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Amyloid-β peptide enhances tumor necrosis factor-α-induced iNOS through neutral sphingomyelinase/ceramide pathway in oligodendrocytes

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Abstract

Although accumulating evidence demonstrates that white matter degeneration contributes to pathology in Alzheimer's disease (AD), the underlying mechanisms are unknown. In order to study the roles of the amyloid-β peptide in inducing oxidative stress damage in white matter of AD, we investigated the effects of amyloid-β peptide 25–35 (Aβ) on pro-inflammatory cytokine tumor necrosis factor-α (TNF-α)-induced inducible nitric oxide synthase (iNOS) in cultured oligodendrocytes (OLGs). Although Aβ 25–35 by itself had little effect on iNOS mRNA, protein, and nitrite production, it enhanced TNF-α-induced iNOS expression and nitrite generation in OLGs. Aβ, TNF-α, or the combination of both, increased neutral sphingomyelinase (nSMase) activity, but not acidic sphingomyelinase (aSMase) activity, leading to ceramide accumulation. Cell permeable C2-ceramide enhanced TNF-α-induced iNOS expression and nitrite generation. Moreover, the specific nSMase inhibitor, 3-*O*-methyl-sphingomyelin (3-OMS), inhibited iNOS expression and nitrite production induced by TNF-α or by the combination of TNF-α and Aβ. Overexpression of a truncated mutant of nSMase with a dominant negative function inhibited iNOS mRNA production. 3-OMS also inhibited nuclear factor κB (NF-κB) binding activity induced by TNF-α or by the combination of TNF-α and Aβ. These results suggest that neutral sphingomyelinase/ceramide pathway is required but may not be sufficient for iNOS expression induced by TNF-α and the combination of TNF-α and Aβ.

Keywords: amyloid beta-peptide 25–35, ceramide, inducible nitric oxide synthase, neutral sphingomyelinase, oligodendrocyte, tumor necrosis factor-α.

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The amyloid-β peptide (Aβ), a 39–43 amino acid fragment, derived from β-amyloid precursor protein, forms insoluble fibrillar aggregates that are deposited in the brains of the Alzheimer's disease (AD) patients (Selkoe 1999). These Aβ depositions have been implicated in neuronal and vascular degeneration, potentially contributing to progressive dementia, the characteristic of the disease (Selkoe 2000). Although the majority of previous studies focused on grey matter pathology in AD, accumulating data demonstrate that white matter degeneration contributes to pathology in AD as well. A high percentage of AD patients show evidence of white matter degeneration with severe loss of oligodendrocytes (OLGs) caused by apoptosis (Brown *et al*. 2000). The observed white matter pathology, including loss of myelin and axons (Brun and Englund 1986; Lassmann *et al*. 1995), as well as OLG apoptosis, might be indirect consequences of neuron damage in gray matter. Alternatively, such damage could be mediated directly by Aβ deposition, which has been reported in OLGs (Wilkins *et al*. 2001) and in animal models (Holtzman *et al*. 2000).

It has been shown that Aβ fibrils stimulate the production of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1β and nitric oxide (NO)

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Abbreviations used: Aβ, amyloid-β peptide 25–35; AD, Alzheimer's disease; aSMase, acidic sphingomyelinase; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; nSMase, neutral sphingomyelinase; OLG, oligodendrocyte; 3-OMS, 3-*O*-methyl-sphingomyelin; nitrite/nitrate, nitrite; PCV, packed cell volume.

in microglia and astrocytes, which promote neurodegeneration (Meda *et al*. 1995; Klegeris *et al*. 1997; Weldon *et al*. 1998; Combs *et al*. 1999; Yates *et al*. 2000). Although NO has beneficial roles, such as destruction of pathogens, removal of debris, and promotion of tissue repair, excess production of NO can be deleterious (Banati *et al*. 1993; Hewett *et al*. 1994; Bolanos *et al*. 1997). For example, inducible nitric oxide synthase (iNOS) production is increased in astrocytes surrounding amyloid plaques and significant peroxynitrite damage to neurons has been observed in AD brain (Brown and Bal-Price 2003).

Ceramide, a lipid second messenger, plays a role in mediating cellular responses as diverse as inflammation, differentiation, gene expression, growth suppression, and apoptosis (Andrieu-Abadie and Levade 2002). Ceramide elevation has been found in the brains of early staged AD patients (Gottfries *et al*. 1996; Zhang *et al*. 1999; Han *et al*. 2002). The regulation of ceramide levels is controlled by the activities of enzymes that synthesize and catabolize ceramide. Multiple routes of ceramide generation and removal exist, and these routes are responsive to a variety of cell stimuli (Pettus *et al*. 2002). One pathway of ceramide formation involves sphingomyelin hydrolysis by either neutral sphingomyelinase (nSMase) or acidic sphingomyelinase (aSMase). Another pathway involves ceramide synthase-catalyzed *de novo* ceramide synthesis. Various factors, such as cytokines, growth factors, hormones, ischemia/reperfusion, and radiation, are known to regulate one or more enzymes of ceramide metabolism, resulting in ceramide accumulation (Andrieu-Abadie and Levade 2002). In turn, ceramide regulates many key intracellular effectors, including the ceramide-activated protein kinases (Lozano *et al*. 1994; Yao *et al*. 1995), serine/threonine protein phosphatases (Hannun 1994), and the short-lived gaseous messenger nitric oxide (NO), that mediate the action of ceramide on the apoptosis, inflammation, and cell growth (Hannun 1994).

Although astrocytes and microglia are thought to be the major sources of iNOS expression in the CNS *in vivo*, recent evidence suggests that the myelin-producing OLGs themselves can express iNOS in culture (Merrill *et al*. 1997; Martinez-Palma *et al*. 2003). We have previously reported that Aβ is toxic to OLGs in culture (Xu *et al*. 2001). We have also demonstrated that Aβ-induced OLG death requires the activation of the nSMase/ceramide pathway and involves oxidative stress (Lee *et al*. 2004). Here we investigated the effects of Aβ on proinflammatory cytokine TNF-α-induced iNOS in OLGs. We found that Aβ or C2-ceramide by itself did not induce iNOS/nitrite production in OLGs; however, Aβ or C2-ceramide enhanced TNF-α-induced iNOS/nitrite production. We also showed that neutral sphingomyelinase/ceramide pathway is required but may not be sufficient for iNOS expression induced by TNF-α and a combination of TNF-α and Aβ. These results implicate that OLGs may be involved in free radical production in response to the combination of Aβ deposition and proinflammatory cytokines, contributing to the AD pathology in brain white matter.

Materials and methods

Chemicals and reagents

All chemicals were purchased from Sigma (St Louis, MO, USA) and all cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise specified.

Oligodendrocyte culture

Primary cultures of neural precursor cell (neurosphere) were prepared and maintained according to the methods of Zhang *et al*. (Zhang *et al*. 1999; Lee *et al*. 2004) with some modifications. Briefly, 14-day rat cortex was loosely homogenized in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium with 10 strokes, and centrifuged for 5 min at 350 *g*. The pellets were incubated with 1.5 mL of 0.025% trypsin at 37°C for 30 min followed by addition of 1.5 mL DMEM/F-12 with 20% fetal bovine serum and centrifuged. The pellets were washed with DMEM/F-12 twice. Dissociated cells were layered on a pre-formed Percoll gradient (50% Percoll and 50% DMEM/F-12) and centrifuged for 15 min at 23 500 *g*. The fraction containing glial progenitors located between the myelin and red blood cell fraction was recovered, washed, and then plated on a 75-mm uncoated culture flask with 20 mL of neurosphere medium (DMEM/F-12, N1 supplement, insulin 25 μg/mL, progesterone 130 ng/mL, basic fibroblast growth factor 20 ng/mL, epidermal growth factor 20 ng/mL). Neurospheres, formed 24 h after plating, were fed by adding 5 mL of fresh medium into the flask every other day. After 7 days, neurospheres were split (1:2) by gentle dissociation with a syringe (25 gauge) and plated in the same medium.

For derivation of neurosphere into progenitor, the neurospheres were treated with 0.05% trypsin/0.53 mM EDTA and centrifuged at 350 *g* for 10 min. The cells were resuspended in progenitor medium (69% DMEM/F-12/HEPES containing N1 supplement, 10 μg/mL insulin, 20 nM progesterone, 100 unit penicillin/streptomycin, 30% conditioned medium from B104 cells, and 1% fetal bovine serum) and plated on 100-mm culture dishes pre-coated with poly L-ornithine. Disaggregated oligosphere cells displayed bipolar or tripolar morphology.

For differentiated OLG cultures, progenitor cells were detached with trypsin and cultured on poly L-ornithine-coated dishes in mature OLG medium (DMEM/F-12; N1 supplement, 20 μg/mL biotin; 20 μg/mL triiodo-L-thyronine, T3, and 1% fetal bovine serum). Immunoreactivity to OLG surface markers including GalC, Rip, and CNP was observed in the vast majority of these differentiated cells within 48 h (Lee *et al*. 2004).

Sphingomyelinase assays

Cells were washed twice with phosphate-buffered saline, then lysed with 0.2% Triton X-100 in phosphate-buffered saline for 10 min and sonicated for 30 s in an ice-cold bath. Protein concentrations were determined by Lowry assay (Lowry *et al*. 1951). Methyl-[^{14}C]sphingomyelin (55 mCi/mmole, Amersham, Piscataway, NJ, USA), which served as the substrate for sphingomyelinase, was evaporated and re-suspended in 25 μL of nSMase assay buffer (40 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.2% Triton X-100) or

aSMase assay buffer (250 mM sodium acetate, pH 5.2, 0.2% Triton X-100) and sonicated to form micelles on ice until use. Reaction mixtures containing 25 μL of cell lysate protein (1 mg/mL) and 25 μL of nSMase or aSMase assay buffer containing methyl-[^{14}C] sphingomyelin (0.23 nmol) was incubated for 2 h at 37°C. The reaction was stopped by adding 200 μL $CHCl_3$/methanol (1:1) and 90 μL H_2O followed by rigorous mixing. The samples were then centrifuged at 1000 *g* for 5 min, and radioactive phosphocholine ([^{14}C]phosphocholine) in the aqueous phase (120 μL) was collected for liquid scintillation counting. Phosphocholine is the degraded moiety of sphingomyelin after ceramide is released by nSMase or aSMase. Activity was calculated as pmol of sphingomyelin hydrolysed by 1 mg of total protein per hour, and expressed as percentage of control values.

Thin-layer chromatography

Cells were cultured in medium containing 10 μCi [^{3}H]palmitate (1 mCi/mL, Amersham) for 25 h with or without exposure to Aβ and/or other agents. Labeled cells were collected and washed with phosphate-buffered saline three times to remove the free isotope before lipid extraction (Xu *et al*. 1998). The cell pellet was resuspended in 400 μL methanol : H_2O : 6 N HCl (100:5:1, v/v) followed by addition of 800 μL chloroform and 240 μL water. The mixture was then vortex-mixed and centrifuged at 10 000 *g* for 5 min. The lipid fraction recovered from the lower phase was blown to dryness and re-extracted with 1 mL chloroform: methanol (2:1, v/v) and resolved by TLC plate (Whatman, Maidstone, UK). The TLC solvent was chloroform : methanol : acetic acid : water (85:4.5:5:0.5, v/v) for ceramide. Plates were air-dried and sprayed with 1 M sodium salicylate before autoradiography. Standard lipids were identified separately and in combination with the samples by UV light after rhodamine 6G (Sigma) staining.

Reverse transcription–polymerase chain reaction

RT–PCR for iNOS has been previously reported (Xu *et al*. 1997). Briefly, equal amounts of RNA (2 μg) were reverse transcribed with oligo-dT and 500 μM dNTPs (BRL Life Technologies, Inc., Gaithersburg, MD, USA), 20 U RNasin (Promega, Madison, WI, USA), 200 μM dithiothreitol, and 200 U reverse transcriptase (BRL) for 50 min at 42°C. After incubation, the sample was heated for 5 min at 95°C, diluted and aliquoted. PCR was performed with a reaction mixture containing cDNA transcribed from 50 ng RNA, 100 μM dNTPs, 1 μM of each primer, 1.5 mM $MgCl_2$, and 2.5 U Taq polymerase (BRL). PCR reaction conditions were as follows: 25 cycles (for each cycle: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C) and a delay time of 10 min. The conditions were determined in preliminary studies to be within the linear range in terms of RT input and PCR cycles. The PCR products were electrophoresed through a 1% agarose gel and visualized by Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). The relative mRNA levels of each gene were determined after normalization based on endogenous cyclophilin mRNA.

Western blot analysis

Detection of iNOS expression in OLGs by western blot analysis has been previously reported (Xu *et al*. 1997). Briefly, OLGs were homogenized in western blot buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/mL leupeptin, 5 μg/mL aprotinin, 4 μg/mL pepstatin, pH 7.9) and centrifuged at 10 000 *g* for 15 min. Then 20 μg of protein from the supernatant of each sample was loaded onto an 8% polyacrylamide gel, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes by electrophoresis. The membranes were blocked in Tris-buffered saline/Tween-20 buffer containing 20 mM Tris-HCl, 5% non-fat milk, 150 mM NaCl, and 0.05% Tween-20 (pH 7.5) for 1 h at room temperature. Primary polyclonal antibody against a mouse macrophage iNOS (Transduction Laboratory, Lexington, KY, USA) at a 1:500 dilution was added to the membrane and incubated for 2 h at room temperature. The membranes were washed with Tris-buffered saline/Tween-20 three times at 10-min intervals, incubated with the second antibody (goat against rabbit IgG conjugated with alkaline phosphatase), diluted to 1:5000, for 1 h and then washed three times each at 10-min intervals with Tris-buffered saline/Tween-20 and two times each for 2 min with Tris-buffered saline. The color reaction based on the Blot AP System was carried out as described in the technical manual provided by Promega.

Griess assay

Nitrite was measured by the Griess assay (Misko *et al*. 1993). The reaction was performed by incubating 100 μL of medium and 100 μL of Griess reagents (1.32% sulfanilamide in 60% acetic acid and 0.1% *N*-1-naphthyl-ethylenediamine-HCl 1:1 mixed, freshly made) for 5 min. The absorbance at 540 nm was measured, and the amount of nitrite/nitrate was obtained by extrapolation from a standard curve using sodium nitrite as a standard.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

OLG viability was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Xu *et al*. 1998).

Preparation of nuclear extracts from oligodendrocytes

Following appropriate treatment of OLGs, crude nuclear mini-extracts were prepared according to the method described previously with modifications (Lee *et al*. 1988). Briefly, OLGs in 100-mm dishes were harvested using a cell scraper, pelleted by centrifugation, washed in cold phosphate-buffered saline solution, and incubated in 1 PCV (packed cell volume) of buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 μg/mL leupeptin, and aprotinin at pH 7.9). OLGs were resuspended in buffer A and kept on ice for 10 min, and then loosely homogenized. Crude nuclei were washed twice with buffer A and nuclear proteins were extracted with 2/3 PCV of ice-cold buffer B (20 mM HEPES, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin and aprotinin, and 25% glycerol at pH 7.9) for 30 min on ice. Then 1/3 PCV of ice-cold buffer C (20 mM HEPES, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and aprotinin at pH 7.9) was added, vortexed rigorously and centrifuged at 13 000 *g* for 15 min at 4°C. Nuclear proteins were aliquoted, frozen on dry-ice and stored at −80°C. Protein concentrations were determined by the Lowry method (Lowry *et al*. 1951).

Electrophoretic mobility shift assay

Nuclear factor κB (NF-κB) consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) from Promega was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmole) from Amersham according to Promega *Technical Bulletin* number 106. The binding reaction was performed in 20 μL of binding buffer (10 mM Tris-HCl, 20 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, pH 7.6) containing 15 μg nuclear protein, 0.0175 pmol of labeled probe (> 30 000 cpm), and 1 μg of poly dIdC. After incubation for 20 min at room temperature, the mixture was subjected to electrophoresis on a non-denaturing 6% polyacrylamide gel at 180 V for 2 h. The gel was dried and autoradiographed.

Production of retrovirus in packaging cells and infection of target cells

A truncated cDNA of rat nSMase was generated by RT–PCR. The following primer pair was used to generate a truncated nSMase protein with 44 amino acids deleted from the C-terminus of full length of nSMase: 5′-CGGGAATTCATGAAGCACAACTTTTCTCTGC-3′ and 5′-CGGTACGTATTACATCACATAGCCAAAGAGTGC-3′. The PCR products were cloned into a pGEM-T easy vector (Promega), sequenced and then subcloned into *Eco*R1–*Sna*B1 sites of the pMX-IRES-GFP retrovirus vectors (Royer *et al.* 2004). Then 60-μg cDNA constructs of pMX-mutant-nSMase-IRES-EGFP or pMX-IRES-EGFP were transfected into Phoenix retroviral producer cells (Clontech, Palo Alto, CA, USA) in a 100-mm dish (3×10^6 cells/100-mm dish) using the calcium phosphate method (Calphos Mammalian transfection kit, BD Biosciences, Franklin Lakes, NJ, USA). The packaging cells were kept in a 5% CO_2 incubator at 32°C for 48 h to produce retrovirus. The retroviral supernatant was then harvested, and concentrated by centrifugation at 6000 *g* for 16 h at 4°C. The retroviral titer of 10^7 ifu/mL was obtained. Target cells were infected with retrovirus and incubated at 37°C for 3 h. The retroviral supernatant was then removed and growth medium were added. The cells were used for experiments after 48 h of post-transfection.

Statistical analyses

Results are expressed as mean ± SD based on at least two or three separate experiments performed in triplicate. Differences among groups were statistically analyzed by one-way ANOVA followed by Bonferroni's *post hoc t*-test. Comparison between two experimental groups was based on 2-tailed *t*-test. A *p*-value less than 0.05 was considered significant.

Results

Amyloid-β peptide 25–35 or C2-ceramide enhanced tumor necrosis factor-α-induced inducible nitric oxide synthase expression and nitrite production

We studied the effect of Aβ and TNF-α on iNOS/nitrite production in OLGs. We found that TNF-α increased iNOS mRNA (Fig. 1a), protein expression (Fig. 1b) and nitrite production (Fig. 1c). Aβ 25–35 (Aβ, 10 μM) or C2-ceramide (25 μM) on their own had little effect on iNOS mRNA, protein expression or nitrite production in OLGs. However, Aβ or C2-ceramide enhanced TNF-α-induced iNOS mRNA, protein expression, and nitrite production (Fig. 1).

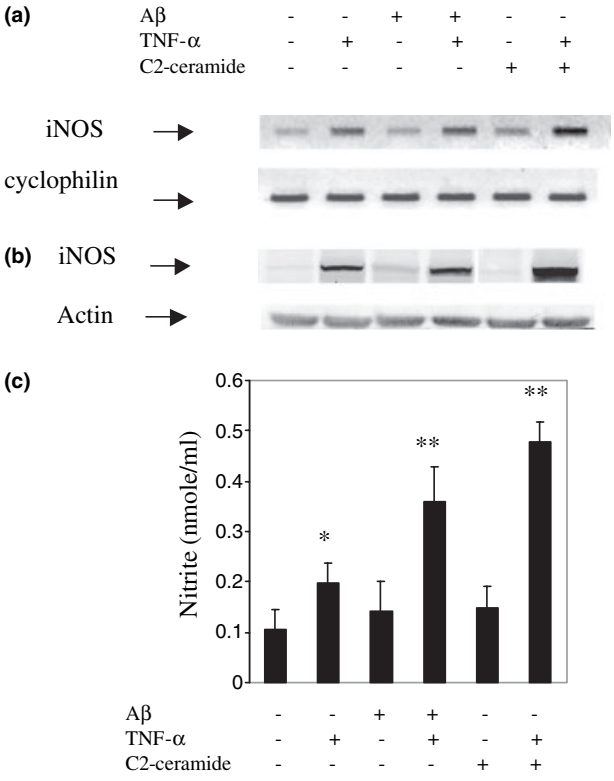

Fig. 1 Amyloid-β peptide 25–35 (Aβ) or C2-ceramide enhanced tumor necrosis factor-α (TNF-α)-induced inducible nitric oxide synthase (iNOS) expression and nitrite production. Oligodendrocytes (OLGs) were treated with TNF-α (20 ng/mL), Aβ (10 μM), C2-ceramide (25 μM), combination of TNF-α and Aβ, or combination of TNF-α and C2-ceramide for either 6 h (for iNOS mRNA analysis) or 24 h (for iNOS protein expression and nitrite production). The medium was collected for nitrite measurement. Cells were harvested for RT–PCR and western blot analysis. (a) iNOS mRNA expression by RT–PCR. Cyclophilin was used as internal control for equal RNA loading. (b) iNOS protein expression by western blot. Actin is used as internal control for equal protein loading. (c) Nitrite production by Griess assay. The values of nitrite are expressed as $x \pm$ SD nmole/mL in three independent experiments with triplicates ($p < 0.05$; *compared to the control; **compared to TNF-α only).

Amyloid-β peptide 25–35 and tumor necrosis factor-α increased neutral sphingomyelinase activity and ceramide formation in oligodendrocytes

Previously we have shown that Aβ increased OLG cell death at least in part through the sphingomyelinase/ceramide pathway (Lee *et al.* 2004). In this study, we observed that Aβ enhanced TNF-α-induced iNOS/nitrite production (Fig. 1). Hence we hypothesized that Aβ enhancement of TNF-α-induced iNOS/nitrite production may be mediated in part by the sphingomyelinase/ceramide pathway in OLGs. To

investigate this possibility, we first tested whether sphingomyelinase activity and ceramide formation were elevated in OLGs in response to the treatments of Aβ, TNF-α or the combination of both. OLGs were treated with Aβ (10 μM), TNF-α (20 ng/mL) or the combination of both for 16 h, and were then assayed for SMase activities and ceramide levels. We found that Aβ and TNF-α increased nSMase activity by 42% and 18%, respectively (Fig. 2a). However, both treatments did not affect acidic SMase activity. These results suggested that Aβ and TNF-α specifically stimulated nSMase activity in OLGs. Ceramide levels were also increased when treated with Aβ (10 μM), TNF-α (20 ng/mL) or the combination of both (Fig. 2b). Furthermore, the specific neutral sphingomyelinase inhibitor, 3-*O*-methyl-sphingomyelin (3-OMS, 1 μM), significantly decreased ceramide generation caused by Aβ and TNF-α (Fig. 2b). These data suggest that both Aβ and TNF-α increase nSMase activities, which in turn increase ceramide generation.

Pharmacological inhibitors of neutral sphingomyelinase reduced amyloid-β peptide 25–35 and/or tumor necrosis factor-α-induced inducible nitric oxide synthase and nitrite production and cytotoxicity.

In order to determine whether TNF-α/Aβ-induced iNOS/nitrite production is mediated by the nSMase/ceramide pathway, OLG cultures were pre-treated with a nSMase inhibitor (3-OMS, 1 μM). OLGs were then treated with TNF-α (20 ng/mL), Aβ (10 μM) or the combination of both for 6 h (for iNOS mRNA expression) or 24 h (for iNOS protein expression and nitrite production). The culture medium was collected to determine nitrite using the Greiss assay (Kleinert *et al.* 1996). iNOS mRNA and protein expression were examined by RT–PCR and western blot, respectively. Our data showed that 3-OMS reduced nitrite production (Fig. 3a), iNOS mRNA (Fig. 3c), and iNOS protein (Fig. 3d) expression when induced by TNF-α or the combination of Aβ and TNF-α. The effects of 3-OMS suggest that Aβ and/or TNF-α induced iNOS and nitrite production by activating the nSMase/ceramide cascade. *N*-Acetyl-cysteine (10 mM), the precursor of GSH, has been shown to decrease the oxidative status and inhibit nSMase activity (Liu *et al.* 1998; Lee *et al.* 2004). Our results showed that *N*-acetyl-cysteine reduced nitrite production (Fig. 3b) and iNOS mRNA (Fig. 3c) expression induced by TNF-α or the combination of Aβ and TNF-α. These results suggest that the nSMase/ceramide cascade is required for iNOS expression induced by TNF-α or the combination of Aβ and TNF-α in OLGs.

We also examined effects of TNF-α and/or Aβ on cytotoxicity in OLGs and whether inhibition of nSMase affected the cytotoxicity. Figure 3(e) showed that Aβ (20 μM) was cytotoxic to OLGs and that TNF-α (20 ng/mL) by itself had little cytotoxicity. The combination of Aβ and TNF-α elicited the same cytotoxicity as Aβ alone. Moreover, the nSMase inhibitor (3-OMS, 1 μM) suppresses cytotoxicity elicited by Aβ and the combination of Aβ and TNF-α. These data suggested that nSMase mediated cytotoxicity of Aβ and the combination of Aβ and TNF-α.

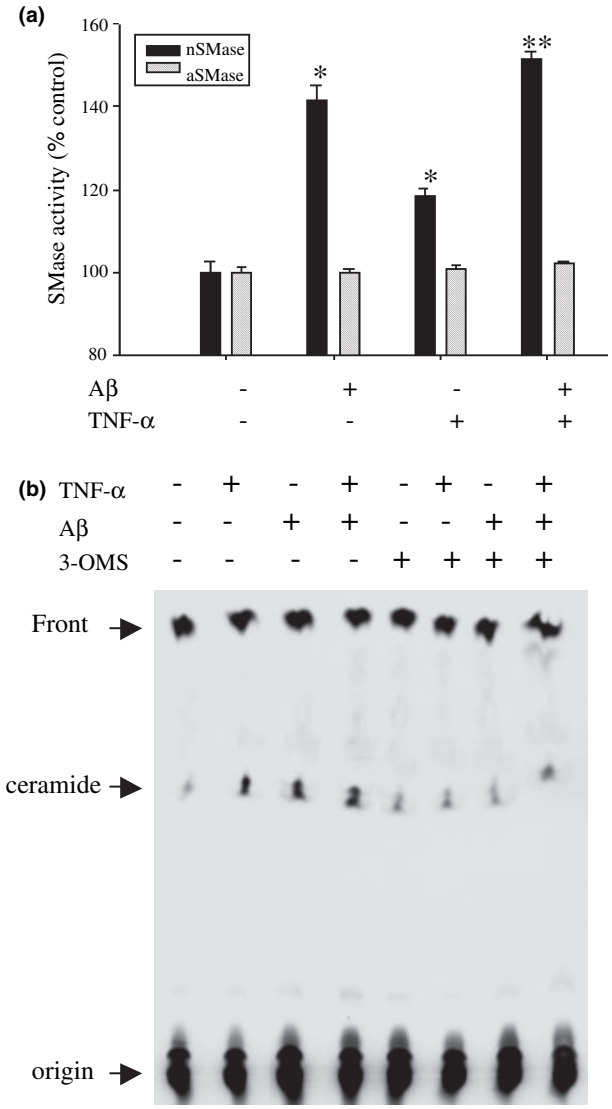

Fig. 2 Amyloid-β peptide 25–35 (Aβ) and/or tumor necrosis factor-α (TNF-α) elevated neutral sphingomyelinase (nSMase) activity and ceramide formation. (a) nSMase and aSMase (acidic sphingomyelinase) activity determination. Oligodendrocytes (OLGs) were treated with TNF-α (20 ng/mL), Aβ (10 μM), or combination of both for 16 h. The cell lysates was subject to enzyme activity assay (see methods). The enzyme activities are expressed as percentage of the control in two separate experiments in duplicates ($x \pm$ SD). ($p < 0.05$; *compared to the no treatment control; **compared to TNF-α or Aβ only). (b) Ceramide levels determined by TLC. OLGs were incubated with 10 μCi [^{3}H]palmitate (1 mCi/mL) 16 h prior to addition of TNF-α (20 ng/mL), Aβ (10 μM), or combination of both for 24 h with or without nSMase inhibitor, 3-*O*-methyl-sphingomyelin (3-OMS) (1 μM). Cell lipids were extracted and subject to TLC and visualized by autoradiography.

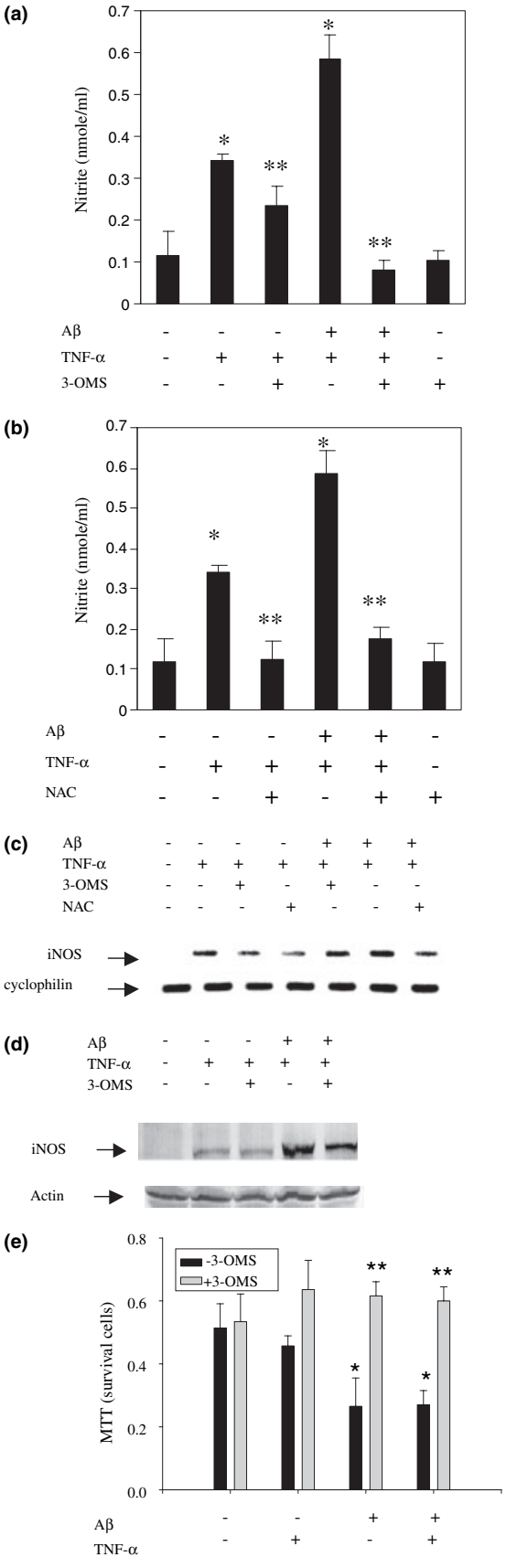

Fig. 3 The specific neutral sphingomyelinase (nSMase) inhibitor (3-*O*-methyl-sphingomyelin, 3-OMS) and antioxidant (*N*-acetyl-cysteine, NAC) inhibited the amyloid-β peptide 25–35 (Aβ) enhanced TNF-α-induced nitrite production, inducible nitric oxide synthase (iNOS) mRNA and protein expression. Oligodendrocytes (OLGs) were treated with nSMase inhibitor, 3-OMS (1 μM) (a) or antioxidant NAC (10 mM) (b) for 1 h followed by treatment of tumor necrosis factor-α (TNF-α) (20 ng/mL), Aβ (10 μM) or the combination of both for either 6 h (for iNOS mRNA analysis) or 24 h (for iNOS protein expression, nitrite production and cytotoxicity). The medium was collected for nitrite determination. Cells were harvested for RT–PCR (c), western blot (d, actin as an internal control for equal protein loading), and cytotoxicity (e). The cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see Methods). The values of nitrite are expressed as $x \pm$ SD nmole/mL in triplicates of three independent experiments. $p < 0.05$; *compared to no treatment control; **compared to no 3-OMS or NAC treatment.

NF-κB has been reported to be involved in the induction of iNOS by various cytokines in astroglia (Da Silva *et al*. 1997; Pahan *et al*. 1998; Hua *et al*. 2002; Marcus *et al*. 2003). In order to examine whether nSMase/ceramide exists up-stream or down-stream of NF-κB, we performed a DNA electrophoretic mobility shift assay. Our data showed that TNF-α induced NF-κB binding activity (Fig. 4). Aβ or C2-ceramide enhanced TNF-α-induced NF-κB binding

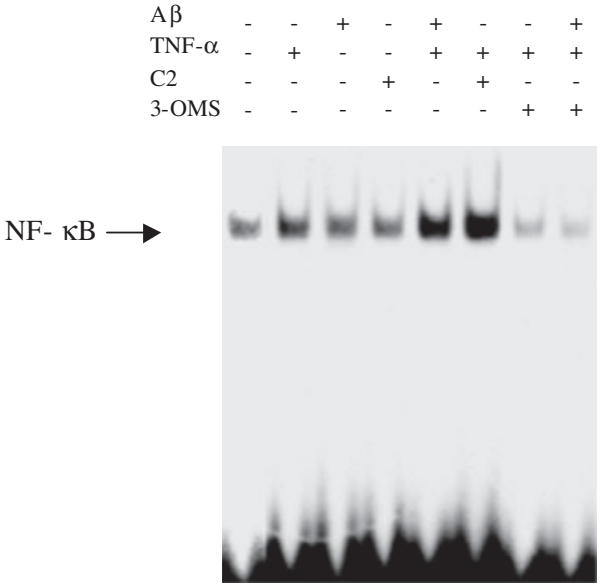

Fig. 4 Neutral sphingomyelinase (nSMase) inhibitor (3-*O*-methyl-sphingomyelin, 3-OMS) inhibited nuclear factor κB (NF-κB) DNA binding activity induced by tumor necrosis factor-α (TNF-α) or the combination of amyloid-β peptide 25–35 (Aβ) and TNF-α. Oligodendrocytes (OLGs) were treated with nSMase inhibitor, 3-OMS (1 μM) for 1 h followed by treatment of TNF-α (20 ng/mL), Aβ (10 μM), C2 (25 μM), the combination of TNF-α and Aβ or the combination of TNF-α and C2 for 6 h. Cells were harvested for electrophoretic mobility shift assay analysis.

activity. nSMase inhibitor, 3-OMS, reduced the NF-κB binding activity induced by TNF-α and the combination of TNF-α and Aβ. These data suggest that the nSMase/ceramide pathway is up-stream of activation of NF-κB.

Ceramide can be generated via multiple routes such as hydrolysis of sphingomyelin by either nSMase or aSMase, and *de novo* synthesis by ceramide synthase. To examine if enzymes other than nSMase are also involved in iNOS and nitrite production, we used the aSMase inhibitor, desipramine (2 μM), or the ceramide synthase inhibitor, fumonisin B1 (2 μM) to treat OLGs and examined their effects on iNOS and nitrite production. Our results showed that desipramine and fumonisin B2 had no effect on iNOS and nitrite production (data not shown), suggesting that aSMase and ceramide synthase are not involved in mediation of iNOS and nitrite production.

A truncated mutant of neutral sphingomyelinase inhibited inducible nitric oxide synthase mRNA

To infect OLGs, 10^7 ifu/mL of retrovirus carrying green fluorescent protein (GFP) and a truncated mutant of nSMase were used. After 48 h of infection, OLGs were treated with Aβ (20 μM), TNF-α (20 ng/mL), or a combination of both. Infection efficiency is 30–40%, estimated by counting GFP-positive cells out of total cells. We first examined the functional outcome of the truncated nSMase. When GFP alone was overexpressed in OLGs, Aβ increased nSMase activity. When the truncated nSMase was overexpressed, it inhibited Aβ-induced nSMase activity (Fig. 5a). The data suggest that the truncated mutant of nSMase has a dominant negative function. Then we studied whether the truncated nSMase inhibited Aβ/TNF-α-induced iNOS expression. The RT–PCR results showed that the truncated mutant of nSMase inhibited iNOS mRNA as compared to the GFP control (Fig. 5b). These data provide the direct evidence that nSMase may be required for iNOS expression.

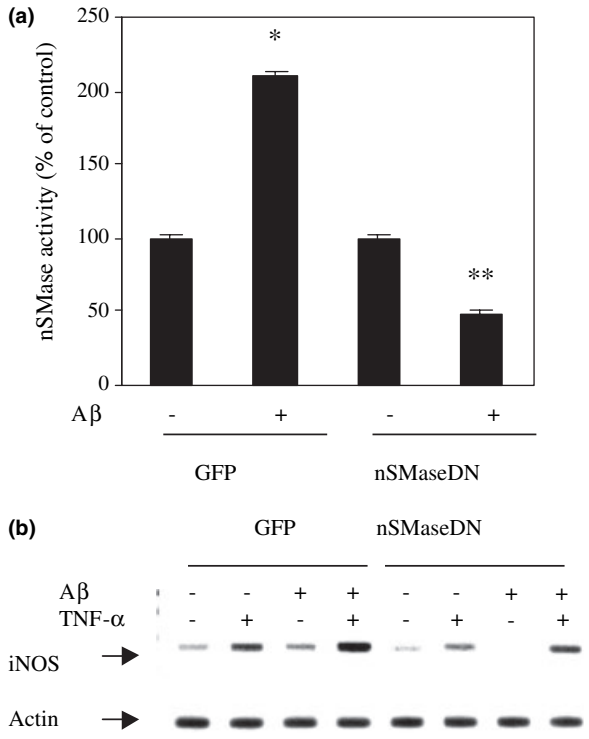

Fig. 5 A dominant negative mutant of neutral sphingomyelinase (nSMase) inhibited inducible nitric oxide synthase (iNOS) mRNA. Oligodendrocytes (OLGs) were infected with 10^7 ifu/mL of retrovirus carrying either green fluorescent protein (GFP) or a dominant negative mutant of nSMase (nSMaseDN) for 48 h, followed by treatment of tumor necrosis factor-α (TNF-α) (20 ng/mL), amyloid-β peptide 25–35 (Aβ) (10 μM), or combination of both. Cells were harvested after 16 h of the treatment for nSMase activity assay (a) or 6 h of the treatment for RT–PCR analysis (b). $p < 0.05$; *compared to no treatment; **compared to GFP.

Discussion

We have made novel observations that although Aβ by itself did not induce expression of iNOS/nitrite, it enhanced TNF-α-induced iNOS/nitrite in OLGs. In this study we investigated the intracellular signaling pathways involved in Aβ enhancement of TNF-α induced iNOS/nitrite production. We have shown that TNF-α and the combination of TNF-α and Aβ induced iNOS mRNA, protein expression, and nitrite production. TNF-α, Aβ, and the combination of both induced nSMase activities and enhanced ceramide levels. We have also demonstrated that both nSMase inhibitor (3-OMS) and a dominant negative mutant of nSMase reduced the effects of TNF-α induction and the Aβ enhancement on iNOS expression and nitrite production. These data suggest that the neutral sphingomyelinase/ceramide pathway is required for iNOS expression induced by TNF-α and the combination of TNF-α and Aβ in OLGs. On the other hand, our data showed that Aβ by itself induced comparable nSMase activities and ceramide levels to those elicited by TNF-α, but only TNF-α rather than Aβ induced iNOS expression and nitrite production. The data suggest that neutral sphingomyelinase/ceramide pathway by itself may not be sufficient for iNOS expression. Moreover, the nSMase inhibition did not completely block the enhancing effect of Aβ (Figs 3c and d, Fig. 5b), implying that other mechanisms than nSMase/ceramide activation contribute to the observed induction of iNOS. Collectively, these multiple lines of evidence suggest that neutral sphingomyelinase/ceramide pathway is required but may not be sufficient for iNOS expression induced by TNF-α and the combination of TNF-α and Aβ in OLGs.

Cytokine-induced iNOS/nitrite expression seems to involve activation of multiple intracellular signaling pathways. In addition to the nSMase/ceramide pathway reported in this study, p38 MAPK (mitogen-activated protein kinase) and NF-κB may be involved in the cytokine-induced iNOS and

nitrite production in OLGs (Bhat *et al*. 1999). In the OLG progenitor cell line, TNF-α has been shown to activate p38 MAPK in a time- and dose-dependent manner (Bhat *et al*. 1999). In the present study, we observed that TNF-α increased NF-κB binding activity in OLGs by electrophoresis mobility shift assay. We also observed that nSMase inhibitor, 3-OMS, inhibited NF-κB DNA binding activity indicating that the nSMase/ceramide pathway is involved in NF-κB activation and nitrite production induced by the combination of TNF-α and Aβ. The multiple pathways exist in astroglial cells upon induction of iNOS by cytokines as well. In fact, studies from astrocytes and C6 glial cells indicate that NF-κB, p38 MAPK, and nSMase/ceramide pathway are involved in the induction of iNOS by various cytokines (Da Silva *et al*. 1997; Pahan *et al*. 1998; Hua *et al*. 2002; Marcus *et al*. 2003; Ayasolla *et al*. 2004). In astrocytes the previous studies found that specific nSMase inhibitor, 3-OMS, reduced cytokine-induced iNOS expression suggesting that the nSMase/ceramide pathway is involved in cytokine-induced iNOS expression (Pahan *et al*. 1998; Ayasolla *et al*. 2004). In fact our data showed that nSMase/ceramide pathway is not sufficient for iNOS expression, suggesting the existence of multiple pathways for cytokine-induced expression of iNOS.

In the current study we directly measured the nSMase and aSMase activity showing that TNF-α, Aβ, and the combination of both induced nSMase activity but not acidic SMase activity. We also found that aSMase inhibitor (desipramine) had little effect on iNOS expression induced by TNF-α and the combination of TNF-α and Aβ (data not shown). These data suggest that nSMase, but not aSMase is involved in the expression of iNOS induced by TNF-α and the combination of TNF-α and Aβ. We have shown that nSMase rather than aSMase is involved in Aβ-induced cell death in oligodendrocytes in the previous study (Lee *et al*. 2004). Our data are consistent with the recent report that TNF-α induces nSMase, but not aSMase activity in oligodendrocytes (Testai *et al*. 2004).

Figure 3(e) showed that TNF-α did not have significant cytotoxicity at 20 ng/mL and did not increase Aβ toxicity. nSMase inhibitor, 3-OMS significantly reduced cytotoxicity induced by Aβ and the combination of TNF-α and Aβ indicating that nSMase/ceramide pathway mediated cytotoxicity induced by TNF-α and/or Aβ. Both aSMase and nSMase activities have been described to be stimulated during apoptosis (Andrieu-Abadie and Levade 2002). Which SMase is responsible for cytotoxicity appears to depend on cell type and stimulus. For example, overexpression of recombinant human nSMase in human aortic smooth muscle cells markedly stimulates apoptosis (Obeid *et al*. 1993). Fibrillar amyloid-beta peptides kill human primary neurons via NADPH oxidase-mediated activation of nSMase, not aSMase (Jana and Pahan 2004). In contrast in human hepatocytes, TNF-α mediated nSMase activation did not induce apoptosis (Chatterjee 1999).

It has been reported that OLGs are vulnerable to NO (Mitrovic *et al*. 1994). The direct exposure of soluble nitric oxide to OLG ultimately kills OLGs by necrosis (Mitrovic *et al*. 1995). We tested whether NO induced by the combination of Aβ and TNF-α increased OLG cell death. We found that the combination of Aβ (10 μM) and TNF-α (20 ng/mL) did not increase Aβ cytotoxicity (Fig. 3e). Physiological roles of NO produced by OLGs are not clear. It has been reported that OLG-produced NO inhibits the expression of myelin basic protein mRNA in these cells (Mackenzie-Graham *et al*. 1994). Thus, OLG-produced NO may be responsible for loss of myelin in white matter of AD brain. The other possible role of NO produced by OLGs may be toxic to neurons. It has been reported that NO produced by activated microglia and astrocyte can kill neurons (Brown and Bal-Price 2003). The current knowledge suggests that microglia can be activated by Aβ and then produce cytokines such as interleukin-1β and TNF-α. These cytokines induce iNOS and NO, which in turn kill neurons (Brown and Bal-Price 2003). It is possible that OLG-produced NO is toxic to neurons as well. The roles of OLG-produced NO in the brain of the AD patient remain to be determined.

In summary, we have demonstrated that Aβ by itself did not induce iNOS and nitrite; however, Aβ enhanced TNF-α-induced iNOS and nitrite production. Neutral sphingomyelinase/ceramide pathway is required but may not be sufficient for iNOS expression induced by TNF-α and a combination of TNF-α and Aβ. Other mechanisms than nSMase/ceramide activation may in part contribute to the observed induction of iNOS as well.

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