Amyloid beta protein (Aβ) in Alzheimer’s disease induces oxidative stress through several mechanisms, including stimulation of nitric oxide synthase (NOS) activity. We examined NOS activity and expression in the senescence-accelerated mouse P8 (SAMP8) line. The SAMP8 strain develops with aging cognitive impairments, increases in Aβ, and oxidative stress, all reversed by amyloid precursor protein antisense or Aβ antibody treatment. We found here that hippocampal NOS activity in 12-month-old SAMP8 mice was nearly double that of 2-month-old SAMP8 or CD-1 mice, but with no change in NOS isoenzyme mRNA and protein levels. Antisense or antibody treatment further increased NOS activity in aged SAMP8 mice. Antisense treatment increased inducible NOS (iNOS) mRNA levels, decreased neuronal NOS mRNA and protein levels, but did not affect endothelial NOS (eNOS) or iNOS protein or eNOS mRNA levels. These results suggest a complex relation between Aβ and NOS in the SAMP8 that is largely mediated through posttranslational mechanisms.


ALZHEIMER’S disease (AD) is a devastating neurological disorder that affects about 4.5 million persons (1). Amyloid β protein (Aβ), a major component of the senile plaque, is central to the pathogenesis of AD (2) and is formed by cleavage of the transmembrane domain of amyloid precursor protein (APP) by enzymes including the gamma secretases (3). One mechanism by which Aβ produces its neurotoxicity is by oxidative stress (4). Oxidative stress can be mediated through release of nitric oxide, which reacts with superoxide radicals to form peroxynitrite (ONOO⁻), a highly reactive oxidizing anion with a half-life of less than 1 second. Exposure to significant levels of ONOO⁻ induces protein oxidation and significant protein conformational changes (5).

However, nitric oxide might also play a protective role in AD. A series of studies have suggested that nitric oxide may act as a second messenger that enhances memory (6–8). Nitric oxide is produced by three nitric oxide synthase (NOS) isoenzymes: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). Each of the isoenzymes produces the same product, nitric oxide, but have unique locations. Therefore, differences in biologic action among the isoenzymes likely reflect these differences in location and distribution. Mice with the isoenzyme nNOS knocked out have impaired memory (9), whereas eNOS knockout mice have enhanced memory (10). Studies suggest that nitric oxide plays a complex role in the regulation of learning and memory. Depending on the isoenzyme, length of exposure, and possible hormetic effects, nitric oxide could play on balance either a protective or a destructive role in cognition and amyloid beta production.

Overall NOS activity and the level of iNOS are elevated in some brain regions of patients with AD (11–14). Aβ has been postulated to cause the elevated NOS levels in AD. Aβ has been shown to affect NOS in some cells through various mechanisms, generally associated with an increase in NOS activity and nitric oxide production. For example, in vitro studies show that Aβ stimulates NOS activity through a nuclear factor κ B pathway in astrocytes (15) and iNOS activity through a tumor necrosis factor-dependent pathway in oligodendrocytes (16) and through activator protein 1/extra-cellular signal-related kinase/mitogen activated protein kinase-dependent pathways in PC12 cells (17). The more neurotoxic 1–42 form of Aβ more robustly stimulates iNOS mRNA and nitric oxide levels than does the less toxic 1–40 form (11). In vivo, injection of Aβ into the brain increases eNOS protein levels (18). In the Swedish double mutation of the APP transgenic mouse, nitric oxide levels were elevated and could be reduced by gamma secretase inhibition (19). At least some of the toxicity of Aβ is likely mediated through nitric oxide because the toxicity can be blocked by inhibition of NOS (20,21). However, other studies indicate that Aβ may inhibit eNOS activity through suppression of the Akt signaling pathway (22,23). In addition, there is some evidence from a brain hypoperfusion model that increases in constitutive
NOS increases Aβ and impairs memory (24). Thus, a complex feedback may exist between Aβ and NOS.

Nitric oxide and NOS activity are also elevated in both the blood (25) and the brain (26) of the senescence-accelerated mouse P8 (SAMP8) mouse, which is used as an animal model of AD. The SAMP8 is a natural mutation that has an age-related overexpression of APP with elevated brain levels of Aβ that produces an age-related impairment in cognition (27). Either antibody directed against Aβ (28) or antisense directed against APP (29) reverses the cognitive impairment of the aged SAMP8 mice. The brains of aged SAMP8 mice show signs of oxidative stress. Both oxidative stress and cognitive impairment are reversed with antisense or antioxidant treatment (30–32). Here, we examined NOS activity and isoenzyme mRNA and protein expression in young and old SAMP8 mice and examined the effects of antibody and antisense treatments on NOS activity and isoenzyme expression.

**Materials and Methods**

**Animals**

Male 2-month-old CD-1 mice (a standard white laboratory mouse), male 2-month-old SAMP8 mice, and male 12-month-old SAMP8 mice from our in-house colonies were housed in groups of four. Animals were kept on a 12-hour light–dark cycle, with lights on at 6 AM. Food and water were available at all times. The SAMP8 mouse strains were originally developed from the AKR/J strain of mice in the laboratory of Professor Toshio Takeda in Kyoto, Japan. The mice were tested regularly to ensure that they are virus and pathogen free. All protocols were approved by the local Institutional Animal Care and Use Committee and were conducted at an Association for the Assessment and Accreditation of Laboratory Animal Care-approved facility.

**Experimental Design**

NOS activity was measured in the hippocampus of 2-month-old CD-1 and 2-month-old SAMP8 mice, and male 12-month-old SAMP8 mice from our in-house colonies were submitted to mRNA and protein isoenzyme measures. Hemibrains from other 12-month-old SAMP8 mice treated with this same antisense regimen (n = 9 antisense; n = 8 controls) were submitted to mRNA and protein isoenzyme measures.

**Intracerebroventricular Injections**

Forty-eight hours prior to an injection, mice were anesthetized with tribromoethane and a hole 1.0 mm lateral to and 0.5 mm posterior to the bregma was made into the skull. Twenty-four hours later, mice were anesthetized with isoﬂurane and antibody to Aβ was injected into the lateral ventricle of the brain at a depth of 2.0 mm; an IgG antibody was injected into controls.

**Measurement of NOS Activity**

Prior to harvesting brains, mice were anesthetized with a single intraperitoneal injection (0.15–0.2 mL) of 40% ethyl carbamate. The mice were then decapitated and the hippocampus was dissected on ice from the rest of the brain and kept on dry ice at −70°C until processed. The hippocampus was homogenized in buffer (10 mL of 1% NP40 in 1× phosphate buffered saline, 37 mg of iodoacetamide, 10 μL of 1 mg/mL pepstatin A, and 100 μL of 200 mM phenylmethylsulfonyl fluoride or phenylmethylsulfonyl fluoride in 100% ethanol). After homogenization, samples were kept on wet ice for 30 minutes and then centrifuged at 4°C for 20 minutes. Protein was assayed by a BCA protein assay kit (Pierce, Rockford, IL).

NOS activity was quantified on the hippocampal homogenate by measuring the conversion of [14C] L-arginine into [14C] L-citrulline and NO. NO and citrulline are produced in equimolar amounts. 14C arginine and 14C citrulline were purchased from PerkinElmer Life Sciences, Inc. Equal amounts of samples were incubated with 50 μCi/mL of L-arginine, 300 mM Heps pH 7.0, 20 mM β-nicotinamide adenine dinucleotide phosphate, 10 mM CaCl2, 1 mM flavin adenine dinucleotide, 1 mM tetrahydrobiopterin, and 8.3 μg/mL calmodulin. Samples were incubated for 60 minutes at 37°C. The reaction was stopped by adding 2.5 volumes of cold methanol and samples were than left on ice for 20 minutes. Samples were centrifuged for 10 minutes at 18,000g at 4°C. Supernatant was spotted in 5 μL aliquots up to 25–25 μL final volume on silica gel thin layer chromatography (TLC) plate (Whatman Ltd, Piscataway, NJ). TLC was performed using NH4OH:CHCl3:CH3OH:H2O (2:0.5:4.5:1) till the solvent ran halfway up the plate. The plate was air dried and exposed to x-ray film for 24 hours. Radioactivity in each spot was counted on the Ambis auto analytical scan and quantified and results expressed as pmol/mg/h (33).
Measurement of NOS Isoenzyme mRNAs: Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from hemibrains using the (Qiagen, Valencia, CA) RNeasy Lipid Tissue Mini Kit protocol. Total complementary DNA (cDNA) was produced by reverse transcription using the (Applied Biosystems, Foster City, CA) TaqMan reverse transcription system of 0.2 \( \mu \)L of purified RNA, 3 \( \mu \)L 10× RT buffer, 6.6 \( \mu \)L MgCl\(_2\), 6 \( \mu \)L 2.5 mM deoxyribonucleotide triphosphates, 1.5 \( \mu \)L random hexamers, 0.6 \( \mu \)L RNase inhibitor, and 0.75 \( \mu \)L Multiscribe RT. Samples were incubated for 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C. Quantitative real-time polymerase chain reaction (RT-PCR) was performed in an Applied Biosystems 7300 Real-Time PCR System. Amplification was carried out in 25-\( \mu \)L reaction mixtures containing 1 \( \mu \)L of template cDNA, 0.5 \( \mu \)L of each 5 mM primer, 12.5 \( \mu \)L 2× SYBR green master mix, and 10.5 \( \mu \)L PCR water. Cycling conditions were as follows: one cycle at 95°C for 10 minutes; 50 cycles of 95°C for 15 seconds and 60°C for 1 minute; followed by one cycle at 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. Primers for quantitative RT-PCR were made using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and primer efficiency was between 95% and 105%. Sequences were as follows: iNOS forward 5′-GACGAGACGGATAGGCAGAG, reverse 5′-CTTCAAACGCCTCCAGGAAC; eNOS forward 5′-CACCCAGAGCTTTCTTTCTTTGC, reverse 5′-GCTTACAGACCACAGGATTAGGA; nNOS forward 5′-TTCTCCCTCCTTGGAGAAAGAG, reverse 5′-TGCCACAGGATTCCATA. The relative amount of gene copies was extrapolated using the comparative Ct method with beta-actin as a normalizer and statagene mouse standard RNA as a calibrator.

Measurement of NOS Isoenzyme Proteins: Western Blotting

Whole brains were removed and weighed, and hemibrains frozen at −80°C until they were processed. The brains were homogenized on ice in 5 volumes (5× the weight in grams) of lysis buffer containing (in mM) 20 Tris–HCl, 150 NaCl, 2 ethylenediaminetetraacetic acid, 1 ethylene glycol tetraacetic acid, 0.5% Triton X-100, and 600:1 protease inhibitor cocktail (Sigma, St. Louis, MO). To remove cellular debris, the samples were centrifuged at 1,000 \( g \) for 10 minutes at 4°C and the supernatant was saved. The supernatant was shaken for 30 minutes at 4°C, followed by centrifugation at 18,000 \( g \) for 20 minutes at 4°C. The supernatant was saved and protein concentrations were determined using the Pierce BCA Protein Assay Kit. For sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis, proteins were separated on 3%–8% Tris acetate gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose. NOS isoenzymes were detected by chemiluminescence (Pierce) using rabbit anti-nNOS (1:1,000), rabbit anti-iNOS (1:1,000), rabbit anti-eNOS (1:1,000), and horseradish peroxidase-conjugated anti-rabbit (1:10,000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Semiquantifiable analysis of the protein was done using Image J Software downloaded from the
National Institutes of Health. Results were expressed as a percentage of values found in 2-month-old SAMP8 mice. NOS protein levels were not normalized with actin (tested at antibody dilution of 1:5,000) as levels were statistically lower in 12-month-old antisense-treated SAMP8 mice than in 2-month-old SAMP8 mice. The nNOS was retested with normalization to rabbit anti-gamma-tubulin (1:1,000; sc-7396; Santa Cruz).

Statistical Analysis
Means are reported with their standard errors. Results were compared by t test using the Prism 5.0 statistical package software.

RESULTS
No differences in NOS activity (Figure 1, upper panel), NOS isoenzyme mRNA levels, or NOS isoenzyme protein

Figure 3. Effects of antisense treatment on nNOS expression. Antisense directed against APP decreased nNOS protein either when expressed as absolute values (upper left panel) or when corrected to gamma-tubulin (lower left panel). mRNA for nNOS was also decreased (upper right panel). Lower right panel shows representative western blots for nNOS and gamma-tubulin proteins.

Note: APP = amyloid precursor protein; nNOS = neuronal nitric oxide synthase.

Figure 4. Effects of antisense treatment on iNOS and eNOS expression. Antisense treatment resulted in an increase in iNOS mRNA levels (upper left panel) but no change in iNOS protein levels or eNOS mRNA or protein levels.

Note: iNOS = inducible nitric oxide synthase; eNOS = endothelial NOS.
levels were found between 2-month-old CD-1 and 2-month-old SAMP8 mice. NOS activity increased with age in the SAMP8, with the 12-month-old SAMP8 having activity levels about twice as high as the 2-month-old SAMP8 (t = 3.11, df = 16, p < .01; Figure 1, lower panel). There were no differences between 2-month-old and 12-month-old SAMP8 mice in mRNA or protein levels for any of the isoenzymes.

Treatment of 12-month-old SAMP8 mice with antibody directed against Aβ produced an increase of about 43% in NOS activity: 0.173 ± 0.011 (control); 0.248 ± 0.015 (antibody); t = 3.11, df = 16, p < .01. Antisense treatment of 12-month-old SAMP8 mice produced an increase of about 76% in NOS activity (Figure 2): t = 2.62, df = 16, p < .05. The 12-month-old antisense-treated SAMP8 mice decreased in nNOS mRNA (t = 3.23, df = 4, p < .05; Figure 3, upper right panel) and nNOS protein levels regardless of whether assessed as absolute levels (t = 3.74, df = 15, p < .01; Figure 3, upper left panel) or corrected to gamma-tubulin (t = 2.88, df = 5, p < .05; Figure 3, lower left panel). The lower right panel of Figure 3 shows representative gels for nNOS and gamma-tubulin. Antisense treatment of 12-month-old SAMP8 mice increased iNOS mRNA (t = 3.24, df = 5, p < .05; Figure 4, upper left panel) but had no effect on iNOS protein, eNOS mRNA, or eNOS protein levels (Figure 4).

**DISCUSSION**

We investigated the levels of NOS activity and isoenzyme expression in the SAMP8 mouse, an animal model of AD. NOS activity levels in 2-month-old SAMP8 mice, an age at which the SAMP8 does not show cognitive impairment, were similar to levels seen in 2-month-old CD-1 mice, a strain of common white laboratory mice. Twelve-month-old SAMP8 mice had NOS activity levels that were more than twice as high as 2-month-old SAMP8 mice. The age of 12 months is a time when SAMP8 mice have elevated levels of APP, Aβ, and oxidative stress and are cognitively impaired (27,31). This is consistent with four other reports on NOS levels in SAMP8 (25,26,34,35). However, we found no differences between 2-month-old and 12-month-old SAMP8 mice in their eNOS, iNOS, or nNOS mRNA or protein levels. This suggests that the increase in NOS activity is mediated through posttranslational mechanisms.

Treatment of 12-month-old SAMP8 mice with antibody (36) or antisense regimens known to reduce brain levels of APP or Aβ, reverse oxidative stress, and improve cognition (29,31) were found to increase NOS activity further. This would be consistent with reports showing that Aβ inhibits the production of nitric oxide. In vitro, Aβ has been shown to decrease constitutive nitric oxide production (37). Here, the increase in NOS activity was accompanied by an increase in iNOS mRNA but not nNOS protein levels. The increase in iNOS mRNA suggests that Aβ may be exerting its inhibitory effects on NOS at the transcriptional level, but the lack of effect on iNOS protein levels again suggests that posttranslational mechanisms dominate. One such posttranslational mechanism is the ability of Aβ to alter NOS phosphorylation, a mechanism by which Aβ inhibits eNOS activity (22,23). Other posttranslational mechanisms include the ability of Aβ to oxidize proteins (38), a mechanism by which Aβ inhibits ATPase activity (39). Aβ can also bind directly to proteins, a mechanism by which it alters the activity of the alpha7 nicotinic receptor (40), and to ionic cofactors such as copper (41).

A decrease with antisense treatment in nNOS mRNA and protein levels suggests that Aβ could increase nNOS levels. Most neurotransmitters that modulate memory are hormetic, with low doses improving and high doses inhibiting memory (42,43). Our preliminary studies have suggested that Aβ shows similar qualities (44). This would suggest that at low amounts nNOS would improve memory, but with further increases, it would inhibit memory.

In conclusion, we found that the aged SAMP8 mouse has increased levels of NOS activity in the hippocampus. Antibody and antisense treatments aimed at lowering Aβ levels increased NOS activity further. These increases in NOS activity were not reflected in changes in mRNA or protein levels for the isoenzymes and, in fact, nNOS mRNA and protein levels decreased with antisense treatment. These results show a complex relation between Aβ and NOS activity and suggest that the major interactions of Aβ on NOS are mediated through posttranslational mechanisms.

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**CONFLICT OF INTEREST**

No author has a conflict of interest.

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