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ORIGINAL RESEARCH COMMUNICATION

NOX Activity Is Increased in Mild Cognitive Impairment

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Abstract

This study was undertaken to investigate the profile of NADPH oxidase (NOX) in the clinical progression of Alzheimer's disease (AD). Specifically, NOX activity and expression of the regulatory subunit $p47^{phox}$ and the catalytic subunit $gp91^{phox}$ was evaluated in affected (superior and middle temporal gyri) and unaffected (cerebellum) brain regions from a longitudinally followed group of patients. This group included both control and late-stage AD subjects, and also subjects with preclinical AD and with amnestic mild cognitive impairment (MCI) to evaluate the profile of NOX in the earliest stages of dementia. Data show significant elevations in NOX activity and expression in the temporal gyri of MCI patients as compared with controls, but not in preclinical or late-stage AD samples, and not in the cerebellum. Immunohistochemical evaluations of NOX expression indicate that whereas microglia express high levels of $gp91^{phox}$, moderate levels of $gp91^{phox}$ also are expressed in neurons. Finally, *in vitro* experiments showed that NOX inhibition blunted the ability of oligomeric amyloid beta peptides to injure cultured neurons. Collectively, these data show that NOX expression and activity are upregulated specifically in a vulnerable brain region of MCI patients, and suggest that increases in NOX-associated redox pathways in neurons might participate in the early pathogenesis of AD. *Antioxid. Redox Signal.* 12, 1371–1382.

Introduction

Alzheimer's disease (AD) is a progressive and irreversible loss of cognitive function and is the most common dementing disorder of the elderly (19). The etiology of AD is poorly understood, and existing treatments unfortunately have only limited efficacy in slowing clinical decline. A major research emphasis in AD has recently been placed on early evaluation, with the hope of identifying the earliest clinical manifestations of disease onset. The clinical diagnosis of mild cognitive impairment (MCI) has arisen from these efforts. The diagnosis of MCI is applied to aged persons expressing consistent, measurable memory impairments without dementia or significant disability related to activities of daily living (ADLs) (50, 68). Many AD subjects often are first seen clinically with amnestic MCI, which is thought to be a transition between normal aging and early dementia and, at present, likely represents the best opportunity for pharmacologic interventions. Current data suggest that conversion from MCI to dementia occurs at a rate of 10 to 15% per year, with ~80% conversion by the sixth year of follow-up, although about 5% of MCI subjects remain stable or convert to normal (3, 10).

Although considerable emphasis has been placed on the study of MCI to define early mechanisms of neurodegeneration in AD, the drive to identify the earliest manifestations of AD also has led to the concept of preclinical AD (PCAD). Although no precise definition exists for PCAD, which is also called presymptomatic AD or prodromal AD, the concept has arisen from neuropathologic analyses of nondemented subjects who nonetheless demonstrate some degree of AD pathology at autopsy. Specifically, the label PCAD is given to subjects who fall within the normal range on antemortem psychometric tests, but show sufficient AD pathologic hallmarks to meet National Institute on Aging–Reagan Institute (NIA-RI) intermediate- or high-likelihood criteria (Braak stage III or higher and moderate or frequent neuritic plaque scores). Although these patients do not quite meet clinical

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diagnostic criteria for MCI or dementia, some PCAD subjects perform less well (still within the normal range) on immediate paragraph recall and word-list delayed recall than do those without significant AD-like pathology (54) and frequently exhibit an absent or attenuated "practice effect" on repeated cognitive measures (13).

The exact mechanisms of AD neurodegeneration are not fully understood, but AD brains are typified by ample pathologic evidence of oxidative stress in affected regions, including the cerebral cortex and hippocampal formation (38). Markers of oxidative stress also are elevated in MCI (25, 67) and PCAD (42), consistent with the hypothesis that pathways that include the production of free radicals participate in the progression of AD.

Although the physiologic source of AD-related oxidative stress is not known, free radicals are produced in mammalian cells as secondary by-products by many different systems, including mitochondrial electron transport, xanthine oxidase, cyclooxgenases, and monoamine oxidases [reviewed in (12, 51)]. However, the enzyme NADPH oxidase (NOX) is noteworthy, as it is dedicated to the specific and deliberate production of free radicals. NOX is a superoxide-producing enzyme system consisting of membrane ($gp91^{phox}$ and $p22^{phox}$) and cytosolic ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) components (1, 11). On activation, the cytosolic regulatory component $p47^{phox}$ becomes heavily phosphorylated, and the entire cytosolic complex migrates with the small GTPase Rac to the membrane, where all components assemble to form the active oxidase. The membrane-integrated protein $gp91^{phox}$ is the catalytic core of the enzyme responsible for the electron transfer from NADPH to molecular oxygen for superoxide production. Although NOX was first described in phagocytic cells such as microglia, it is now well established that NOX subunits are expressed in neurons and astrocytes (26, 45). Experimental evidence points to a role for NOX in neuronal physiology, particularly in functions relating to hippocampal electrophysiology (29, 62). These data raise the possibility that alterations to NOX might participate in perturbations to neurons, and many reports have proposed that NOX may be involved in AD pathogenesis [reviewed in (5, 66)]. For example, published reports show that AD brains are associated with increased membrane localization of $p47^{phox}$ (58). Other recent reports show that NOX activity *in vitro* can be increased by β-amyloid peptides ($A\beta$), and that NOX may be involved in $A\beta$-induced neuronal injury (20, 43).

Finally, genetic deletion of $gp91^{phox}$ was recently shown to decrease neurovascular dysfunction and cognitive decline in transgenic mice overexpressing the Swedish mutation of the human amyloid precursor protein (49). Thus, ample support indicates that NOX could participate in AD, and this study was undertaken to delineate the profile of NOX enzymatic activity and subunit expression in both vulnerable and spared regions of AD and control brains. These studies used a very well characterized group of age- and gender-matched samples with exceptionally low postmortem interval autopsies (less than 4 h).

Additionally, as few studies have investigated the putative early forms of AD, our studies also included samples with MCI and PCAD to span the clinical progression of AD. Finally, *in vitro* studies were conducted to understand better the physiologic implications of increased NOX activity in neurons.

Materials and Methods

Human subjects

All subjects were followed up longitudinally at the University of Kentucky Alzheimer's Disease Center (ADC) with annual neuropsychologic, physical, and neurologic examinations. A total of 10 control, 10 PCAD, 7 MCI, and 10 late-stage AD subjects were used to quantify NOX activity and expression. The diagnoses of MCI, PCAD, AD, and normal cognitive function were based on integration of clinical and neuropathologic data and were defined by consensus conference. All control subjects had neuropsychological scores in the normal range with no evidence of memory decline.

Histopathologic examination of normal control subjects showed only age-associated changes and Braak staging scores of 0 to II, meeting the NIA-Reagan Institute low-likelihood criteria for the histopathologic diagnosis of AD. As a precise set of clinical criteria do not exist for the diagnosis of PCAD, PCAD subjects were distinguished as those individuals with antemortem psychometric test scores in the normal range, and yet still showed sufficient AD pathologic alterations to meet intermediate or high NIA-Reagan Institute (NIA-RI) criteria [Braak scores of III to V, with moderate or frequent neuritic plaque score according to Consortium to Establish a Registry for AD (CERAD)].

All MCI patients were normal clinically on enrollment into the longitudinal study, and MCI developed during follow-up. The clinical criteria for diagnosis of amnestic MCI included (a) memory complaints, (b) objective memory impairment for age and education, (c) normal general cognitive function, (d) intact activities of daily living, and (e) failure to meet criteria for dementia. The neuropathologic findings of MCI subjects were previously described (39) and demonstrated Braak staging scores of III to V.

All AD subjects demonstrated progressive intellectual decline, met NINCDS-ADRDA Workgroup criteria for the clinical diagnosis of probable AD (41), and also met accepted criteria for the histopathologic diagnosis of AD with typical Braak scores of VI. Clinical progression to AD was diagnostically characterized by (a) a decline in cognitive function from a previous higher level, (b) decline in one or more areas of cognition in addition to memory, (c) a clinical dementia rating scale score of 0.5 to 1, (d) impaired activities of daily living, and (e) a clinical evaluation that excludes other causes of dementia. Demographic and pathologic data for all subjects in the study are shown in Table 1.

Measures of NOX activity

Frozen samples taken from the cerebellum (CBLM) and superior and middle temporal gyri (SMTG) of control, PCAD, MCI, and AD patients were homogenized in protease inhibitor–containing buffer at 4°C and then subjected to differential centrifugation to isolate membranes. Membrane samples (10 to 25 μg total protein) were incubated with 5 μM lucigenin and 100 μM NADPH, and NOX activity was measured immediately by documenting the light produced by each sample at 37°C. Light emission was recorded from each sample in 10-s intervals for exactly 3 min. The specific role of NOX in the measured luminescence was determined by subtracting the background level of luminescence for each sample [generated by the inclusion of 1 μM diphenyleneio-

Table 1. Patient Demographics and Information

	Control	*PCAD*	*MCI*	*AD*
Age (yr)	87.4 ± 4.24	84.4 ± 4.92	89.0 ± 6.2	79.1 ± 8.0
Number of samples (M/F)	10 (3 M/7 F)	10 (2 M/8 F)	7 (3 M/4 F)	10 (3 M/7 F)
Postmortem interval (h)	3.13 ± 0.85	2.74 ± 0.67	2.81 ± 0.58	3.1 ± 0.72
Brain weight (g)	1,138 ± 110	1,074.6 ± 356	1,117.9 ± 143	1,045.0 ± 108
Braak stage	0.6 ± 0.69	3.9 ± 0.57[a]	3.71 ± 0.76[a]	6.0 ± 0.0[a]

All subjects were followed up longitudinally at the University of Kentucky Alzheimer's Disease Center (ADC). All data were assessed by one-way ANOVA followed by Tukey's *post hoc* analyses, as described in Methods. Statistical comparison of age, postmortem interval, and brain weight showed no significant differences between control, PCAD, MCI, or AD subjects.

[a]Significantly increased ($p < 0.001$) Braak staging scores for PCAD, MCI, and AD subjects, as compared with control subjects.

donium (DPI) within the sample]. NOX activity is presented as average luminescent counts per minute (CPMs) per microgram protein.

Measures of p47^{phox} and gp91^{phox} expression

Brain samples taken from the same patients used for NOX activity were homogenized in a Tris-buffered saline (pH 7.4) lysis buffer containing 0.1% Triton X-100, 5 m*M* EDTA, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Samples were then denatured in SDS, and equivalent amounts of protein were electrophoretically separated in polyacrylamide gels and blotted onto nitrocellulose. Blots were processed by using the following primary antisera: anti-p47^{phox} (1:500; Millipore, Billerica, MA); anti-gp91^{phox} (1:500, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), and anti-tubulin (1:100, Wako Chemicals USA, Inc., Richmond, VA). After incubation with primary antibodies, blots were washed and exposed to horseradish peroxidase–conjugated secondary antibodies and visualized by using a chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Blot images were scanned and densitometrically analyzed for quantification. To ensure accurate quantification across multiple blots, samples from all groups (control, PCAD, MCI, and AD) were included in each individual blot, and all data are presented as a ratio of expression over that of tubulin, which was included as a loading control.

Histologic evaluation of gp91^{phox} and neuronal and microglial markers

For histology, postmortem brain specimens taken from the SMTG cortex of selected control and MCI patients were immersion-fixed in 4% paraformaldehyde for 24 h and processed for paraffin embedding, and 6-μm sections were cut and collected for immunohistochemical analysis by using antibodies to gp91^{phox} (1:100; Santa Cruz Biotechnology). To visualize the cellular distribution of NOX expression, sections were double-labeled for gp91^{phox} and neuronal or microglial cell markers by using the following primary antisera: anti-Iba-1 (1:100, Wako Chemicals) and anti-NeuN (1:100; Abcam Inc., Cambridge, MA). For overall qualitative evaluations of the patterns of immunoreactivity, sections were incubated with biotinylated or peroxidase-linked secondary antibodies, and then visualized by using diaminobenzidine (DAB, for gp91^{phox}) or NOVAred (for NeuN and Iba1) as chromagens by following manufacturer's instructions (Vector Laboratories, Burlingame, CA). To document nonspecific staining, the primary antibodies were omitted from the staining protocol.

Preparation of synthetic soluble Aβ (1-42) oligomers

Stable Aβ (1-42) (rPeptide) oligomers were generated as described previously (35, 36). In brief, Aβ was solubilized (100 μg/ml) from a dried hexafluoroisopropanol film in DMSO and added to 50 m*M* NaPi/150 m*M* NaCl, pH 7.5 in 5 ml at a final concentration of 20 μg/ml in a polypropylene container, vortexed briefly, and incubated at room temperature for 2 h. Oligomer formation was stopped by the addition of BSA to 1%. The oligomers were isolated by size exclusion on a 125-ml Sephadex G-75 column equilibrated in culture medium, collecting 5-ml fractions. The oligomers were eluted in the void volume of the G-75 column (greater than 70 kDa) and the Aβ concentration was quantified with ELISA (6E10/bio4G8 sandwich ELISA).

Establishment of primary rat neuron cultures

Neuronal cultures were established as described previously by our laboratory (23, 64). Primary rat cortical neuronal cells were cultured from E18 Sprague–Dawley rats and maintained in 5% CO_2 at 37°C in MEM/Neurobasal medium containing 5% fetal bovine serum (heat inactivated), N2 supplement, B27 supplement, and 1% antibiotic. Cells were used in experiments between days 5 and 8 after plating. All cell-culture supplies were obtained from GIBCO Life Sciences (Gaithersburg, MD).

Analysis of neuronal injury in vitro

Neuronal viability was quantified as described previously by our laboratory (23, 64). In brief, viable neurons were counted in premarked microscope fields (five distinct fields per dish) before treatment and 24 h after treatment, with the viability of neurons assessed by morphologic criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable, whereas neurons with fragmented neurites and vacuolated or swollen soma (or both) were considered nonviable, as were neurons that detached from the dish over the course of treatment. The number of viable neurons both before and after treatment was determined, and survival was expressed as the percentage of total neurons present before treatment that remained viable after 24 h. These morphologic analyses of viability were confirmed with additional measures of cell viability determined by quantifying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion, as described previously (24, 63). In brief, after treatment *in vitro*, cells were exposed to 0.5 mg/ml MTT in serum-free and

phenol red–free medium for 1 to 4 h at 37°C. After exposure, medium was aspirated, and formazan precipitates were extracted by addition of 100 μl dimethylsulfoxide. The amount of formazan formation was analyzed within an hour by determination of optical density at 570 nm with reference wavelength of 630 nm on a spectrophotometric UV/VIS plate reader (Molecular Devices, Sunnyvale, CA). At least eight cultures were used for each data point.

Statistical analyses

All data are shown as mean ± standard error of the mean. Data were analyzed with one-way analyses of variance (ANOVA) followed by Tukey's Multiple Comparisons Tests to determine differences in clinically affected groups as compared with controls. Statistical significance was accepted at $p < 0.05$.

Results

Patient-cohort characteristics

Subject demographic and pathologic data are shown in Table 1. Braak-staging scores for PCAD, MCI, and LAD subjects were significantly higher than those for control subjects, but statistical comparison of age, postmortem interval, and brain weight showed no significant differences in these parameters between control, PCAD, MCI, and AD subjects.

NOX activity in samples that span the clinical spectrum of AD

Although reports have demonstrated that NOX is activated by amyloid beta in cell-culture and animal models (20, 43, 49), the profile of NOX enzymatic activity in AD has not been evaluated, nor has NOX been specifically evaluated in cases of MCI or PCAD. Initial experiments were thus designed to determine NOX activity in affected and nonaffected brain regions across the clinical spectrum of AD. Specifically, samples were taken from the CBLM, which is generally not associated with AD pathology or dysfunction, and the SMTG, which are significantly affected by AD, of control, PCAD, MCI, and late-stage AD patients and evaluated for NOX activity, as described in Methods. One-way ANOVA of NOX activity in the SMTG indicated significant differences between groups ($F_{(3,\ 33)} = 5.5$; $p = 0.0035$). Post hoc analyses show that although no significant difference in NOX activity was found in PCAD or AD samples compared with controls, NOX activity in MCI samples was significantly elevated compared with activity in control samples taken from the SMTG (Fig. 1A). Conversely, one-way ANOVA of NOX activity in the CBLM indicated no significant differences in mean NOX activity between groups ($F_{(3,\ 33)} = 0.94$; $p = 0.7988$), and post hoc analyses did not reveal any significant differences in NOX activity between PCAD, MCI, and AD samples compared with controls (Fig. 1B).

NOX subunit expression in samples that span the clinical spectrum of AD

Experiments were then designed to determine whether the observed increases in NOX activity were associated with enhanced expression of key NOX subunits. To this end, the expression profile of the regulatory subunit $p47^{phox}$ and the catalytic subunit $gp91^{phox}$ in the CBLM and SMTG of control, PCAD, MCI, and late-stage AD patients was measured with Western blot, as described in Methods. Evaluation of blots representative of SMTG samples indicated that $p47^{phox}$ expression relative to tubulin was increased in MCI compared with control samples (Fig. 2A). Quantification of all samples across multiple blots confirmed specific increases in $p47^{phox}$ expression in MCI samples (Fig. 2B). Specifically, one-way ANOVA of $p47^{phox}$ expression in the SMTG indicated significant differences between groups ($F_{(3,33)} = 5.166$; $p = 0.0049$), and post hoc Tukey's analyses indicated significant increases in $p47^{phox}$ expression in MCI samples as compared with control samples (Fig. 2B). Conversely, no observable difference was noted in $p47^{phox}$ expression in the CBLM (Fig. 2C). One-way ANOVA of $p47^{phox}$ expression in the CBLM confirmed that no significant differences in mean $p47^{phox}$ expression existed between groups ($F_{(3,33)} = 0.1459$; $p = 0.9316$), and post hoc analyses did not reveal any significant differences in NOX activity between PCAD, MCI, or AD samples compared with controls (Fig. 2D).

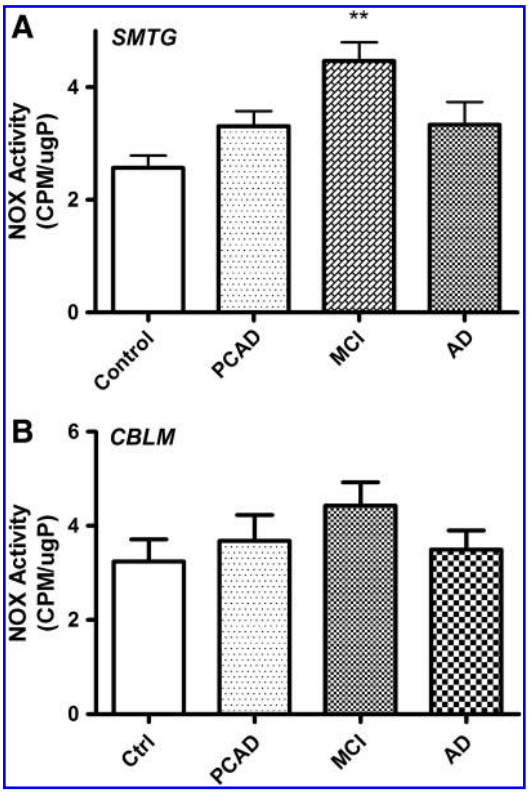

FIG. 1. NOX activity in affected and nonaffected brain regions of control, PCAD, MCI, and AD patients. Tissue samples from the (**A**) superior/middle temporal gyri (SMTG) and (**B**) cerebellum (CBLM) were collected from normal control subjects, subjects with preclinical Alzheimer's disease (PCAD), subjects with amnestic mild cognitive impairment (MCI), and subjects with definitive, late-stage Alzheimer's disease (AD) and analyzed for NOX activity, as described in Methods. All data were analyzed with one-way ANOVA followed by Tukey's *post hoc* analyses, as described in Methods. **Significantly increased ($p < 0.01$) NOX activity in SMTG of MCI samples as compared with control SMTG samples.

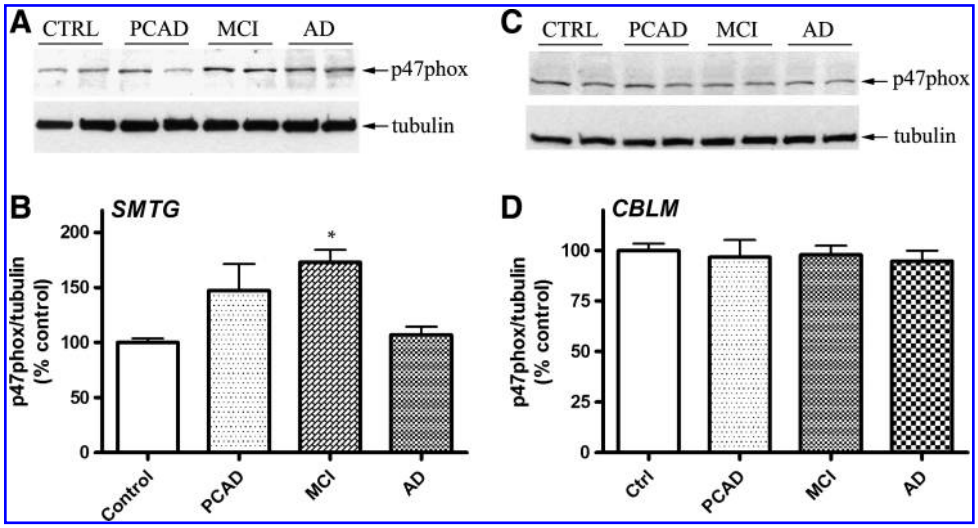

FIG. 2. p47^{phox} expression in affected and nonaffected brain regions of control, PCAD, MCI, and AD patients. Tissue samples from the (**A, B**) superior/middle temporal gyri (SMTG) and (**C, D**) cerebellum (CBLM) were collected from normal control subjects, subjects with preclinical Alzheimer's disease (PCAD), subjects with amnestic mild cognitive impairment (MCI), and subjects with definitive, late-stage Alzheimer's disease (AD) and analyzed for p47^{phox} expression with Western blot, as described in Methods. (**A**) Representative blots illustrating p47^{phox} and tubulin expression in SMTG from control, PCAD, MCI, and AD samples. (**B**) p47^{phox} expression in SMTG was quantified with Western blot, and data were analyzed with one-way ANOVA followed by Tukey's *post hoc* analyses, as described in Methods. *Significantly increased ($p < 0.05$) p47^{phox} expression in MCI samples as compared with control samples. (**C**) Representative blots illustrating p47^{phox} and tubulin expression in CBLM from control, PCAD, MCI, and AD samples. (**D**) Quantification of p47^{phox} expression in cerebellar tissue homogenates.

Expression of the catalytic subunit gp91^{phox} in control, PCAD, MCI, and late-stage AD patients revealed a pattern similar to that observed for p47^{phox}. Representative blots of gp91^{phox} expression in SMTG samples indicated increased expression relative to tubulin in MCI samples (Fig. 3A). Quantification across multiple blots confirmed alterations in gp91^{phox} expression across groups (Fig. 3B), with one-way ANOVA demonstrating significant differences in mean gp91^{phox} expression in the SMTG ($F_{(3,31)} = 3.694$; $p = 0.0221$). Post hoc Tukey's analyses likewise indicated significant increases in gp91^{phox} expression in MCI samples as compared with control samples (Fig. 3B). Conversely, only modest observable differences were seen in CBLM gp91^{phox} expression in MCI, PCAD, and AD samples compared with controls (Fig. 3C). Whereas one-way ANOVA of CBLM gp91^{phox} expression did indicate significant differences in mean gp91^{phox} expression between groups ($F_{(3,33)} = 3.5$; $p = 0.026$), post hoc analyses did not reveal any significant differences in NOX activity between PCAD, MCI, and AD samples specifically compared with controls (Fig. 3D).

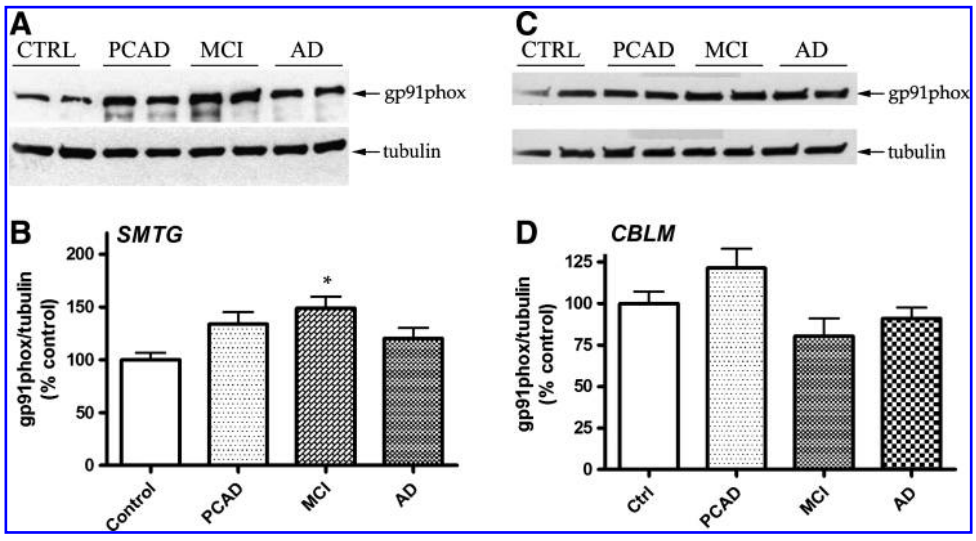

FIG. 3. gp91^{phox} expression in affected and nonaffected brain regions of control, PCAD, MCI, and AD patients. Tissue samples from the (**A** and **B**) superior/middle temporal gyri (SMTG) and (**C** and **D**) cerebellum (CBLM) were collected from normal control subjects, subjects with preclinical Alzheimer's disease (PCAD), subjects with amnestic mild cognitive impairment (MCI), and subjects with definitive, late-stage Alzheimer's disease (AD) and analyzed for gp91^{phox} expression with Western blot, as described in Methods. (**A**) Representative blots illustrating gp91^{phox} and tubulin expression in SMTG from control, PCAD, MCI, and AD samples. (**B**) gp91^{phox} expression in SMTG was quantified with Western blot, and data were analyzed with one-way ANOVA followed by Tukey's *post hoc* analyses, as described in Methods. *Significantly increased ($p < 0.05$) gp91^{phox} expression in MCI samples as compared with control samples. (**C**) Representative blots illustrating gp91^{phox} and tubulin expression in CBLM from control, PCAD, MCI, and AD samples. (**D**) Quantification of gp91^{phox} expression in cerebellar tissue homogenates.

Histologic pattern of gp91^{phox} expression

Although these data indicate that NOX activity and expression are increased in MCI compared with control, these data do not give any indication as to which cells are carrying this signal. This issue is noteworthy as, whereas the pathophysiologic consequences of increased NOX activity/expression in the brain are frequently thought to reflect toxic microglial overactivation, NOX subunits are expressed in neurons and astrocytes (26, 45). Indeed, NOX has been shown to participate in neuronal synaptic physiology (29, 62), raising the possibility that altered NOX activity or expression or both in neurons could perturb their function. To resolve whether neuronal or astrocytic NOX could participate in the increased

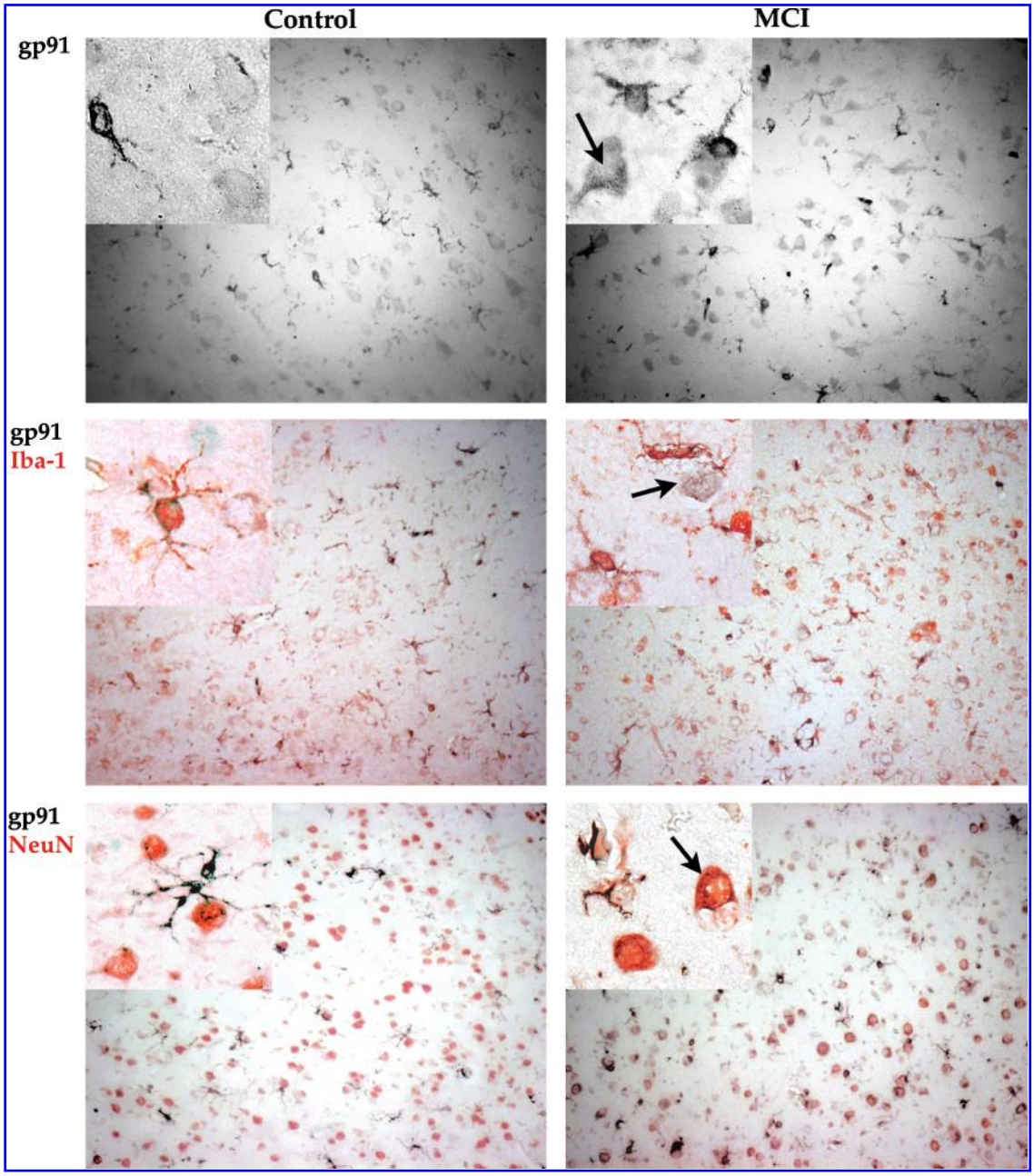

FIG. 4. Cell-type–specific expression of cortical gp91^{phox} in control and MCI patients. Brain sections from the cortex were collected from normal control subjects and subjects with amnestic mild cognitive impairment (MCI) and analyzed for gp91^{phox} expression with immunocytochemistry, as described in Methods. (**Top**) Representative low-magnification and high-magnification (insert) images depict the pattern of gp91^{phox} staining in cortex of control (left) and MCI (right) patients. *Arrow*, gp91^{phox} immunoreactivity in a cell with neuronal morphology. (**Middle**) Representative low-magnification and high-magnification (insert) images depict the pattern of gp91^{phox} staining (black DAB stain) specifically in Iba-1–positive microglia (NOVAred stain) in cortex of control and MCI patients. *Arrow*, gp91^{phox} immunoreactivity not associated with Iba-1 expression. (**Bottom**) Representative low-magnification and high-magnification (insert) images depict the pattern of gp91^{phox} staining (black DAB stain) specifically in NeuN-positive neurons (NOVAred stain) in cortex of control and MCI patients. *Arrow*, gp91^{phox} immunoreactivity in NeuN-positive cells.

NOX expression and activity detected in MCI patients, the expression of gp91^{phox} was evaluated immunohistochemically in control and MCI brain tissue, as described in Methods. Qualitative analyses of gp91^{phox} expression in the SMTG sections demonstrated prominent gp91^{phox} immunoreactivity in cells with morphology typical of microglia (Fig. 4, top panel). However, gp91^{phox} immunoreactivity was also consistently noted in larger cells that resembled neurons, particularly in MCI samples (Fig. 4, top panel). To more accurately confirm the cell-type–specific pattern of gp91^{phox} expression, control and MCI sections were double-labeled for gp91 and cell markers specific for either microglia (Iba-1) or neurons (NeuN). Evaluation of tissue sections double-labeled for gp91^{phox} and Iba-1 showed very prominent microglial localization of gp91^{phox} expression (Fig. 4, middle panel). However, a less intense but consistent pattern of gp91^{phox} staining that was not associated with Iba-1–positive cells was observed. Evaluation of tissue sections double-labeled for gp91^{phox} and NeuN then confirmed that neurons express detectable levels of gp91^{phox}, particularly in MCI brains (Fig. 4, bottom panel).

NOX inhibition decreases oligomeric Aβ-induced neurotoxicity

Although many investigators have proposed a role for NOX in promoting neuronal injury [reviewed in (5, 33)], cause-and-effect relations between NOX activation and neuronal injury in AD have not been fully established. It is thus possible that the increased NOX activity noted in MCI patients could be a compensatory mechanism to stabilize or maintain cognitive function, as NOX is a key regulator of ROS generation in synaptic plasticity and memory formation [reviewed in (28)]. To determine how NOX activity might relate to neuronal injury in AD brain, cell-culture experiments were designed that use small, oligomeric Aβ species. A soluble, oligomeric form of Aβ was specifically chosen, as emerging data increasingly indicate that rather than deposited Aβ, small oligomeric forms are the amyloid species associated with AD neuropathology, neuronal viability, and synaptic dysfunction [reviewed in (44)]. For these experiments, primary hippocampal neurons were isolated and plated, as described in Methods, and neuronal viability was determined after exposure to 36 nM oligomeric Aβ in the presence or absence of the NOX inhibitor apocynin (100 μM), which acts by preventing the assembly of the NADPH oxidase subunits (60). Data show that oligomeric Aβ caused significant decreases in cell viability, as measured with either MTT or morphologic cell counts (Fig. 5A–C). Conversely, cell death was significantly attenuated when neurons were exposed to oligomeric Aβ in the presence of apocynin (Fig. 5A–C). Additional experiments confirmed the effects of apocynin by using specific decoy peptides (gp91ds-tat) that also specifically prevent the assembly of NADPH oxidase (48, 52) (data not shown).

Discussion

In this report, data obtained from human brain samples that span the clinical spectrum of AD progression are described. Specifically, samples were taken from a group of age-and gender-matched normal control, PCAD, MCI, and late-stage AD patients. By using brain regions that are vulnerable to AD (SMTG) and generally unaffected by AD (CBLM), we designed experiments to determine whether NOX activity and expression increased with the clinical progression of AD. With a luminescent assay to detect NADPH-dependent free radical production, data show that NOX activity is increased over control levels in the SMTG of MCI brains, but not in PCAD or late-stage AD samples. Likewise, no significant changes in NOX activity over control were found in the CBLM of PCAD, MCI, or AD patients. Data also show that expression of the catalytic and regulatory subunits of NOX is increased in vulnerable regions of MCI brains. Finally, immunohistologic data suggest that the increases in NOX expression in MCI samples could reflect alterations in microglial or neuronal expression (or both) of NOX subunits. Overall, these data are in general agreement with published studies suggesting a role for NOX in the pathogenesis of AD (5, 66) and further extend these studies by demonstrating increased NOX activity and expression specifically associated with early disruptions of cognitive function in human patients. Collectively, these findings indicate that increased NOX activity is associated with the very early manifestations of dementia and suggest either that NOX might actively participate in disruptions in neuronal function, or that increased NOX activity is triggered in response to the pathophysiologic event(s) disrupting cognition.

Although the etiology of AD-related pathology and cognitive decline has not been elucidated, advanced age is the strongest risk factor for AD. Among the many hypotheses of aging, the free radical theory of aging (15) describes a mechanistic rationale for the slow decline in body function with aging that may have particular relevance to the development of AD. This oxidative stress–based theory describes a redox imbalance, whereby the production of free radicals overtakes endogenous antioxidant capacity, leading to oxidative damage to critical cellular elements (16). Oxidative stress has been implicated in brain aging and AD (6, 8, 9, 38, 59). Finally, markers of oxidative stress are elevated in cases of MCI (25, 67) showing that even the most early manifestations of dementia are associated with oxidative stress. Thus, ample evidence indicates that oxidative stress could drive the onset and progression of AD, but the source of AD-related oxidative stress is not fully understood. Although evidence supports a role for a "mitochondrial cascade hypothesis," in which age-related increases in basal mitochondrial ROS production lead to neuronal dysfunction and pathologic hallmarks of AD (61), data in this article support the hypothesis that increased NOX might participate with mitochondria in AD-associated oxidative stress, which is in keeping with data presented in previous publications (20, 43, 49, 58). Although NOX has been proposed to participate in other neurodegenerative syndromes, the detrimental actions of NOX appear to be most strongly associated with age-related chronic disease processes, such as Parkinson disease and atherosclerosis, in addition to AD [reviewed in (33)]. Based on these observations, a concept termed "antagonistic pleiotropy" has been proposed to explain a potentially dual role of NOX in the brain, describing a scenario in which the physiologic production of reactive oxygen species (ROS) garners an advantage in early life, but the sustained or aberrant activation of NOX culminates in harmful effects with age later in life (33).

The immunohistochemical data presented here show that microglia express high levels of gp91^{phox}, suggesting that the observed increases in NOX activity might reflect altered or enhanced microglial reactivity. This potential scenario is

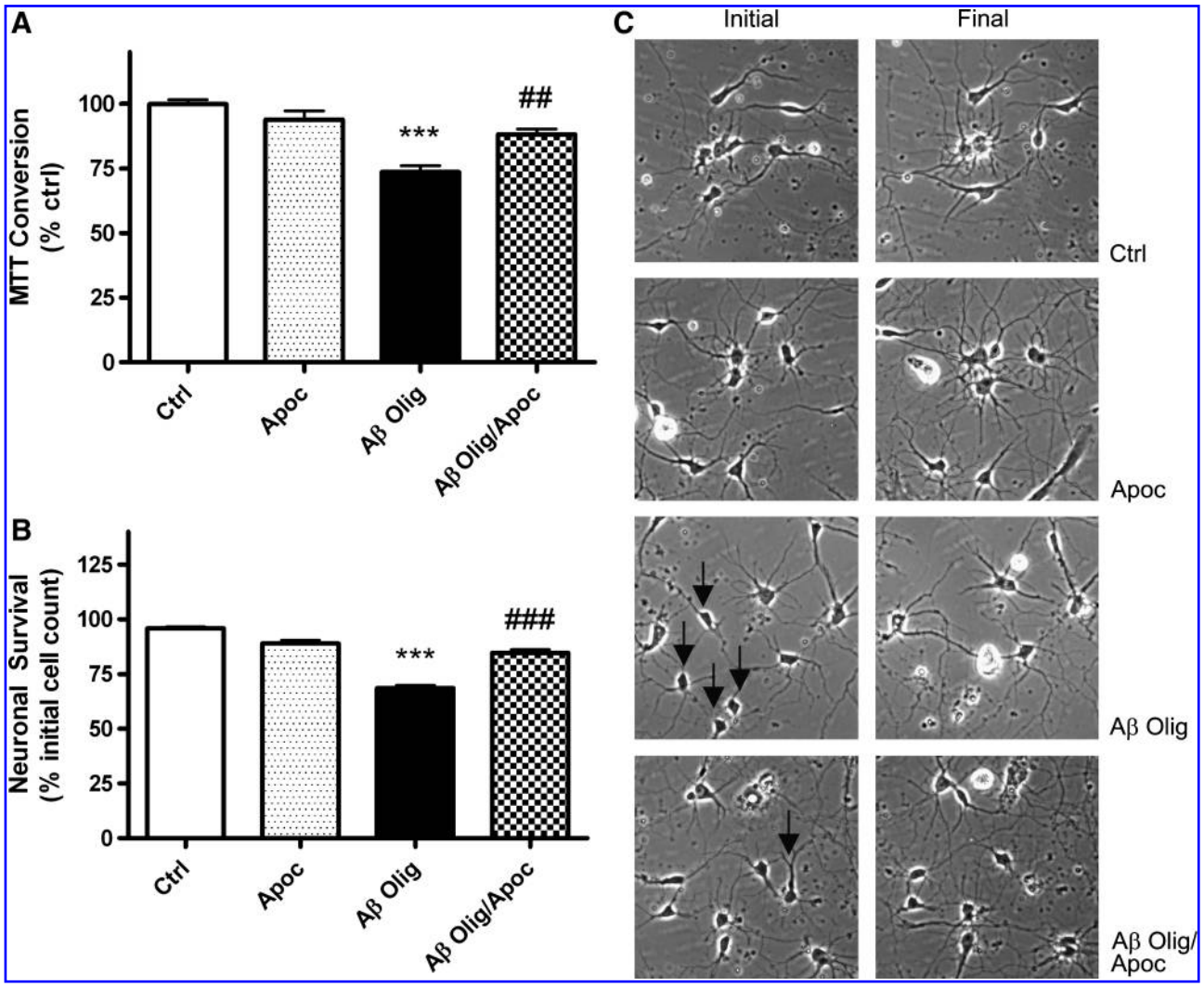

FIG. 5. Effects of NOX inhibition on oligomeric Aβ-mediated neurotoxicity *in vitro*. Primary hippocampal neurons were treated with 36 n*M* oligomeric Aβ (Aβ Olig) in the presence or absence 100 μM apocynin (**Apoc**) for 24 h, and neuronal injury was determined with MTT and cell counts, as described in Methods. (**A**) At the end of the exposure period, cells were treated with 0.5 mg/ml MTT for 2 h, after which the medium was aspirated, the formazan precipitates dissolved in DMSO, and the amount of formazan conversion analyzed at 570 nm. Results were generated from three separate experiments with at least four dishes per treatment group in each. Data are presented as percentage of MTT conversion in control cultures and were analyzed with one-way ANOVA. ***Statistically significant decrease ($p < 0.001$) in neuronal survival after exposure to oligomeric Aβ, as compared with control neurons. ##Statistically significant increase ($p < 0.01$) in neuronal survival in neurons treated with oligomeric Aβ in the presence of apocynin, as compared with cells treated with oligomeric Aβ alone. (**B**) Blinded cell counts based on repeated measures of morphology were conducted to quantify neuronal survival, as described in Methods. Results were generated from three separate experiments with at least four dishes per treatment group in each. Data reflect the number of cells remaining at the end of the exposure period and are expressed as the percentage of cells counted in each field initially. Data were analyzed with one-way ANOVA. ***Statistically significant decrease ($p < 0.001$) in neuronal survival after exposure to oligomeric Aβ as compared with control neurons. ##Statistically significant increase ($p < 0.01$) in neuronal survival in neurons treated with oligomeric Aβ in the presence of apocynin, as compared with cells treated with oligomeric Aβ alone. (**C**) Representative images of primary hippocampal neurons taken either immediately before (Initial) or after (Final) exposure to oligomeric Aβ in the presence or absence of apocynin. *Arrows*, Specific neurons that were healthy at the onset of exposure but did not survive exposure to oligomeric Aβ; they illustrate the decrease in cell loss noted in cells co-treated with apocynin.

supported by an existing body of literature implicating the aberrant or excessive activation of microglia in the pathogenesis of AD [reviewed in (53)]. Activation of NOX is a characteristic feature of microglial activation, both *in vitro* and *in vivo*, and experimental evidence suggests that ROS generated by activated microglia in AD could directly contribute to brain injury by inducing lipid peroxidation, DNA fragmentation, and protein oxidation in surrounding cells, a phenomenon called "bystander lysis" (40). In addition to direct oxidative stress, NOX has been shown to drive intracellular inflammatory signaling and the promulgation of neurotoxic inflammatory cascades in addition to contributing to local

concentrations of free radicals (64). For instance, NOX activity has been specifically implicated in the activation of NF-κB and the synthesis of TNF-α (22), which may be important mediators of age- and AD-related neuronal damage. Likewise, NOX activity has also been shown to be critical for the microglial efflux of glutamate (2), which is especially interesting in light of the use of the NMDA-receptor antagonist memantine in clinical settings of AD. Although the potential clinically therapeutic effects of memantine have not been critically evaluated in MCI, it may be possible that NOX-based increases in glutamate release, especially in concert with free radicals and inflammatory cytokines, could lead to disruptions in synaptic signaling and thus alter cognition. Overall, many potential mechanisms exist whereby increases in microglial NOX could undermine neuronal function and viability, and they are not mutually exclusive. However, although microglial activation is well associated with AD, few studies have specifically addressed the number or phenotype of microglia in MCI. Interestingly, recent PET imaging studies using carbon-11–linked (R)-PK11195 have documented enhanced microglial activation in many MCI patients (7, 46), although other groups have not been successful in detecting enhanced PK11195 binding in MCI subjects (65).

NOX expression was initially described in phagocytic cells like microglia, but it is now well established that NOX subunits are expressed in nonphagocytic brain cells like neurons (26, 45). Thus, the possible role of neuronal NOX in mediating MCI highlighted by neuronal expression of $gp91^{phox}$ in MCI brains is not without merit. The widespread expression of NOX subunits has led to the recognition that deliberate ROS production by NOX plays an important role in biologic events, including neuronal signaling (29, 62), in addition to well-established roles in host defense. Substantial evidence suggests that ROS are important signaling molecules involved in synaptic plasticity and memory formation, and that NOX is a key regulator of ROS generation in synaptic plasticity and memory formation [reviewed in (28)]. For example, published reports have documented cognitive dysfunction in human patients with chronic granulomatosis (47), a clinical condition caused by mutation in the $gp91^{phox}$ gene. Furthermore, mice deficient in either $gp91^{phox}$ or $p47^{phox}$ have been shown to have disrupted cognitive function and memory (27). Thus, these data raise the rather unforeseen possibility that the increased NOX activity noted in this report could be a compensatory mechanism actually to preserve cognitive function. However, selective experimental data appear to indicate a delicate balance of ROS required for signaling, with either too little or too much ROS resulting in impairments in long-term potentiation (LTP) and memory (30). In further relation to aging and the development of AD, data suggest an age-related shift in the role of ROS signaling in hippocampal LTP and memory formation (18, 21, 30). For example, overexpression of the antioxidant enzyme superoxide dismutase has been shown to impair LTP in young mice, but preserves LTP in aged mice (18, 21), whereas long-term treatment of mice with low-molecular-weight antioxidant enzyme mimetics can reverse hippocampus-dependent learning deficits in aged mice (37). Thus, these data support the hypothesis that aberrant or sustained NOX activation in neurons can directly disrupt cellular and synaptic function, particularly in aging.

The physiologic mechanisms of NOX activation in brain are not well understood, and indeed, the molecular sources of oxidative stress in AD are still subject to debate and investigation. Although the specific role that amyloid β peptides (Aβ) play in causing AD is subject to controversy, it is clear that Aβ-containing senile plaques are associated with degenerating neurons, and that Aβ peptides are potently bioactive both *in vitro* and *in vivo*. Thus, evidence suggests that AD may be mediated at least in part *via* the overproduction of Aβ [reviewed in (55)], and data convincingly demonstrate that Aβ can increase NOX activity in cultured neurons and microglia (4, 20, 57). However, the specific association of increased NOX with MCI and not with PCAD, even though both syndromes share similar Braak scores, and not AD, which is typified by extensive neuritic amyloid plaques, argues against an obvious role for amyloid deposits in driving NOX activation. However, the simple deposition of amyloid has been generally shown to be dissociated with many key biologic alterations, including the behavioral deficits in human and rodents (17). Evidence suggests that smaller oligomeric Aβ species correlate well with behavioral deficits and further are potent mediators of synaptotoxicity and neuronal death and [reviewed in (14, 44)]. Furthermore, the neurotoxic effects of Aβ oligomers occur in the low-nanomolar range, raising the possibility that even low concentrations of Aβ oligomers that may exist in the context of MCI, which precedes extensive Aβ deposition, could participate in neuronal injury and behavioral alterations. In support of this scenario, Aβ oligomers have been shown to colocalize with postsynaptic densities, and associate specifically with dendritic spine collapse and synapse loss (31). Further data indicate that small Aβ oligomers induce synaptic changes and dendritic spine loss *in vitro* (32, 56) and memory loss in Tg2576 mouse models of AD (34). Whereas the ability of small or soluble Aβ species to increase NOX activity was not specifically evaluated, the ability of NOX inhibitors to prevent oligomeric Aβ-induced neurotoxicity suggests that the increases in NOX activity noted in MCI patients might reflect the early neurodegenerative processes and loss of synaptic function that participate in the progression of AD. Studies are currently under way to document the ability of oligomeric Aβ to activate NOX in neurons and microglia, and also to document the presence and quantity of Aβ oligomers in samples spanning the clinical spectrum of AD. Collectively, these studies will lead to a better understanding of the role of NOX and soluble oligomeric Aβ in the pathogenesis of AD.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

Aβ = amyloid beta peptides
AD = Alzheimer's disease
CBLM = cerebellum
CERAD = Consortium to Establish a Registry for AD
DAB = diaminobenzidine
DPI = diphenyleneiodonium
LTP = long-term Potentiation
MCI = mild cognitive impairment
NIA-RI = National Institute of Aging–Reagan Institute
NOX = NADPH oxidase
PCAD = preclinical Alzheimer's disease
ROS = reactive oxygen species
SMGT = superior and middle temporal gyri

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