INFLAMMATORY MEDIATOR AND \( \beta \)-AMYLOID (25–35)-INDUCED CERAMIDE GENERATION AND \( \text{iNOS} \) EXPRESSION ARE INHIBITED BY VITAMIN E

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Abstract—To investigate the putative role of \( \beta \)-amyloid peptide (A\( \beta \)) in inducing oxidative stress damage in Alzheimer disease (AD), we studied the effects of proinflammatory cytokines and A\( \beta \) peptide on the induction of inducible nitric oxide synthase (iNOS). A\( \beta \)(25–35) upregulated the cytokine (TNF-\( \alpha \)/IL-1\( \beta \))-induced expression of iNOS and the production of nitric oxide (NO) in astrocytes, which were inhibited by vitamin E. A\( \beta \) treatment of C6 glial cells (together with LPS and IFN-\( \gamma \)), in addition to inducing iNOS, enhanced the oxidative stress as measured by increased expression of manganese superoxide dismutase and an increase in 2,7’-dichlorofluorescein diacetate fluorescence. We also observed that LPS, IFN-\( \gamma \), and A\( \beta \)(25–35) treatment led to the activation of the sphingomyelin–ceramide (SM-Cer) cascade with an increase in cellular ceramide. Inhibition of the SM-Cer cascade either by vitamin E treatment or by the neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin also resulted in alteration of the transcriptional binding activities of C/EBP, NF\( \kappa \)B, AP-1, and CREB in C6 glial cells. Hence, these findings suggest a role for ceramide in iNOS induction and NO production in A\( \beta \)-induced AD pathobiology and provide a possible explanation for the beneficial effects of vitamin E therapy. © 2004 Elsevier Inc. All rights reserved.

Keywords—Alzheimer disease, Inducible nitric oxide synthase, Vitamin E, \( \beta \)-Amyloid peptide, Ceramide, Reactive oxygen species, CCAAT enhancer binding proteins, Free radicals

INTRODUCTION

Alzheimer disease (AD) is characterized by the excessive accumulation of \( \beta \)-amyloid peptides (A\( \beta \)) and by the appearance of neurofibrillary tangles and senile plaques in the brain. Inflammatory responses induced by A\( \beta \) are suggested to play a role in the loss of cholinergic neurons in the AD brain, which is hypothesized to be the cause of early memory loss and other cognitive deficits that are hallmarks of AD. The observed increased expression of inducible nitric oxide synthase (iNOS) in the microglia and astrocytes surrounding the A\( \beta \) plaques and the significant increase in peroxynitrite in neurons suggest a role for peroxynitrite (ONOO\(^{-}\); a reaction product of NO\(^{-}\) and O\(_2\)\(^{-}\))-mediated pathology in the AD brain [1–4]. A\( \beta \) (1–42 fibrous form) was reported to upregulate the induction of iNOS in astrocytes treated with the proinflammatory cytokines interleukin 1\( \beta \) (IL-1\( \beta \)) and tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) [2]. Microglia associated with A\( \beta \) plaques stain positively for IL-1\( \beta \), and increased numbers of IL-1\( \beta \)-expressing microglia were found to be associated with AD progression [5]. These findings suggest the possible role of proinflammatory cytokines in the pathophysiology of AD.

Cytokines as well as A\( \beta \) peptides are known to induce oxidative stress in vitro in cell cultures [5,6]. Disturbance of the cellular redox balance in favor of reactive oxygen species (ROS)-generating systems results in cellular oxidative stress, which is thought...
to have deleterious effects on various intracellular events [6]. Cytokine-induced ROS were reported to activate the sphingomyelin (SM)–ceramide, signal transduction pathway, generating ceramide, which, in turn, was reported to mediate the induction of manganese superoxide dismutase (MnSOD), suggesting a role of oxidative stress in these events [7]. Ceramide-mediated upregulation of iNOS by NF-κB activation and its inhibition by antioxidants such as N-acetylcysteine (NAC) suggest a role for cellular redox in the SM–ceramide cascade and induction of MnSOD and iNOS [7,8].

Vitamin E is known for its antioxidant functions [11]. Vitamin E is a generic term for lipid-soluble compounds called tocopherols and tocotrienols that have antioxidant properties, as documented by protection against disease conditions associated with oxidative stress and in inflammatory disease [9,10]. Most of these effects have been attributed to the ability of these compounds to scavenge reactive oxygen and nitrogen species and to suppress radical-chain propagation reactions [11,12].

Here we report that the Aβ(25–35) upregulates the cytokine- or lipopolysaccharide (LPS)- plus cytokine-initiated induction of iNOS and MnSOD and the production of NO with an increase in the transcriptional binding activity of related transcription factors such as C/EBP, NF-κB, AP-1, and CREB. These events were coincident with an increase in ceramide levels and an increase in cellular oxidative stress. However, the Aβ/cytokine-induced alterations in cellular redox were attenuated by Vitamin E, resulting in the reduction of nitrite and ceramide levels with decreased induction of iNOS, MnSOD, and reduced activation of transcription factors.

MATERIALS AND METHODS

Reagents DMEM and fetal bovine serum were purchased from Life Technologies, Inc (Rockville, MD, USA). Rat recombinant IFN-γ, IL-1β, and TNF-α were obtained from R & D systems (Minneapolis, MN, USA). LPS (from Escherichia coli) and antibodies against iNOS were from Transduction Labs (Palo Alto, CA, USA). Antibody to MnSOD was obtained from Upstate Biotech (Waltham, MA, USA). β-Actin, Aβ(25–35) fragment, and the reverse peptide (35–25) were from Sigma (St. Louis, MO, USA). The neutral sphingomyelinase inhibitor 3-O-methylsphingomyelin, the ceramide synthase inhibitor fumonisin B1, and the acidic sphingomyelinase inhibitor desipramine were all from Biomol (Plymouth Meeting, PA, USA). All other reagents were obtained either from Calbiochem (La Jolla, CA, USA) or from Sigma unless otherwise mentioned, and all other antibodies and DNA sequences (C/EBP, NFκB, AP-1, and CREB) used for electrophoretic mobility shift assays (EMSA) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alzheimer brain pathology samples (Nos. 543, 591, and 673) from three women with confirmed AD diagnosis and control brain tissue samples (1068, 458, 464, and 627) from men who were cognitively normal were supplied by the Kathleen Price Bryan Brain Bank (Durnham, NC, USA). However, these samples were not matched by age or by postmortem delay.

Preparation of aged Aβ(25–35) and induction of cells with β-amyloid peptide

Both the Aβ(25–35) and the reverse peptide Aβ(35–25) were purchased from Sigma. They were solubilized in phosphate-buffered saline (PBS) at a concentration of 1 mM, incubated in a capped vial at 37°C for 4 days, and stored frozen at −20°C until use [13]. They were used at a final concentration of 7.5 μM or in higher amounts (wherever indicated).

Cell culture and treatment of astrocytes and C6 glial cells

Astrocytes were isolated from rat cerebral tissue as described earlier, according to the procedure by McCarthy and DeVellis [8]. Cells were maintained and induced with different stimuli in DMEM medium (Manassas, VA, USA) containing 4.5 g/L glucose and 10% fetal bovine serum. For isolation of astrocytes, after 10 days of culture, microglia and oligodendrocytes were removed by shaking for 24 h in an orbital shaker at 240 rpm. To ensure complete removal of all oligodendrocytes and microglia before subculturing, the shaking was repeated twice after 1 or 2 days. C6 glial cultures were maintained in DMEM/F-12 medium containing 10% fetal bovine serum. Cells were trypsinized, subcultured, and stimulated with LPS (0.25 μg/ml), IFN-γ (25 ng/ml), or any other cytokines with or without Aβ in serum-free medium and were also maintained and induced with different stimuli (wherever indicated). After treatment, cells were harvested after 18-h incubation at 37°C in 5% CO2 unless stated otherwise. Vitamin E, trolox (a water-soluble derivative of vitamin E), or any other additions were added 2 h prior to addition of stimuli and again at the time of addition of the stress stimuli.

Assay for NO synthesis

Synthesis of NO was determined by assaying culture supernatants for nitrite, a stable reaction product of NO and molecular oxygen. Briefly, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent and incubated at room temperature for 15 min. The
optical density of the assay samples was measured spectrophotometrically (570 nm). Fresh culture medium served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay [14].

**SDS–PAGE**

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the procedure described by Laemmli [15] and followed by immunoblot analysis. Briefly, after incubation in the presence or absence of different stimuli, C6 glial cells (2 × 10⁶/ml) were scraped off and washed with cold PBS, and the cell lysate was prepared by incubating the cells for 30 min on ice in 0.5 ml of lysis buffer containing 20 mM Hepes, pH 7.4; 2 mM EDTA; 250 mM NaCl; 0.1% Nonidet P-40; 0.1% Triton X-100; 2 µg/ml leupeptin; 2 µg/ml aprotinin; 1 mM phenylmethylsulfonyl fluoride; 0.5 µg/ml benzamidine; and 1 mM dithiothreitol for 30 min. The lysate was centrifuged, and the supernatant was collected. Cell extract proteins (50 µg) were resolved on 4–10% SDS–PAGE, electrotransferred onto a nitrocellulose membrane, blotted with indicated antibodies, and then detected by immunoblotting using an Imaging Densitometer (Model GS-670; Bio-Rad, Hercules, CA, USA).

**Preparation of nuclear extracts and electrophoretic mobility shift assay**

Nuclear extracts from treated or untreated cells (1 × 10⁷) were prepared using the method of Dignam et al. [16] with slight modification. Cells were harvested, washed twice with ice-cold PBS, lysed in 400 µl of buffer A (10 mM Hepes, pH 7.9; 10 mM KCl; 2 mM MgCl₂; 0.5 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 5 µg/ml aprotinin; 5 µg/ml pepstatin A; and 5 µg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pellet was resuspended in 40 µl of buffer B (20 mM Hepes, pH 7.9; 25% (v/v) glycerol; 0.42 M NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 5 µg/ml aprotinin; 5 µg/ml pepstatin A; and 5 µg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 µl of modified buffer C (20 mM Hepes, pH 7.9; 20% (v/v) glycerol; 0.05 M KCl; 0.2 mM EDTA; 0.5 mM dithiothreitol; and 0.5 mM phenylmethylsulfonyl fluoride) and stored at 70°C until further use. Nuclear extracts were used for the electrophoretic mobility shift assay using the NFκB DNA–binding protein detection system kit (Life Technologies, Inc.) according to the manufacturer’s protocol. Briefly, the protein binding DNA sequences (previously labeled with ³²P) of C/EBP, NFκB, AP-1, and CREB were incubated with nuclear extracts prepared after various treatments of C6 glial cells. The DNA–protein binding reactions were performed at room temperature for 20 min in a buffer containing 10 mM Trizma base, pH 7.9; 50 mM NaCl; 5 mM MgCl₂; 1 mM EDTA; and 1 mM dithiothreitol plus 1 µg of poly(dl-dC), 5% (v/v) glycerol, and ~0.3 pmol of ³²P-labeled C/EBP, NFκB, AP-1, or CREB (all from Santa Cruz Biotechnology). Protein–DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels at room temperature in 50 mM Tris, pH 8.3, 2 mM EDTA and were detected by autoradiography. For supershift analysis, 1 µg of the respective antibody (as indicated) was included in the DNA–protein binding reaction.

**Quantification of sphingomyelin by high-performance thin-layer chromatography (HPTLC) and densitometry**

Sphingomyelin was resolved from total lipid extracts by HPTLC (LHPK plates from Whatman, Clifton, NJ, USA) as described by Ganser et al. [17], for extraction of phospholipids with the following modification: the plates were overrun for 30 min during development and were dried overnight in a vacuum desiccator. Sphingomyelin was quantitated with densitometric scanning using an Imaging Densitometer (Model GS-670; Bio-Rad), and software provided with the instrument by the manufacturer.

**Quantification of ceramide levels by diacylglycerol kinase assay**

The total lipids were extracted either from the brain tissue or from cells exposed to LPS (10 µg/ml) and IFN-γ (25 units/ml) with or without β-amyloid peptide (25–35) fragments (5–30 µM), and with or without vitamin E treatment, for 18 h according to the method described earlier [7]. Ceramide content was measured according to Preiss et al. [18] using diacylglycerol kinase and [γ-³²P]ATP. Briefly, dried lipids were solubilized in a cocktail of octyl-β-D-glucopyranoside/cardiolipin solution (20 µl) and the kinase reaction was carried out using an imidazole buffer mix and [γ-³²P]ATP (sp act 1–5 × 10⁵ cpm/nmol) for 30 min at room temperature. The labeled ceramide 1-phosphate was extracted and resolved on HPTLC with a solvent system consisting of methyl acetate, n-propanol, chloroform, methanol, 0.25% KCl, and acetic acid (100:100:100:40:36:2) as described earlier [7]. Quantification of ceramide 1-phosphate was performed by autoradiography and densitometric scanning with an Imaging Densitometer (Model GS-670; software provided with the instrument by the manufacturer). Data are expressed either as arbitrary units (absorbance) or as percentage change.
Measurement of intracellular peroxide formation

Formation of intracellular peroxides was detected by using the nonfluorescent cell permeant compound 2,7'-dichlorofluorescein diacetate (DCDFA; Molecular Probes, Eugene, OR, USA) [19,20]. After deesterification by endogenous esterases to their polar metabolite, carboxy dichlorofluorescein, which diffuses out of the cell slower than its parent compound, it can be oxidized by ROS and peroxides to form 2,7-dichlorofluorescein. However, its oxidation may not necessarily discriminate between various ROS species. The method described by Mattson et al. [20], with minor modification, was used. After different treatments, cells were washed with TBS (Tris-buffered saline: 1 ml, 2 min) with DMEM containing 1% FBS (DMEM/FBS) and incubated with 80 μM DCDFA (stock solution, 80 mM in dimethyl sulfoxide) in DMEM/FBS for 60 min at 37°C. Cells were washed twice with TBS at 37°C and were disrupted by addition of NaOH (0.1 N) in 50% MeOH and a uniform suspension was prepared with a pipette by pipetting in and out several times. Generation of ROS/peroxides was measured in a fluorescence plate reader (excitation, 485 nm; emission, 520 nm; gain, 10). Blanks (identically processed wells containing no cells, loaded with DCDFA) were subtracted.

Cell viability

Cytotoxicity of the inhibitors was determined by measuring the metabolic activity of cells using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.

Statistical analysis of the data

All data are expressed as means ± SEM. All necessary comparisons were carried out using the Tukey–Kramer multiple comparison test. Statistical differences at P < 0.05 were considered significant. The densitometric data for MnSOD by Western blot (Fig. 3B) and the nitric oxide data in Fig. 9A are expressed on an arbitrary scale.

RESULTS

Vitamin E attenuates Aβ(25–35)-enhanced iNOS gene expression in rat primary astrocytes and in C6 glioma cells

Rat primary astrocytes are known to produce iNOS in response to cytokines [1,23]. Aβ(25–35) upregulated the expression of iNOS protein and stimulated the production of nitrite in rat astrocytes in response to treatment with LPS (our unpublished observations) and to cytokines such as TNF-α and IL-1β (Fig. 1). Vitamin E treatment attenuated the Aβ-cytokine-induced expression of iNOS and production of nitric oxide (Figs. 1A and 1B). We performed the above experiments using the reverse peptide Aβ(35–25) as control for Aβ(25–35) peptide stimulation of iNOS. However, we did not observe any change in iNOS induction or in nitric oxide release by using this reverse peptide with any cytokine combination (results not shown).

Similar cytokine treatment of C6 glial cells showed sensitivity to LPS and IFN-γ treatment for induction of iNOS and MnSOD expression and production of nitric oxide (Fig. 2). This cytokine-induced iNOS expression and nitric oxide production were dose and time dependent. We observed iNOS expression as well as nitric oxide production 6 h after cytokine and Aβ treatment and an increase in expression of the iNOS protein and nitric oxide production up to 18 h (results not shown). Vitamin E blocked the induction of iNOS and attenuated MnSOD expression. Aβ(25–35) peptide enhanced iNOS gene expression, and its inhibition by vitamin E was also confirmed by the activity of an iNOS–luciferase (iNOS-Luc) construct in C6 glial cells after stimulation with cytokine in the presence of Aβ and in the presence or absence of vitamin E or trolox. We performed a dose–
response induction of iNOS expression and nitrite production in response to cytokine and A\(\beta\)(25–35) (Fig. 2C). Cells respond to the addition of increasing amounts of A\(\beta\). Although we observed enhanced iNOS protein expression that parallels nitrite production, higher doses of A\(\beta\) did not lead to a corresponding increment in nitrite. In conclusion, cytokine treatment of astrocytes or C6 glial cells induced iNOS expression, and nitric oxide production is further enhanced by A\(\beta\) addition and is attenuated by vitamin E treatment.

**Vitamin E attenuated A\(\beta\)(25–35)-induced ROS accumulation**

Studies from various laboratories describe cytokine-induced alterations in cellular redox and suggest a possible role for ROS in A\(\beta\)-mediated induction of
iNOS and production of NO [5,6,20–23]. To measure ROS/peroxide formation, cells were loaded with the nonfluorescent dye DCDFA, which upon interaction with ROS/peroxides, particularly H$_2$O$_2$, is converted to a fluorescent compound, DCF. As shown in Fig. 3A, treatment of C6 glial cells with LPS and IFN-γ led to generation of fluorescence. This fluorescence was increased several fold by treatment of C6 glial cultures with cytokines together with Aβ(25–35) (7.5 and 15 μM) in a concentration-dependent manner, whereas cultures treated with reverse peptide (35–25) showed weak or no fluorescence (data not shown). The induction of Aβ-mediated fluorescence was prevented by co-treatment with 10 μM vitamin E. These observations

![Graph showing DCFDA fluorescence](image)

**Fig. 3.** Vitamin E inhibits LPS- (0.25 μg/ml), IFN-γ- (25 U/ml), and Aβ(25–35)-induced cellular redox and MnSOD expression in C6 glial cells. (A) Aβ/LPS- and IFN-γ-induced ROS generation in C6 glial cells using the fluorescent compound DCDFA is inhibited by vitamin E (see Materials and Methods for details). The individual numbers at the bottom represent the different quantities of cytokines added to the cultures in concentrations as shown. The DCDFA fluorescence produced by cytokine (LPS, IFN-γ) and Aβ+ vitamin E is shown in lighter bars for clarity (lanes 9, 10, 13, and 14). Experiments were performed in triplicate and data are means (±SEM). *p < .05. The blot (in B) for MnSOD was exposed either for a shorter time interval, 30 sec (Sx), or for a longer time, 5 min (Lx), for the purpose of clarity. The protein band was quantitated using an imaging system (Bio-Rad imaging densitometer; Model GS-670) and represented as a histogram. Data are means ± SEM. *p < .05 was considered significant.
indicate that Aβ and cytokine treatment produced oxidative stress and that vitamin E blocked these effects. This conclusion is consistent with the observed increase in expression of MnSOD (Figs. 2 and 3B), where we observed at least a 3-fold increase in the MnSOD protein levels (a stress-response gene) under these conditions. Thus, vitamin E treatment attenuates the production of DCDFA fluorescence as well as induction of MnSOD (Figs. 3A and 3B). This effect of vitamin E was temperature sensitive, whereas the heat-denatured vitamin had no effect (results not shown).

Ceramide generated by Aβ(25–35)-induced degradation of sphingomyelin upregulates the induction of iNOS expression

Earlier reports indicate that cytokine-mediated alteration in cellular redox activates the sphingomyelin–ceramide signal transduction cascade by conversion of sphingomyelin to ceramide with an increase in gangliosides (sialic acid-containing glycosphingolipids), such as Gd3 [7,24,25]. The observed generation of peroxides by cytokine and Aβ treatment (Fig. 3A) may therefore reflect a possible perturbation in cellular ceramide homeostasis under these conditions. Hence, we measured the cellular levels of ceramide in response to LPS and IFN-γ and Aβ(25–35). Treatment of C6 glial cell cultures with cytokine (LPS and IFN-γ) together with varying concentrations of Aβ(5–30 μM) resulted in a dose-dependent increase in nitrite and a corresponding increase in ceramide with a decrease in sphingomyelin (Figs. 4A and 4B), suggesting a role of cytokine Aβ-induced ceramide generation in the induction of iNOS and production of NO. Pretreatment of C6 cells with 10 μM vitamin E reduced the LPS- and IFN-γ-induced ceramide production significantly (Fig. 4A) and the cytokine- and Aβ-induced ceramide to lesser extent.

To further understand the intracellular source of Aβ-induced generation of ceramide for iNOS induction, we tested the effect of 3-O-methyl sphingomyelin, a novel inhibitor of neutral sphingomyelinase, desipramine (an acidic sphingomyelinase inhibitor), and fumonisin B1 (ceramide synthase inhibitor) on the production of NO (Figs. 5A, and 5B). Treatment of cells with 3-O-methyl sphingomyelin, and to a lesser extent fumonisin B1, ameliorated the LPS-, IFN-γ- and Aβ-induced iNOS induction as well as the production of nitrite, thereby indicating that degradation of sphingomyelin by neutral sphingomyelinase activity and a biosynthetic pathway for ceramide may contribute to the ceramide pool (probably in a 60:40 ratio) and leading to the observed iNOS gene expression and increased production of NO. We also tested other compounds known to block neutral sphingomyelinase activity, such as Manumycin A [26] and 3,4-dichloroisocoumarin [27], which were effective in blocking iNOS expression as well as nitric oxide production (data not shown). However, these compounds are not specific for neutral sphingomyelinase, Manumycin A is also a potent farnesyl transferase inhibitor [28], and 3,4-dichloroisocoumarin [29] is a potent protease inhibitor as well as a granzyme inhibitor. Previous studies from this laboratory have reported the induction of the MnSOD gene by ceramide or by ceramide generated from sphingomyelin [7]. MnSOD is a mitochondrial enzyme for converting the superoxide anion to H2O2, and its expression was increased by LPS, IFN-γ, and Aβ treatment (Figs. 2, 3B, and 5B) and was decreased almost to control levels either by vitamin E or by treatment with 3-O-methyl sphingomyelin, indicative of mitochondrial stress, which correlated with increased iNOS induction. Hence, we conclude that 3-O-methyl sphingomyelin and fumonisin B1 attenuation of the LPS,IFN-γ/Aβ-induced expression of MnSOD and iNOS suggests a role for ceramide and is also indicative of the possible generation of free radicals during these cellular events.

Aβ activates transcription factors C/EBP, NFκB, CREB, and AP-1 in cytokine- and Aβ-induced inflammatory processes in C6 glial cells and is inhibited by vitamin E and neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin

Transcription factors NFκB, C/EBP, and AP-1 play an important role in the transactivation of iNOS gene expression and are important for cytokine as well as COX-2 expression [31–33]. Recent reports indicate a critical role for a family of transcription factors, the C/EBPs, in interactions with other transcription factors such as NFκB, Stat3, c-Myb, PU.1, SP-1, ATF-2, PPARs, and Runx-1 in regulating the transcription of various genes [33]. C/EBP binding activity showed activation with LPS, IFN-γ, and Aβ(25–35) treatment, and inhibition of activation of C/EBP was evident upon treatment of C6 glial cells with either vitamin E or 3-O-methyl sphingomyelin or trolox (Fig. 6A). Supershift analysis performed with nuclear extract obtained after treatment with LPS, IFN-γ, and Aβ with various antibodies for C/EBP (lanes 9–14) showed a clear supershifted band with C/EBPβ and a modest supershift with C/EBPδ.

NFκB is an important transcription factor activated under various stress conditions and is responsible for transcription of iNOS as well as several other genes associated with the inflammatory process. Vitamin E has been reported to attenuate NFκB activation in microglial cells [30]. Therefore we characterized the binding activity of NFκB by EMSA in nuclear extracts obtained from these glial cells. There was a significant increase in NFκB binding activity to NFκB consensus sequence, in response to LPS, IFN-γ, and Aβ(25–35) (Fig. 6B). This NFκB binding activity was reduced upon treatment with
3-O-methyl sphingomyelin (indicating a role for ceramide in Aβ-induced activation of NFkB) and to some extent with 10 μM vitamin E or trolox (1.5 mM). However, unlike the complete inhibition observed upon treatment with neutral sphingomyelinase inhibitor 3-O-methyl SM, use of a higher concentration of vitamin E did not appear to block its activation.

Transcription factors AP-1 and CREB play a significant role in antioxidant and redox regulation [30,32]. Hence, similar experiments were performed with the same nuclear extracts (as mentioned above) for AP-1 (Fig. 6B) and CREB, and these transcription factors showed a similar trend in their activation in the presence of cytokine and Aβ and inhibition by 3-O-methyl sphingomyelin.
Again, the effect of inhibition by vitamin E does not seem to be clear with CREB binding activity. However, none of these inhibitors alone had an effect on the activation of these transcription factors (results not shown).

Elevated levels of ceramide in Alzheimer’s brain compared to normal human brain

To evaluate the possible role of ceramide in the neuroinflammatory process in AD, levels of ceramide in control and in AD brains were measured. Total lipids were extracted from the frontal cortex region of control and Alzheimer brain samples and diacyl glycerol (DAG) kinase assay was performed to detect total ceramide and DAG levels. AD brain showed elevated levels of ceramide and DAG in comparison to normal control brain (Figs. 7A and 7B). We tested ceramide levels from three control and four Alzheimer brain samples. On average, the Alzheimer samples showed a 4-fold increase in ceramide levels in comparison to control brain samples. In Alzheimer, the atrophy is seen morphologically in the frontal and parietal regions with characteristic narrowed gyri and widened sulci. Portions of frontal cortex were analyzed for ceramide analysis, and tissue was also sectioned for microscopy and staining with Bielschow-

Fig. 5. Neutral sphingomyelinase inhibitor (3-O-methyl sphingomyelin) and ceramide synthase inhibitor (fumonisin B1) inhibit Aβ- and LPS- and IFN-γ-stimulated iNOS and MnSOD protein expression in C6 glial cells. C6 glial cells were treated with inhibitor of neutral sphingomyelinase 3-O-methyl sphingomyelin (3-O-methyl SM) and ceramide synthase inhibitor (fumonisin B1) and acidic sphingomyelinase inhibitor (desipramine) in the presence of LPS (0.25 μg/ml), IFN-γ (25 ng/ml), and Aβ (7.5 μM). Cell lysates were Western blotted after separation on 10% SDS–PAGE for iNOS and MnSOD detection after treatment, as indicated. (B) Almost complete inhibition of NO production and iNOS and reduction of MnSOD protein expression with higher concentrations of 3-O-methyl sphingomyelin (25 μM). Data are means (±SEM). *p < .05 was considered significant (values being compared with relative control values).
silver stain, which showed the characteristic neurofibrillary tangles and Alzheimer plaques, said to be rich in Aβ deposits (results not shown) [21,22].

**DISCUSSION**

The detection of activated glial cells (astrocytes and microglia) near the amyloid plaque deposits and the expression of proinflammatory mediators (e.g., TNF-α, IL-1β, iNOS) in the AD brain indicate that inflammatory disease plays a definite role in the pathobiology of AD [2,5]. Here we document the roles of cellular redox and bioactive lipid ceramide in the Aβ-induced expression of iNOS in C6 glial cells and astrocytes. Treatment of glial cells with vitamin E or the neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin can downregulate the expression of iNOS as well as production of NO, induced by cytokine and Aβ. These conclusions are based on the following observations (Fig. 8): (1) Aβ(25–35) upregulated the cytokine-mediated induction of iNOS and altered the cellular redox in glial cells. (2) Aβ(25–35) and cytokine treatment activated the sphingomyelin–ceramide signaling cascade by conversion of sphingomyelin to ceramide. (3) Inhibition of neutral sphingomyelinase by 3-O-methyl sphingomyelin and by the ceramide synthesis inhibitor fumonisin blocked the induction of iNOS, indicating a role of ceramide in Aβ-mediated induction of iNOS. (4) The increase in ceramide and MnSOD expression parallels the cytokine/Aβ-induced oxidative stress as measured by DCDFA. (5) Vitamin E treatment downregulates ceramide generation, MAP kinase activation (Fig. 9), and transcriptional binding activities as well as iNOS and MnSOD expression.

**Fig. 6.** Vitamin E and neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin (3-O-methyl SM) inhibit the Aβ-enhanced, LPS- and IFN-γ-mediated activation of transcription factors C/EBP, NFκB, AP-1, and CREB in C6 glial cells. (A) Aβ upregulates the C/EBP binding activity as seen by EMSA. EMSA was carried out using the nuclear extracts prepared from C6 glial cells after treatment with LPS and IFN-γ for 4 h either in the presence or in the absence of Aβ. In lanes 9–13 polyclonal IgG’s specific for C/EBP α, β, δ, and ε were used in supershift experiments with nuclear extracts from LPS, IFN-γ/Aβ-treated (4 h) C6 glioma cells for binding to γ-32p-labeled C/EBP oligomer. For clarity, the dried gel was exposed for autoradiography either for longer (24 h) or for shorter (6 h) periods. The top shows the longer exposure time (Lx) and the bottom shows the shorter exposure time (Sx). Note the supershifted complexes in lanes 10 and 11 (top; correspond to β and δ proteins). ε antibody from two different stocks was tested (in lanes 12 and 13). (B) Similar EMSA for detection of NFκB, AP-1, and CREB with the same nuclear extracts. Lane 8 is same as lane 3, for the binding assay, but with added cold probe as control to demonstrate the specificity of protein binding to the DNA oligomer (see Materials and Methods for details).

**Fig. 7.** Elevated levels of ceramide in Alzheimer brain in comparison to normal control human brain. The levels of ceramide and DAG were measured as described under Materials and Methods. Autoradiogram of HPTLC showing levels of ceramide (A) and its image (B).

The detection of activated glial cells (astrocytes and microglia) near the amyloid plaque deposits and the expression of proinflammatory mediators (e.g., TNF-α, IL-1β, iNOS) in the AD brain indicate that inflammatory disease plays a definite role in the pathobiology of AD [2,5]. Here we document the roles of cellular redox and bioactive lipid ceramide in the Aβ-induced expression of iNOS in C6 glial cells and astrocytes. Treatment of glial cells with vitamin E or the neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin can downregulate the expression of iNOS as well as production of NO, induced by cytokine and Aβ. These conclusions are based on the following observations (Fig. 8): (1) Aβ(25–35) upregulated the cytokine-mediated induction of iNOS and altered the cellular redox in glial cells. (2) Aβ(25–35) and cytokine treatment activated the sphingomyelin–ceramide signaling cascade by conversion of sphingomyelin to ceramide. (3) Inhibition of neutral sphingomyelinase by 3-O-methyl sphingomyelin and by the ceramide synthesis inhibitor fumonisin blocked the induction of iNOS, indicating a role of ceramide in Aβ(25–35)-mediated induction of iNOS. (4) The increase in ceramide and MnSOD expression parallels the cytokine/Aβ-induced oxidative stress as measured by DCDFA. (5) Vitamin E treatment downregulates ceramide generation, MAP kinase activation (Fig. 9), and transcriptional binding activities as well as iNOS and MnSOD expression.
The NO produced by iNOS and ONOO⁻, a reaction product of NO and O₂⁻, is known to be cytotoxic because it inactivates various cellular processes by nitrosylating proteins, lipids, and nucleic acids. The involvement of reactive oxygen species (O₂⁻, H₂O₂, OH⁻, etc.) in the pathogenesis of AD is indicated by lipid peroxidation [21,34] and increased activities of catalase, superoxide dismutase, and glutathione reductase in the hippocampus and amygdala (regions of brain that are affected most in AD pathogenesis) [34,35]. The Aβ/cytokine-induced intracellular signaling events leading to alteration of cellular redox, production of ceramide, induction of MnSOD and iNOS, and production of NO can be attenuated by treatment with antioxidants such as vitamin E or trolox. Accordingly, the Aβ-induced alterations in cellular redox and induction of MnSOD in glial cells in culture document a role for Aβ in altering the cellular redox in glial cells (Figs. 2–4) [20]. The possible role of ROS is proposed in plaque formation leading to neurodegeneration [36]. We observed a parallel relationship between the alteration in cellular redox and the induction of iNOS (Figs. 2–4). Moreover, the inhibition of induction of iNOS by vitamin E (a known antioxidant) documents the involvement of cellular redox in the Aβ/cytokine-induced proinflammatory signal transduction pathway in glial cells.

We have previously reported that the sphingomyelin–ceramide cascade is redox sensitive and that cytokine-induced alteration in cellular redox activates the neutral sphingomyelinase for production of ceramide [7,24]. Reduction in the levels of glutathione either by treatment with proinflammatory cytokines (TNF-α, IL-1β, IFN-γ) or by diame or exogenous H₂O₂ or increased endogenous H₂O₂ by inhibition of catalase activated the conversion of sphingomyelin to ceramide in astrocytes [7]. Moreover, addition of exogenous ceramide or ceramide generated by the action of exogenous sphingomyelinase upregulates the cytokine-induced induction of iNOS [24]. A 3.5-fold increase in

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**Fig. 8.** Flow chart showing possible signaling cascades in the Aβ-stimulated astroglial cells leading to neuronal stress/dysfunction and cell death. Inflammatory mediators (cytokines) together with Aβ protein are likely to activate the sphingomyelin–ceramide cascade, leading to sphingomyelin hydrolysis and generation of ceramide. Induction of reactive oxygen species (ROS) leads to enhanced expression of the mitochondrial antioxidant enzyme MnSOD. The activation of Ras and MAP kinases (ERKs, p38 kinase, and the stress-activated SAPK/JNKs) leads to activation of transcription factors such as AP-1, C/EBP, CREB, and NFκB, resulting in increased expression of iNOS and other proinflammatory genes, including Cox-1 and 2 and cytokines TNF-α, IL-1β, and IL-6 (our unpublished data). These events result in the possible alteration of the microenvironment of the neurons in terms of alteration in cellular redox (peroxynitrite formation, superoxide generation), imbalance in Ca²⁺ homeostasis, and DNA and protein oxidation, causing stress and thereby leading to neuronal cell death.
ceramide levels and parallel induction of iNOS by treatment of cells with Aβ (Fig. 4) document the role of Aβ-generated ceramide in the induction of iNOS. In situ, ceramide generation can occur by three different metabolic pathways: (1) as a result of de novo synthesis from serine and palmitoyl-CoA, (2) by degradation of sphingomyelin to ceramide by acid sphingomyelinase, or (3) by degradation by neutral sphingomyelinase [37,38]. The inhibition of induction of iNOS by inhibitors of neutral sphingomyelinase and that of synthesis of ceramide but not by inhibitors of acid sphingomyelinase indicate that the ceramide produced in Aβ/cytokine treatment originated from both the degradation of sphingomyelin by neutral sphingomyelinase and the synthetic pathway (Figs. 5A and 5B). The observed increase (greater than 3-fold) in ceramide by Aβ treatment with a parallel increase in iNOS and NO production (Figs. 4 and 5) and inhibition of iNOS by blocking the intracellular increase in ceramide docu-
ment a possible role of these lipid secondary messengers in the NO-mediated pathogenesis of neurodegenerative disorders such as AD. Several studies support a role for the hydrolysis of sphingomyelin to ceramide as a stress-activated signaling mechanism due to the effects of either cytokines, chemotherapeutic agents, or radiation, causing diverse biological responses that often lead to cell cycle arrest, apoptosis, and cell senescence [7,37–43]. Ceramide-activated kinases and phosphatases play a role in these events [7,41,44]. Our studies document a role for intracellular ceramide in Aβ-induced proinflammatory events in glial cells. Consistent with these in vitro data, the AD brain was found to contain higher amounts of ceramide than age-matched controls (Fig. 7). Recent reports from two laboratories, one demonstrating an increase in intracellular levels of ceramide in Alzheimer disease and the other showing that ceramide can promote β-peptide biogenesis, further substantiate the possible role of ceramide in the pathobiology of AD [45,46].

Finally, as demonstrated in the above studies, iNOS expression, ceramide generation, transcriptional factor binding activity, etc., were all blocked by the neutral sphingomyelinase inhibitor 3-O-methyl sphingomyelin. Interestingly, these were also blocked by Vit E. We performed our studies using α-tocopherol. However, our unpublished observations showed that other tocopherols and tocotrienols, i.e., other similar compounds with vitamin E activity, had similar effects on inhibition of iNOS and nitric oxide production. α-Tocopherol, the major vitamin E component, is known to quench free radicals and exhibit antioxidant effects [47]. As an antioxidant, vitamin E reduces the degeneration of hippocampal cells after cerebral ischemia and delays neurological deterioration in transgenic mice expressing human variants of β-amyloid precursor protein [48,49]. Moreover, at least one clinical trial suggests that vitamin E may help prevent the development of AD or slow further deterioration [50]. Hence, the above studies demonstrating attenuation/downregulation of cytokine- and Aβ-induced ceramide generation leading to iNOS expression elucidate a potential therapeutic mechanism of vitamin E. More significant, however, are our findings that signaling events transduced by ceramide and Aβ under inflammatory conditions and during aging might play a role in the AD disease process.

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**ABBREVIATIONS**

Aβ --- beta-amyloid peptide  
MnSOD --- manganese superoxide dismutase  
SM --- sphingomyelin  
C/EBP --- CCAAT enhancer binding protein  
DCDFA --- 2',7'-dichlorofluorescein diacetate  
EMSA --- electrophoretic mobility shift assay  
CREB --- cyclic AMP response element binding protein  
IFN-γ --- interferon-γ  
ROS --- reactive oxygen species