3233
19. Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins

- NA, Copeland NG, Price DL, Sisodia SS: Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 1997, 19:939–945
20. Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D, Sanders S, Zehr C, O'Campo K, Hardy J, Prada CM, Eckman C, Younkin S, Hsiao K, Duff K: Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nat Med* 1998, 4:97–100
21. Reaume AG, Howland DS, Trusko SP, Savage MJ, Lang DM, Greenberg BD, Siman R, Scott RW: Enhanced amyloidogenic processing of the β -amyloid precursor protein in gene-targeted mice bearing the Swedish familial Alzheimer's disease mutations and a "humanized" A β sequence. *J Biol Chem* 1996, 271:23380–23388
22. Siman R, Reaume AG, Savage MJ, Trusko S, Lin YG, Scott RW, Flood DG: Presenilin-1 P264L knock-in mutation: differential effects on A β production, amyloid deposition and neuronal vulnerability. *J Neurosci* 2000, 20:8717–8726
23. Skovronsky DM, Doms RW, Lee VM: Detection of novel, intraneuronal pool of insoluble A β that accumulates with time in culture. *J Cell Biol* 1998, 141:1031–1039
24. Turner RS, Suzuki N, Chyung AS, Younkin SG, Lee VM: Amyloids β_{40} and β_{42} are generated intracellularly in cultured human neurons and their secretion increases with maturation. *J Biol Chem* 1996, 271:8966–8970
25. Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos Jr L, Eckman C, Golde TE, Younkin SG: An increased percentage of long amyloid β -protein secreted by familial amyloid β -protein precursor (β APP717) mutants. *Science* 1994, 264:1336–1340
26. Spitz DR, Oberley LW: An assay for superoxide dismutase activity in mammalian tissue homogenates. *Anal Biochem* 1989, 179:8–18
27. MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA: Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* 1996, 93:11853–11858
28. Lamb BT, Sisodia SS, Lawler AM, Slunt HH, Kitt CA, Kearns WG, Pearson PL, Price DL, Gearhart JD: Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice. *Nat Genet* 1993, 5:22–30
29. LaFerla FM, Tinkle BT, Bieberich CJ, Haudenschild CC, Jay G: The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nat Genet* 1995, 9:21–30
30. Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G: Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Science* 1996, 274:99–102
31. McGowan E, Sanders S, Iwatsubo T, Takeuchi A, Saido T, Zehr C, Yu X, Uljon S, Wang R, Mann D, Dickson D, Duff K: Amyloid phenotype characterization of transgenic mice overexpressing both mutant amyloid precursor protein and mutant presenilin 1 transgenes. *Neurobiol Dis* 1999, 6:231–244
32. Lamb BT, Bardel KA, Kulnane LS, Anderson JJ, Holtz G, Wagner SL, Sisodia SS, Hoeger EJ: Amyloid production and deposition in mutant amyloid precursor protein and presenilin-1 yeast artificial chromosome transgenic mice. *Nat Neurosci* 1999, 2:695–697
33. Flood DG, Reaume AG, Dorfman KS, Lin TG, Lang DM, Trusko SP, Savage MJ, Annaert WG, Strooper BD, Siman R, Scott RW: FAD mutant PS-1 gene-targeted mice: increased A β 42 and A β deposition without APP overproduction. *Neurobiol Aging* 2002, 23:335–348
34. DeStrooper B, Simons M, Multhaup G, Leuven FV, Beyreuther K, Dotti CG: Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. *EMBO J* 1995, 14:4932–4938
35. Butterfield DA, Howard BJ, LaFontaine MA: Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. *Curr Med Chem* 2001, 8:815–828
36. Markesbery WR: Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* 1997, 23:134–147
37. Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Goldberg MP, Choi DW: Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci* 1995, 15:6377–6388
38. Piantadosi CA, Zhang J: Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 1996, 27:327–331
39. Kroemer G, Zamzami N, Susin SA: Mitochondrial control of apoptosis. *Immunol Today* 1997, 18:44–51
40. Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N: Oxidative damage in Alzheimer's. *Nature* 1996, 382:120–121
41. Ischiropoulos H: Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 1998, 356:1–11
42. Shin CM, Chung YH, Kim MJ, Lee EY, Kim EG, Cha CI: Age related changes in the distribution of nitrotyrosine in the cerebral cortex and hippocampus of rats. *Brain Res* 2002, 931:194–199
43. Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isebe C: Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. *Neurosci Lett* 1999, 269:52–54
44. Sloane JA, Hollander W, Moss MB, Rosene DL, Abraham CR: Increased microglial activation and protein nitration in white matter of the aging monkey. *Neurobiol Aging* 1999, 20:395–405
45. Ischiropoulos H, Zhu L, Chen J, Tasi M, Martin JC, Smith CD, Beckman JS: Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys* 1992, 298:431–437
46. Hsu JL, Hsieh Y, Tu C, O'Connor D, Nick HS, Silverman DN: Catalytic properties of human manganese superoxide dismutase. *J Biol Chem* 1996, 271:17687–17691
47. Cassina A, Radi R: Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch Biochem Biophys* 1996, 328:309–316
48. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, Ma W, Tousson A, White CR, Bullard DC, Brennan ML, Luscis AJ, Moore KP, Freeman BA: Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest* 2001, 108:1759–1770
49. Baldus S, Eiserich JP, Brennan ML, Jackson RM, Alexander CB, Freeman BA: Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med* 2002, 33:1010–1019
50. El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC: Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. *Neurobiol Aging* 1998, 19:S81–S84
51. Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Villalba M, Ferrari D, Rossi F: Activation of microglial cells by beta-amyloid protein and interferon-gamma. *Nature* 1995, 374:647–650
52. Reynolds WF, Rhees J, Maciejewski D, Paladino T, Sieburg H, Maki RA, Masliah E: Myeloperoxidase polymorphism is associated with gender specific risk for Alzheimer's disease. *Exp Neurol* 1999, 155:31–41
53. Grune T, Blasig IE, Sitte N, Roloff B, Haseloff R, Davies KJ: Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J Biol Chem* 1998, 273:10857–10862
54. Souza JM, Choi I, Chen Q, Weisse M, Daikhin E, Yudkoff M, Obin M, Ara J, Horwitz J, Ischiropoulos H: Proteolytic degradation of tyrosine nitrated proteins. *Arch Biochem Biophys* 2000, 380:360–366
55. Gow AJ, Duran D, Malcolm S, Ischiropoulos H: Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett* 1996, 385:63–66
56. Kamisaki Y, Wada K, Bian K, Balabanli B, Davis K, Martin E, Behbod F, Lee YC, Murad F: An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc Natl Acad Sci USA* 1998, 95:11584–11589
57. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP: Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and anti-apoptotic properties. *J Biol Chem* 2001, 276:4588–4596
58. Echtay KS, Murphy MP, Smith RA, Talbot DA, Brand MD: Superoxide activates mitochondrial uncoupling protein 2 from matrix side. Studies using targeted antioxidants. *J Biol Chem* 2002, 277:47129–47135
59. James AM, Cocheme HM, Smith RA, Murphy MP: Interactions of mitochondria-targeted and untargeted ubiquinones with the mitochondrial respiratory chain and reactive oxygen species. Implications

- for the use of exogenous ubiquinones as therapies and experimental tools. *J Biol Chem* 2005, 280:21295–21312
60. Dhanasekaran A, Kotamraju S, Kalivendi SV, Matsunaga T, Shang T, Keszler A, Joseph J, Kalyanaraman B: Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. *J Biol Chem* 2004, 279:37575–37587
 61. Beal MF: Aging, energy and oxidative stress in neurodegenerative diseases. *Ann Neurol* 1995, 38:357–366
 62. Yen HC, Oberley TD, Oberley C, Gairola CG, Szweda LI, St. Clair DK: Manganese superoxide dismutase protects mitochondrial complex I against adriamycin-induced cardiomyopathy in transgenic mice. *Arch Biochem Biophys* 1999, 362:59–66
 63. Mathews CK, van Holde KE: *Biochemistry*. Redwood City, Benjamin/Cummings, 1990
 64. Kokoszka JE, Coskun P, Esposito LA, Wallace DC: Increased mitochondrial oxidative stress in the Sod2 (+/–) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. *Proc Natl Acad Sci USA* 2001, 98:2278–2283
 65. Van Remmen H, Williams MD, Guo Z, Estlack L, Yang H, Carlson EJ, Epstein CJ, Huang TT, Richardson A: Knockout mice heterozygous for Sod2 show alterations in cardiac mitochondrial function and apoptosis. *Am J Physiol* 2001, 281:H1422–H1432
 66. Yen TC, Chen YS, King KL, Yeh SH, Wei YH: Liver mitochondrial respiratory functions decline with age. *Biochem Biophys Res Commun* 1989, 165:994–1003
 67. Cooper JM, Mann VM, Schapira AH: Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. *J Neurol Sci* 1992, 113:91–98
 68. Wei YH: Oxidative stress and mitochondrial DNA mutations in human aging. *Proc Soc Exp Biol Med* 1998, 217:53–63
 69. Maurer I, Zierz S, Moller HJ: A selective defect of cytochrome C oxidase is present in brain of Alzheimer disease patients. *Neurobiol Aging* 2000, 21:455–462
 70. Kish SJ, Mastrogiacomo F, Guttman M, Furukawa Y, Taanman JW, Dozic S, Pandolfo M, Lamarche J, DiStefano L, Chang LJ: Decreased brain protein levels of cytochrome oxidase subunits in Alzheimer's disease and in hereditary spinocerebellar ataxia disorders: a nonspecific change? *J Neurochem* 1999, 72:700–707
 71. Eckert A, Keil U, Marques CA, Bonert A, Frey C, Schussel K, Muller WE: Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. *Biochem Pharmacol* 2003, 66:1627–1634
 72. Richardson JS: Free radicals in the genesis of Alzheimer's disease. *Ann NY Acad Sci* 1993, 695:73–76
 73. Marklund SL, Adolfsson R, Gottfries CG, Windblad B: Superoxide dismutase isoenzymes in normal brains and in brains from patients with dementia of Alzheimer type. *J Neurol Sci* 1985, 67:319–325
 74. Good PF, Werner P, Hsu A, Olanow CW, Perl DP: Evidence of neuronal oxidative damage in Alzheimer's disease. *Am J Pathol* 1996, 149:21–28
 75. Vodovotz Y, Lucia MS, Flanders KC, Chesler L, Xie QW, Smith TW, Weidner J, Mumford R, Webber R, Nathan C, Roberts AB, Lippa CF, Sporn MB: Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J Exp Med* 1996, 184:1425–1433
 76. Dickson DW: Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? *J Clin Invest* 2004, 114:23–27