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Minocycline Attenuates Neuronal Cell Death and Improves Cognitive Impairment in Alzheimer's Disease Models

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Minocycline is a semi-synthetic tetracycline antibiotic that effectively crosses the blood–brain barrier. Minocycline has been reported to have significant neuroprotective effects in models of cerebral ischemia, traumatic brain injury, amyotrophic lateral sclerosis, and Huntington's and Parkinson's diseases. In this study, we demonstrate that minocycline has neuroprotective effects in *in vitro* and *in vivo* Alzheimer's disease models. Minocycline was found to attenuate the increases in the phosphorylation of double-stranded RNA-dependent serine/threonine protein kinase, eukaryotic translation initiation factor-2 α and caspase 12 activation induced by amyloid β peptide$_{1-42}$ treatment in NGF-differentiated PC 12 cells. In addition, increases in the phosphorylation of eukaryotic translation initiation factor-2 α were attenuated by administration of minocycline in Tg2576 mice, which harbor mutated human APP695 gene including the Swedish double mutation and amyloid β peptide$_{1-42}$-infused rats. We found that minocycline administration attenuated deficits in learning and memory in amyloid β peptide$_{1-42}$-infused rats. Increased phosphorylated state of eukaryotic translation initiation factor-2 α is observed in Alzheimer's disease patients' brains and may result in impairment of cognitive functions in Alzheimer's disease patients by decreasing the efficacy of *de novo* protein synthesis required for synaptic plasticity. On the basis of these results, minocycline may prove to be a good candidate as an effective therapeutic agent for Alzheimer's disease.
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INTRODUCTION

Minocycline is a second-generation tetracycline that effectively crosses the blood–brain barrier (Yong *et al*, 2004). In addition to its antibiotic properties, minocycline has been reported to have neuroprotective effects in various experimental models of cerebral ischemia (Yrjanheikki *et al*, 1999), traumatic brain injury (Sanchez Mejia *et al*, 2001), amyotrophic lateral sclerosis (ALS) (Zhu *et al*, 2002), Parkinson's diseases (PD) (Wu *et al*, 2002), kainic acid treatment (Heo *et al*, 2006), Huntington's disease (HD) (Chen *et al*, 2000; Wang *et al*, 2003), and multiple sclerosis (Popovic *et al*, 2002). Additionally, minocycline was reported to attenuate white matter damage in a rat model of chronic cerebral hypoperfusion (Cho *et al*, 2006).

Minocycline exerts its neuroprotective effects via mitochondrial permeability-transition mediated cytochrome c release from mitochondria in ALS mice model (Zhu *et al*, 2002), the inhibition of caspase-1 and -3 expressions in a HD transgenic mouse model (Wang *et al*, 2003), and the suppression of hypoxic activation of rodent microglia (Suk, 2004).

Recently, it has been reported that minocycline treatment suppressed microglial production of IL-1beta, IL-6, TNF, and NGF in *in vitro* as well as amyloid precursor protein (APP) transgenic mice, but did not affect amyloid beta peptide (Aβ) deposition in this Alzheimer's disease (AD) animal model (Seabrook *et al*, 2006).

AD is one of the most popular neurodegenerative disorders characterized neuropathologically by the presence of neuritic plaques composed of amyloid fibrils and neurofibrillary tangles, which primarily contain paired helical filaments of hyperphosphorylated tau (Selkoe, 2001). Although many lines of evidence have confirmed the finding that Aβ exhibits neurotoxicity and induces apoptosis, another hypothesis has emerged to show that synaptic failure and impairment of cognitive function occur in the early phase of AD before the appearance of neuronal degeneration (Selkoe, 2002). In fact, it has been demon-

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strated that oligomeric assemblies of Aβ, observed during the growth of amyloid fibrils, can affect synaptic function including synaptic plasticity and cognitive function *in vivo* (Cleary *et al*, 2005; Walsh *et al*, 2002). As synaptic plasticity underlies cognitive function, such as learning and memory, its disruption would be expected to cause a decline of cognitive ability observed in AD.

It has been recently reported that mice lacking GCN2, one of the four kinases known to be responsible for the phosphorylation of eukaryotic initiation translation factor 2 alpha (eIF-2α), that is double-stranded RNA-dependent serine/threonine protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), heme-regulated eIF2α kinase (HRI), exhibited enhanced long-term potentiation and sustained memory was induced when exposed to weak stimuli and training (Costa-Mattioli *et al*, 2005). Studies on a variety of different forms of synaptic plasticity have suggested a link between messenger RNA translation and learning and memory (Kandel, 2001). Phosphorylated eIF2α (p-eIF2α) reduces general protein synthesis, but facilitates the mRNA translation of the transcriptional modulator ATF4 (Harding *et al*, 2000), which inhibits synaptic plasticity and behavioral learning by repressing CREB activity (Chen *et al*, 2003).

Stress conditions such as ultraviolet, endoplasmic reticulum (ER) stress, and reactive oxygen species (ROS) elicit a cellular adaptive response for the coordinated expression of stress-responding genes. One of these responses is the phosphorylation of eIF2α (Harding *et al*, 1999).

Prolonged ER stress is linked to the pathogenesis of several neurodegenerative disorders, which include cerebral ischemia, PD, and AD (DeGracia and Montie, 2004; Katayama *et al*, 2004; Smith *et al*, 2005).

In this study, we demonstrate that minocycline exerts neuroprotective actions in *in vitro* and *in vivo* model of AD. Moreover, minocycline attenuated the phosphorylation of eIF-2α induced by Aβ_{1-42} treatment or the expression of C-terminal fragments of APP (APP-CTs) in neuronal cells. We also found that minocycline improves the learning and memory impairments in the Aβ_{1-42}-infused AD rat model (Nitta *et al*, 1997). The increases in p-eIF2α were also attenuated by administration of minocycline in Tg2576 mice, which harbor mutated human APP695 gene including the Swedish double mutation as well as Aβ_{1-42}-infused rats. These results suggest that minocycline could be a potential therapeutic agent for AD.

MATERIALS AND METHODS

Reagents and Antibodies

Minocycline was obtained from Sigma (MO, USA). Aβ_{1-42}, from US Peptide (CA, USA). Anti-caspase-12, anti-phospho-eIF-2α, anti-p-PKR antibodies were obtained from Cell Signaling (MA, USA), and eIF2α antibody was from Biosource (CA, USA).

DNA Constructs

The cDNA constructs of APP-C59 and APP-C99 were generated by PCR from human APP695 cDNA and encompassed the last 59 or 99 amino-acid residues, respectively. They were subcloned into a pcDNA3-flag vector with the flag at the N terminus. All constructs were sequenced using an ABI310 Sequencer.

Cell Culture and Transfection

PC12 cells were plated on polyethyleneimine (PEI, 0.2 mg/ml; Sigma, MO, USA) coated plates and maintained in DMEM (Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% FBS (GIBCO BRL, NY, USA) and 0.3% antibiotics at 37°C in 5% CO_2. PC12 cells were treated with nerve growth factor (NGF, 50 ng/ml) from Invitrogen (CA, USA) and allowed to differentiate for 4 days. NGF differentiated PC12 cells were transiently transfected with 2 μg of plasmid DNA and 3 μl of Fugene 6 (Roche Molecular Biochemicals, Germany) in 1 ml of growing medium according to the manufacturer's instructions.

Cell Viability Assay

MTT-metabolizing activity was determined at 48 h post-transfection according to the manufacturer's instructions (Sigma). LDH activities in the conditioned media were measured using a Cytotox96 nonradioactive cytotoxicity assay kit (Promega, WI, USA) according to the manufacturer's instructions. The percentage of LDH release was obtained by comparing to the maximal release of positive control treated with 1% Triton-X 100.

Western Blotting

Protein was resolved in SDS polyacrylamide gel, electrophoresed at 30–50 μg of protein/lane, and transferred onto a nitrocellulose membrane (Amersham Pharmacia, Buckinghamshire, UK). The protein blot was confirmed with appropriate antibodies and detected using horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia). Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia).

Subcellular Fractionation for Microsomes

Cells were resuspended in hypotonic buffer A (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, protease inhibitor) on ice for 30 min. Cells were disrupted by passing through 26-gauge needle 30 times, and then 30-gauge needle 20 times. Cell lysates were centrifuged at 750 *g* for 10 min at 4°C. The supernatant was centrifuged at 10 000 *g* for 20 min at 4°C, the pellet as the mitochondrial fraction. The supernatant was centrifuged at 100 000 *g* for 1 h at 4°C. The pellet was saved as cytosolic fraction and the pellet as the microsome (ER enrichment). The purity of the fractions was checked with subcellular markers, that is calnexin for ER, β-actin for cytosol, and cytochrome *c* oxidase IV for checking mitochondrial contamination.

Aβ_{1-42} Infused AD Rat Model

Male Wistar rats weighing 200–250 g (7-week-old) were housed in a specific pathogen-free room that was auto-

matically maintained on a 12-h light/dark cycle at 25°C and proper humidity. Animals were handled in accordance with 'the Guidelines for Animal Experiments of Ethics Committee of Seoul National University'. They had free access to food and water. The $A\beta_{1-42}$ peptides were dissolved in 35% acetonitrile/0.1% trifluoroacetic acid. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Continuous infusion of $A\beta_{1-42}$ (600 pmol/day) was maintained for a week by attachment of an infusion kit connected to an osmotic mini-pump (Alzet 1007D; Alza, CA, USA). Sham-operated rats were infused with 35% acetonitrile/0.1% trifluoroacetic acid. The infusion kit was implanted into the right ventricle (1.2 mm posterior to the berema, 1.5 mm lateral to the midline, 4.0 mm ventral to the surface of the skull, according to the brain atlas of Paxinos and Watson. Minocycline (45 mg/kg/day) or PBS was then injected i.p. for 3 weeks.

Transgenic Mice

Tg2576 mice harboring the mutated human APP695 gene were obtained from Taconic (German Town, NY, USA). The production, genotyping, and background strain (C57BL/$6 \times$ SJL) of Tg2576 mice used in this study were described previously (Hsiao *et al*, 1996). All experiments were performed in accordance with 'the Guidelines for Animal Experiments from the Ethics Committee at Seoul National University'. Minocycline (10 mg/kg/day) or PBS was administered to Tg2576 mice or wt mice i.p. from 3 months after birth, 5 days per week for 9 months.

Morris Water-Maze Task

The experimental apparatus, which is a circular water tank (140 cm in diameter, 45 cm high) was filled with opaque water made by adding dry milk powder to water at the temperature of 21–23°C and located in a laboratory that contained prominent extra-maze cues. Animals are required to find a submerged platform (15 cm in diameter, 35 cm high) in the pool using the spatial cues. The two starting points were changed daily. Spatial training consisted of five sessions, (two trials per session per day) during which the platform was left in the same position. In each training session, the latency to escape on to the hidden platform was recorded. After final train session, a single probe trial was conducted. The escape platform was removed, and each rat was allowed to swim for 90 s in the maze. The number of times the rat crossed the annulus where the platform had been located was recorded. Data collection was automated by a video image motion analyzer (Ethovision, Noldus Information Technology h.v., Netherlands).

Passive Avoidance Test

Wistar rats were handled for 3 days before the start of the behavioral experiments. The apparatus and experimental procedure used in the passive avoidance test comprised of a two-compartment step-through passive avoidance apparatus made of black Perspex was used. The apparatus consisted of illuminated and dark compartments ($25 \times 25 \times 25$ cm) attached to a grid floor and were separated by a guillotine door. The rat was placed in the illuminated compartment and the door was raised. After entering the dark compartment, the rat was returned to its home cage. After further 30 min, the rat was again placed in the illuminated compartment. When the rat entered the dark compartment, the guillotine door was closed. Scrambled electrical foot shocks with intensity of 0.5 mA were delivered for 3 s through the grid floor using a shock generator. In the test trial made 24 h after the second trial, the rat was placed again in the illuminated compartment and the response latency to enter the dark compartment was measured up to a maximum of 300 s.

Human AD Brains

Paraffin-embedded brain stocks of AD and age-matched control subjects were obtained from the Netherlands Brain Bank (NBB). Coronal sections (4 μm) were cut through the hippocampus and processed for immunohistofluorescence. All experimental procedures were performed in accordance with 'the Guidelines of the Ethics Committee at Seoul National University'.

Immunohistochemistry

Fixed wild-type, Tg2576 mice brains, human AD or age-matched control human brains in 10% neutral buffered formalin for 48 h were dehydrated and embedded in paraffin. Before immunostaining, slides were deparaffinized by oven heating and immersion in xylene. After dehydration through graded alcohols to water, a primary antibody was revealed by incubating the cells for 45 min with Cy™ 3-conjugated secondary antibody (Molecular Probes, Carlsbad, CA). After three washes with permeabilization buffer and one wash with PBS, cells were mounted on microscope slides in mounting medium (DAKO, CA). Confocal microscopic observation was performed using Zeiss LSM510 (Jena, Germany).

Hematoxylin–Eosin (H&E) Staining

The rats were anesthetized with 10% chloral hydrate (350 mg/kg), i.p. Then the rats were perfused with 200 ml 0.9% NaCl solution and subsequently with 4% paraformaldehyde in 0.1 mol/l phosphate buffer at pH 7.4. Brains were removed and post-fixed for 24 h in the same fixative. The post-fixed brains were cryo-protected in 25% sucrose in PBS. Then the brains were removed, paraffin-embedded, and coronally sectioned at 7 μm thickness. The sections were then mounted on slides and stained with H&E. The cell loss of the hippocampal area was assessed and quantified under a microscope. Neuronal damage was expressed as a percentage of the number of eosinophilic cells/the total number of cells in each region in the hippocampal area.

Statistical Analysis

Data are expressed as mean $\pm$ SEM values. One-way ANOVA test was used for statistical significance, where $P < 0.05$ was considered to be significant.

RESULTS

Transient Transfection into Nerve Growth Factor-Differentiated PC 12 Cells

In this study, we investigated the effects of minocycline on APP-C59 or APP-C99-induced cytotoxicities, p-eIF2α, and caspase 12 in differentiated PC 12 cells employing a transient transfection system. Under our experimental conditions, transient transfection efficiencies using Fugene 6 reagent were assessed with GFP vector in differentiated PC 12 cells. The transfection efficiency was $40.0 \pm 4.0\%$ ($n = 5$) in PC 12 cells, as confirmed by the expression of GFP fluorescence. In addition, we confirmed the expression of APP-C59 or APP-C99 by Western blotting after transfection (data not shown).

Minocycline Inhibited the Increases in p-eIF2α and Reduced the Neuronal Cell Death Induced by Aβ_{1-42} Treatment

Aβ plays an important role in the pathogenesis of AD (Yankner, 1996). Initially, we tested whether minocycline affects the reduced viability of NGF-differentiated PC12 cells induced by Aβ_{1-42} treatment, using LDH release, and the MTT-reduction assay. We examined dose-dependent effects of minocycline over Aβ_{1-42} treatment (30 μM, 24 h). The condition of Aβ_{1-42} treatment at 30 μM for 24 h was chosen based on our preliminary study (data not shown) and study by others (Schuster *et al*, 2005), in which 25 μM concentration of Aβ_{1-42} reduced cell viability by about 20%. Minocycline at concentrations of 1, 5, 10, 20 μM minocycline were pretreated in differentiated PC 12 cells 24 h before Aβ_{1-42} treatment. Minocycline significantly inhibited LDH release by Aβ_{1-42}, dose-dependently from 0, 1 to 5 μM, however, there its effect reached plateau at 5 μM (Figure 1b). Based on the results by us and by others (Suk, 2004; Wang *et al*, 2003; Zhu *et al*, 2002), 10 μM concentration of minocycline was chosen for this study.

We found that pretreatment with minocycline at 10 μM for 24 h significantly attenuated Aβ_{1-42}–induced LDH release (30 μM for 24 h), from $14.03 \pm 4.21\%$ to $4.91 \pm 0.70\%$ in NGF-differentiated PC 12 cells (Figure 1a). For the MTT assay, minocycline restored the decrease in MTT reduction by Aβ_{1-42} from $86.02 \pm 4.30\%$ to $96.77 \pm 1.07\%$.

It has been reported that the phosphorylated form of eIF2α and activation of PKR are increased in the degenerating neurons of the brains from AD patients (Chang *et al*, 2002a) and in cultured neuronal cells treated with Aβ (Chang *et al*, 2002b). Here, we checked whether minocycline affects the upregulated p-eIF-2α induced by Aβ_{1-42} treatment, and found that minocycline attenuated the increases in p-eIF-2α at serine 51 by Aβ_{1-42} treatment in minocycline pretreated NGF-differentiated PC 12 cells by about 20%, whereas eIF2α was not affected by Aβ_{1-42} or by minocycline (Figure 1c). Aβ is reported to phosphorylate eIF-2α via PKR activation in neuronal cells (Chang *et al*, 2002a; Suen *et al*, 2003). We investigated the effects of minocycline on PKR phosphorylation caused by Aβ_{1-42}. Aβ_{1-42} treatment increased p-PKR by about 25% and minocycline pretreatment reverted phosphorylation at threonine 446 and 451 (Figure 1d) to the control level, but did not affect PKR (data not shown). In response to ER stress caused by various stimuli such as intracellular calcium homeostasis perturbation, ROS, and protein misfolding, eIF2α becomes phosphorylated (Lindholm *et al*, 2006). Results from Western blots showed that minocycline attenuated activation of caspase 12 induced by Aβ_{1-42} treatment (Figure 1e).

Minocycline Reduced the Upregulation of p-eIF2α and Attenuated Neuronal Cell Death Induced by the C-terminal Fragments of Amyloid Precursor Protein

Next, we examined the effects of minocycline on the cell death induced by the expression of the APP-CTs in NGF-differentiated PC12 cells. Treatment with minocycline at 10 μM for 48 h post-transfection significantly reduced cytotoxicity induced by APP-C59 from 23.18 ± 1.18 to $6.36 \pm 1.82\%$ (Figure 2a). For APP-C99 transfection, LDH release was reduced from $29.09 \pm 1.90\%$ to $6.82 \pm 1.80\%$ (Figure 2a). The MTT reduction assay showed that minocycline pretreatment significantly ameliorated the decrease in MTT reduction by APP-C59 from 75.00 ± 2.08 to $91.67 \pm 2.08\%$ and that of APP-C99 from 68.75 ± 2.08 to $91.80 \pm 1.51\%$ (Figure 2a). In addition, we found that increases in p-eIF-2α induced by APP-CTs expression were significantly attenuated by minocycline pretreatment in NGF-differentiated PC12 cells by about 20% (APP-C59) and 44%(APP-C99), whereas eIF2α was unaffected by either Aβ_{1-42} or by minocycline (Figure 2b). Figure 2b shows that p-eIF2α in the mock control group was slightly reduced by minocycline even though there was no significant difference. This discrepancy remains to be elucidated by further study.

Minocycline Reduced Neuronal Death and Attenuated Learning and Memory Impairment in an Aβ_{1-42} Infused AD Rat Model

Here we assessed the effects of minocycline in *in vivo* AD model. Aβ_{1-42} (600 pmole/day) or the vehicle (35% acetonitrile/0.1% trifluoroacetic acid) was infused into lateral ventricles of male Wistar rats weighing 200–250 g (7-week old) continuously for a week. Minocycline (45 mg/kg/day) or PBS was then injected i.p. for 3 weeks into Aβ_{1-42} (600 pmol/day)- or vehicle (35% acetonitrile/0.1% trifluoroacetic acid)-infused sham-operated groups. The minocycline concentration used in this study (45 mg/kg/day, i.p.) was chosen, based on the report by others (Hunter *et al*, 2004a). Three weeks after completing the i.p. injections, we tested for spatial learning and memory impairment using Morris Water Maze test in the PBS- or minocycline-treated animals of Aβ_{1-42} (600 pmol/day)- or sham-operated groups. The Aβ_{1-42}-infused minocycline-treated group showed shorter latency times than the PBS-injected group from the second day of these learning sessions (ANOVA, $P < 0.05$, Figure 3a). We confirmed no noticeable differences between the sham-operated animals treated with PBS or minocycline (Figure 3a). To confirm whether the memory impairment shown in the Aβ_{1-42} infused rats was actually attenuated by minocycline treatment, we performed the

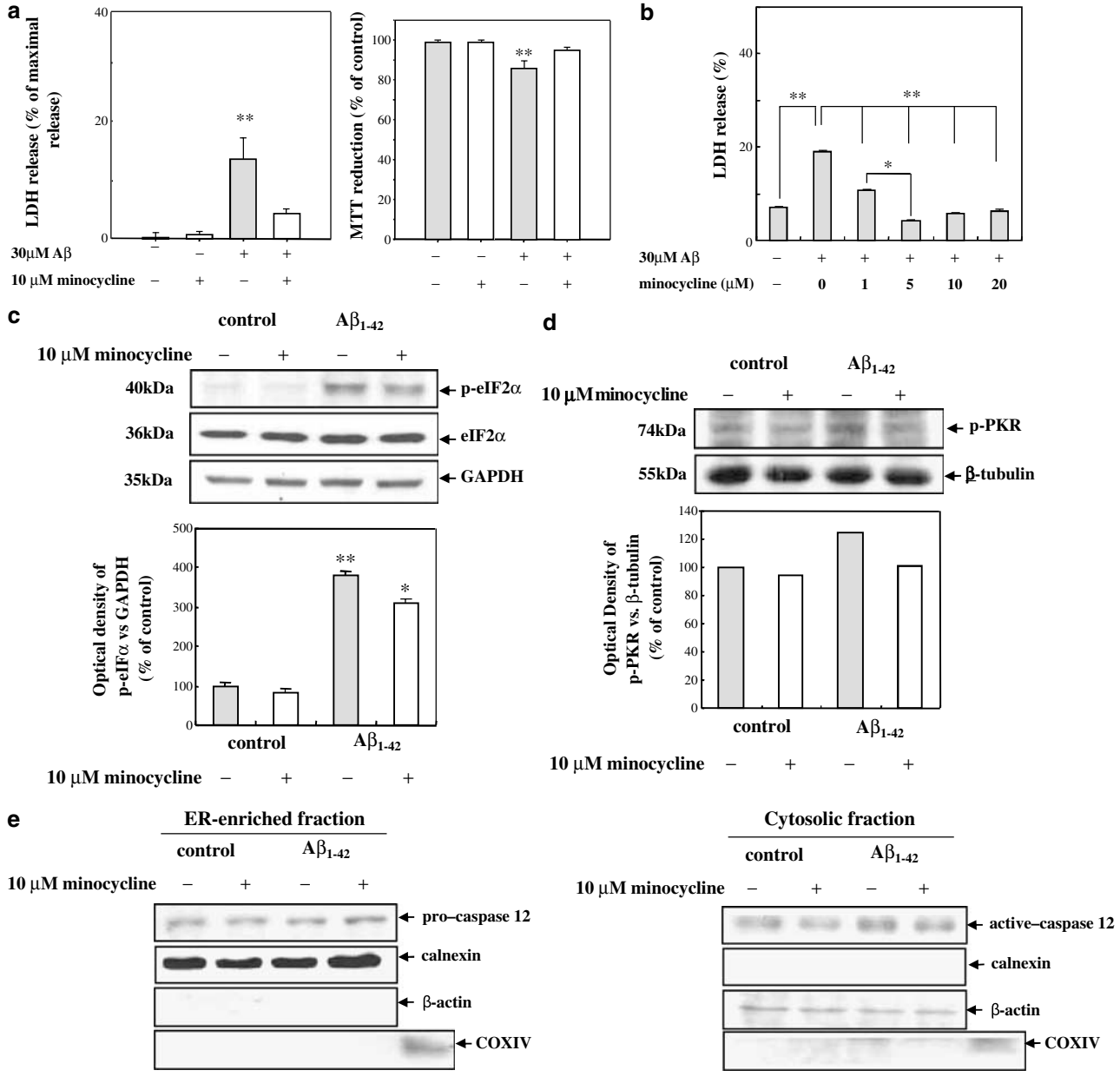


Figure 1 Minocycline inhibited the increases in p-eIF2α and reduced the neuronal cell death induced by $A\beta_{1-42}$ treatment. (a) Differentiated PC12 cells were pretreated with vehicle (PBS) or 10 μM minocycline for 24 h and then treated with 30 μM $A\beta_{1-42}$. Twenty four hours after $A\beta_{1-42}$ treatment, cell viability was measured by LDH or MTT activity *vs* the control (vehicle-treated PC12 cells). Data represent mean ± SEM obtained from 16 culture wells per experiment, determined in four independent experiments. Asterisks indicate significantly different from the PBS-pretreated 30 μM $A\beta_{1-42}$ treated group (**$P<0.01$ by one-way ANOVA). The percentage of LDH release was obtained by comparing to the maximal release of positive control treated with 1% Triton-X 100. (b) Dose-dependent effects of minocycline over $A\beta_{1-42}$ treatment (30 μM, 24 h) were examined. 0, 1, 5, 10, 20 μM concentrations of minocycline was pretreated for 24 h and then treated with 30 μM $A\beta_{1-42}$. Twenty-four hours after $A\beta_{1-42}$ treatment, cell viability was measured by LDH *vs* the control (vehicle-treated PC12 cells). Data represent mean ± SEM obtained from 10 culture wells per experiment, determined in two independent. (c) Differentiated PC12 cells were pretreated with vehicle or 10 μM minocycline and then treated with 30 μM $A\beta_{1-42}$ for 24 h. p-eIF-2α and eIF2α levels were checked by immunoblotting whole cell lysates. GAPDH was used as a loading control. Densitometric analysis was also carried out, and results are presented as means ± SEM of three independent experiments (**$P<0.01$, *$P<0.05$ by one-way ANOVA). (d) Differentiated PC12 cells were pretreated with vehicle or 10 μM minocycline and then treated with 30 μM $A\beta_{1-42}$ for 24 h. p-PKR levels were checked by immunoblotting using whole cell lysates. β-Tubulin was used as a loading control. Relative ratio of the densitometric values of p-eIF2α and eIF2α is normalized using the M4 image analysis program. (e) Changes in procaspase 12 levels in ER-enriched fractions and of activated caspase 12 in the cytosolic fraction were detected by immunoblotting with anti-caspase 12 antibody. The purity of the fractions was checked with subcellular markers, that is calnexin for ER, β-actin for cytosol, and cytochrome c oxidase IV for checking mitochondrial contamination.

probe test, and recorded the average latency during the stay at the zone 1 without platform. Minocycline-treated rats stayed significantly longer in zone 1 than at the other zones (zones 2–4) (ANOVA, $P<0.05$, Figure 3b). However, no significant differences were observed for PBS-treated animals in terms of times spent in different zones

(Figure 3b). There was no noticeable differences in the sham-operated animals treated with PBS or minocycline (Figure 3b).

To determine whether minocycline affects short- and long-term working memory impairment induced by $A\beta_{1-42}$ infusion, we performed passive avoidance testing. The minocycline-treated group showed significantly longer latencies than the PBS treated $A\beta_{1-42}$ infused rats (one-way ANOVA, $P<0.05$, Figure 3c). There was no noticeable difference between the sham-operated animals treated with the vehicle (PBS) or minocycline (Figure 3c). Next, we checked neuronal cell death by H&E staining in the $A\beta_{1-42}$ infused rat model, and found that minocycline treatment significantly reduced neuronal cell death in the hippocampus and dentate gyrus in an $A\beta_{1-42}$ infused rat model (Figure 3d). The percentage of eosinophilic cells *vs* total cells in dentate gyrus was $7.75\pm1.98\%$ for the PBS-treated, and $10.38\pm2.4\%$ in minocycline-administered sham-operated rats and $52.38\pm7.25\%$ for the PBS-treated, and $12.70\pm2.43\%$ in minocycline-administered $A\beta_{1-42}$ infused rats, respectively. In CA3, the percentage of eosinophilic cells *vs* total cells was $17.88\pm2.79\%$ for the PBS-treated, and $19.02\pm3.62\%$ in minocycline-administered sham-operated rats and $67.48\pm6.72\%$ for the PBS and $32.30\pm4.40\%$ in minocycline-administered $A\beta_{1-42}$ infused rats, respectively (Figure 3e). All *in vivo* experiments were performed in accordance with 'the Guidelines for Animal Experiments of Ethics Committee of Seoul National University'.

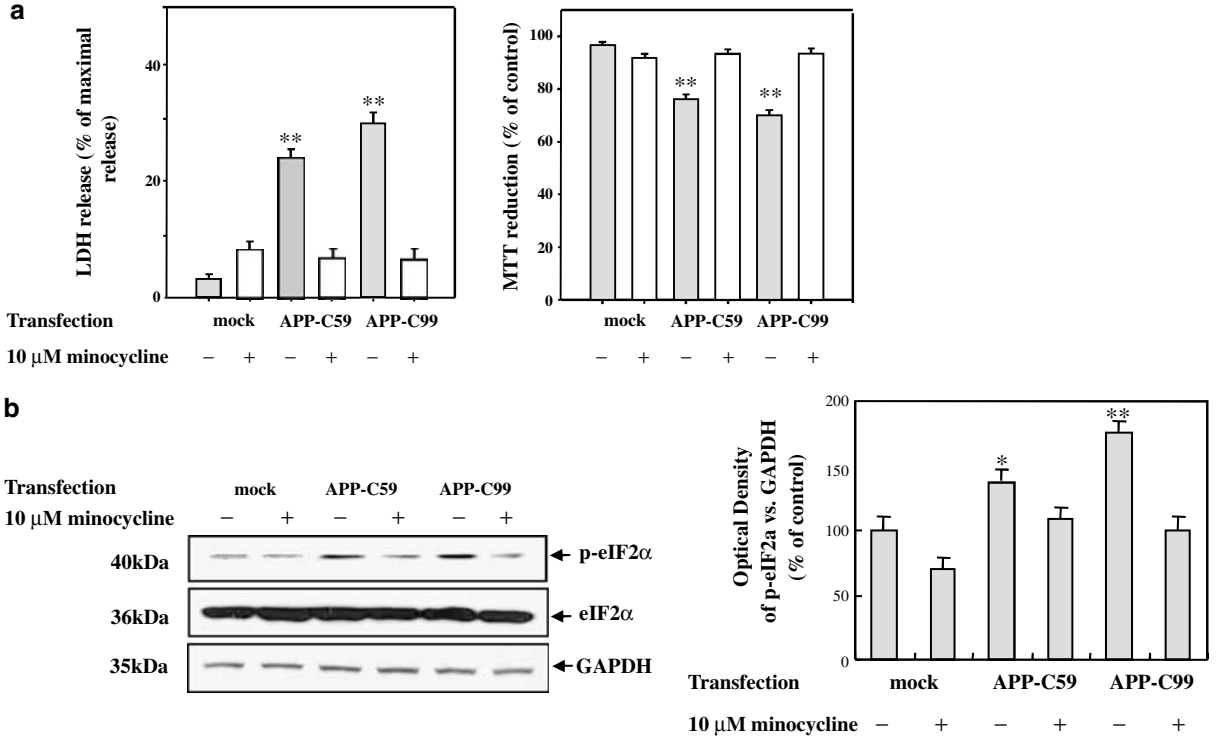
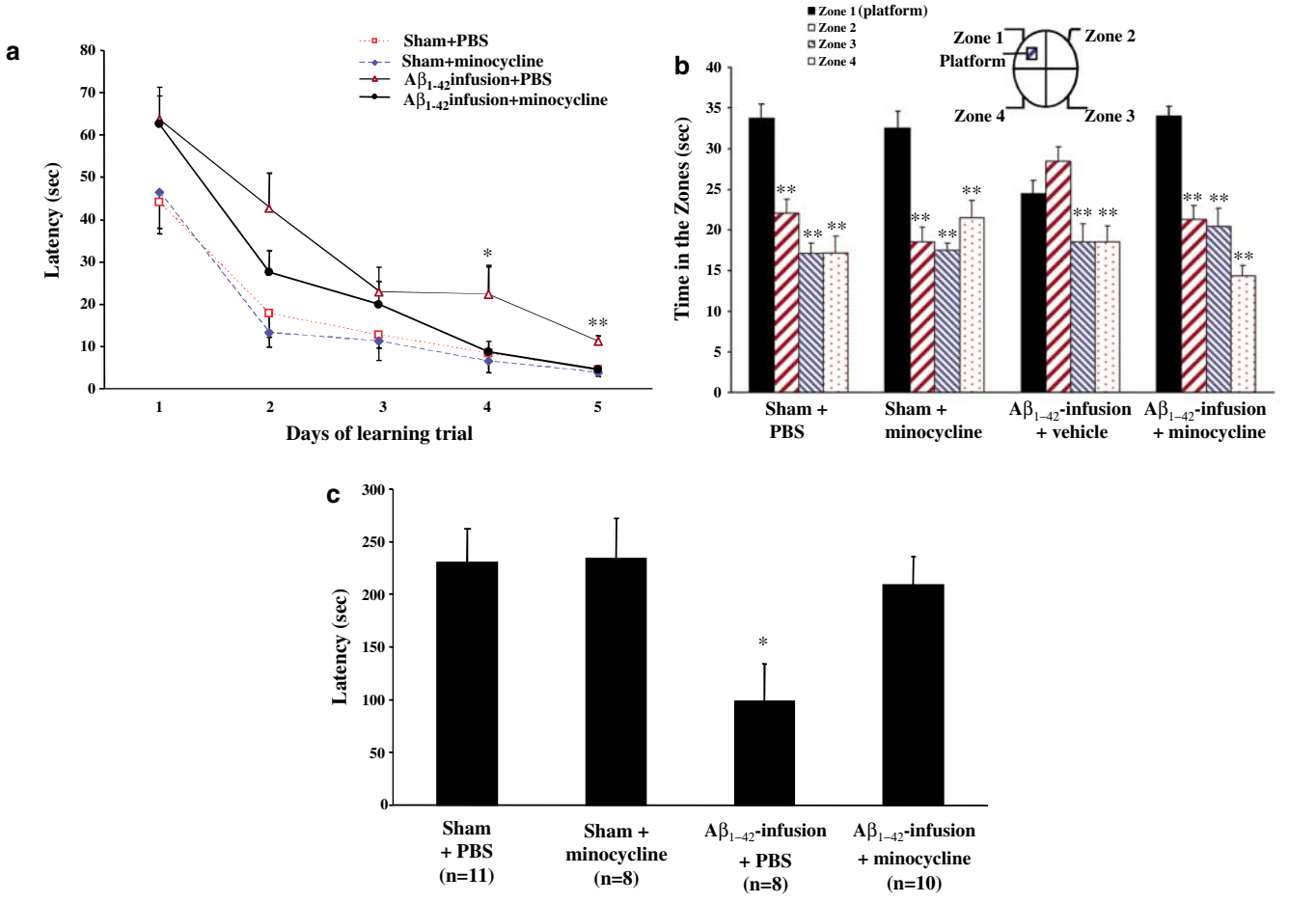


Figure 2 Minocycline reduced the upregulation of p-eIF2α and attenuated the neuronal cell death induced by the C-terminal fragments of amyloid precursor protein. (a) NGF-differentiated PC12 cells were transfected with mock, APP-C59, or APP-C99, and then treated with vehicle (PBS) or 10 μM minocycline 6 h post-transfection. After 48 h, cell viabilities were determined by LDH release and MTT assays. Results are expressed as percentages of peak LDH or MTT activities in the control (non-transfected PC12 cells). Data represent mean ± SEM obtained from 16 culture wells per experiment, determined in four independent experiments. The percentage of LDH release was obtained by comparing to the maximal release of positive control treated with 1% Triton-X 100. Asterisks indicate significantly different from PBS-treated mock-transfected cells (*$P<0.01$ by one-way ANOVA). (b) PC12 cells were transfected with mock, C59, or C99 and then treated with vehicle or 10 μM minocycline 6 h post-transfection. After 48 h, levels of p-eIF2α and eIF2α were examined by immunoblotting. GAPDH was used as a loading control. Densitometric analysis was also performed. The results are presented as means ± SEM of three independent experiments (**$P<0.01$, *$P<0.05$ by one-way ANOVA).

Figure 3 Minocycline reduced neuronal death and attenuated learning and memory impairment in an $A\beta_{1-42}$ infused AD rat model. (a) $A\beta_{1-42}$ (600 pmol/day) dissolved in 35% acetonitrile/0.1% trifluoroacetic acid was infused into lateral ventricles using an osmotic pump for 7 days. Sham-operated rats were infused with 35% acetonitrile/0.1% trifluoroacetic acid only. Minocycline (45 mg/kg/day) or PBS was administered intraperitoneally during the following 3 weeks into sham-operated or $A\beta_{1-42}$ infused rats. After the water maze test training, testing was performed over five sessions after minocycline or vehicle had been administered. Latency times for the animals in the minocycline-treated group were compared to those of PBS-treated animals (one-way ANOVA, $P<0.05$ *vs* vehicle). (b) The probe test was performed after the final training session. The times that rats of the minocycline injected group stayed in zones 1, 2, 3, and 4 were compared to those of the vehicle injected group (one-way ANOVA, $P<0.05$ *vs* vehicle). (c) In the passive avoidance test, the latency times of PBS or minocycline-treated sham-operated or $A\beta_{1-42}$ infused rats were compared with each other (one-way ANOVA, *$P<0.05$, **$P<0.01$ *vs* vehicle). (d) Hematoxylin and eosin-stained sections of the hippocampal areas (dentate gyrus, CA3) of PBS or minocycline administered group after vehicle (35% acetonitrile/0.1% trifluoroacetic acid only) or $A\beta_{1-42}$ infusion were observed. Note that the depth of staining of neurons was reduced by minocycline pretreatment in $A\beta_{1-42}$ infused rats. (e) The percentage of eosinophilic cells *vs* total cells was calculated in dentate gyrus and CA3 of PBS or minocycline administered sham-operated or $A\beta_{1-42}$ infused rats (one-way ANOVA, *$P<0.05$, **$P<0.01$ *vs* vehicle).

The Increases in p-eIF2α were Attenuated by Administration of Minocycline in $A\beta_{1-42}$ Infused Rats and in Tg2576 Mice

From the *in vitro* studies (Figures 1 and 2), p-eIF2α increased by following $A\beta_{1-42}$ treatment or transfection of APP-CTs. Here, we investigated whether the phosphorylation of eIF2α was also altered in the brains of $A\beta_{1-42}$ infused rats compared to sham-operated control rats. To examine the *in situ* distribution of p-eIF2α, sections from the hippocampus of control and $A\beta_{1-42}$ infused rats were stained with p-eIF2α-specific antibody. The immunoreactivities of p-eIF2α in the CA1 and CA3 of hippocampus of $A\beta_{1-42}$ infused rats were found to be increased significantly



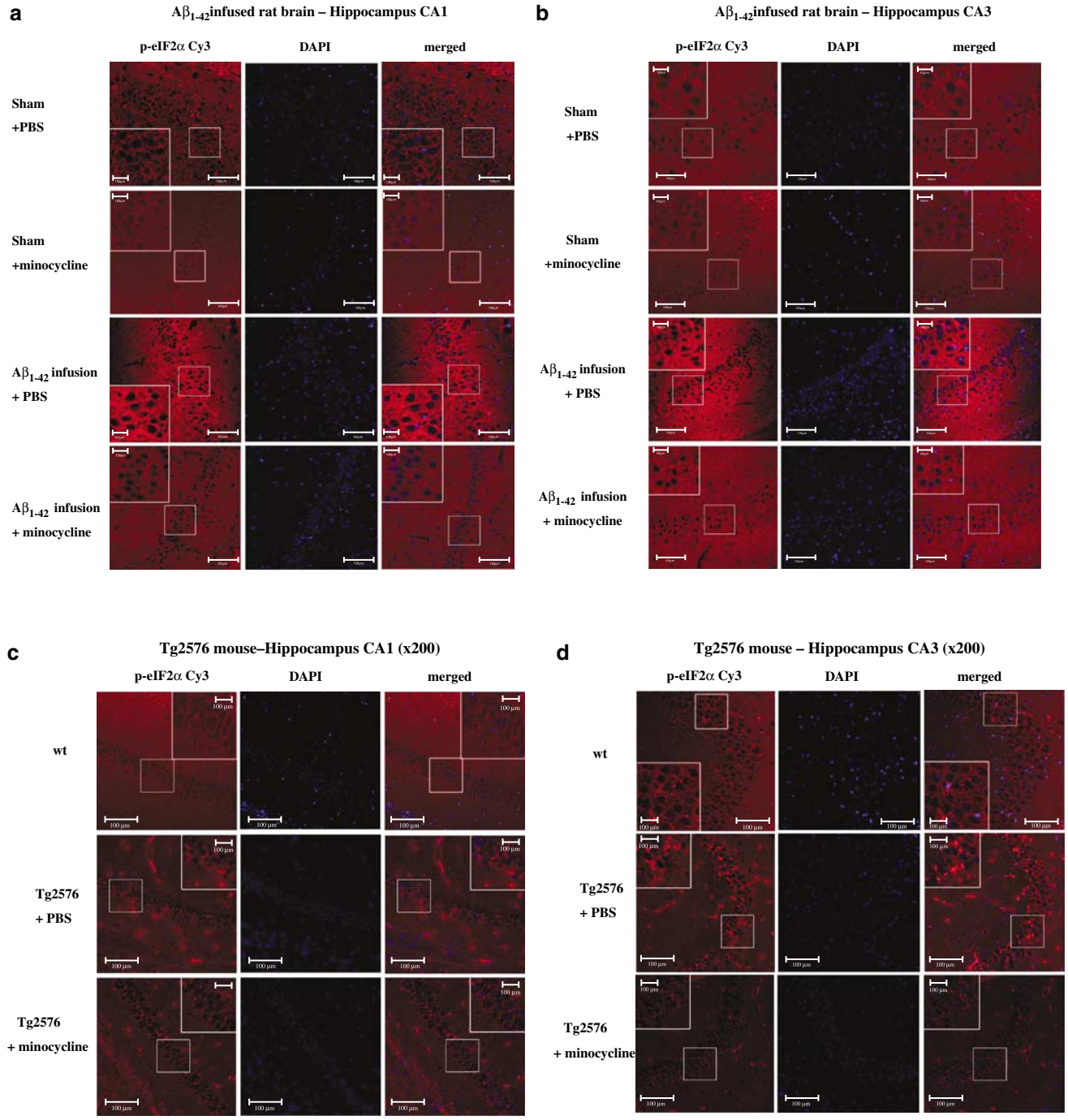


Figure 4 The increases in p-eIF2α are attenuated by administration of minocycline in Aβ_{1-42} infused rats and Tg2576 mice. (a and b) The fixed brains of Aβ_{1-42} infused rats administered vehicle or minocycline ($n=4$), (c and d) the fixed brains of Tg2576 mice administered vehicle or minocycline ($n=4$) in 10% neutral-buffered formalin for 48 h were dehydrated and embedded in paraffin. The fluorescent immunohistochemistry was performed in CA1 (a and c) and CA3 (b and d) with p-eIF2α antibody overnight and visualized using Cy3-conjugated secondary antibody (Jackson, West Grove, PA). DAPI (1 μM) counterstaining was performed. Images were collected using the LSM 510 program on a Zeiss confocal microscope. Scale bar indicates 100 μm; in inset, also 100 μm. The results are representative of four separate experiments performed with different samples, respectively.

compared to control rats (Figure 4a and b), whereas there was no noticeable difference in the immunoreactivities of eIF2α between two animal groups (data not shown). Minocycline administration (45 mg/kg/day) for 3 weeks i.p. after Aβ_{1-42} (600 pmol/day) having been infused into rat lateral ventricles continuously for a week significantly reduced the p-eIF2α immunoreactivities (Figure 4a and b). We confirmed that there was no noticeable difference between PBS- and minocycline-treated sham-operated animals (Figure 4a and b).

Additionally, we examined the effects of minocycline on the phosphorylation status of e-IF2α in Tg2576 mice. Minocycline (10 mg/kg/day) or PBS was administered to Tg2576 mice or wt mice i.p. from 3 months after birth, 5

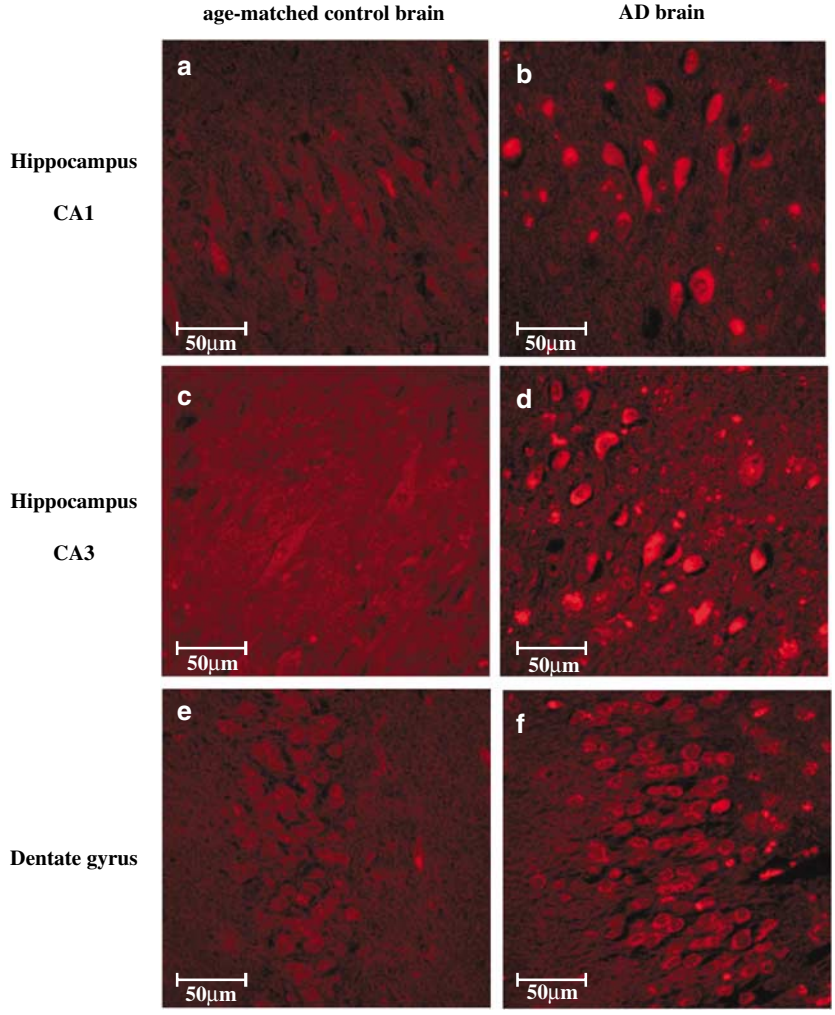


Figure 5 p-eIF2α immunoreactivities are elevated in CA1, CA3, and dentate gyrus from human AD brains. The fixed brains of age-matched control or AD brains ($n=3$) in 10% neutral-buffered formalin for 48 h were dehydrated and embedded in paraffin. The fluorescent immunohistochemistry was performed in CA1 (a and b), CA3 (c and d) and dentate gyrus (e and f) with p-eIF2α antibody overnight and visualized using Cy3-conjugated secondary antibody (Jackson, West Grove, PA). Scale bar indicates 50 μm. The results are representative of three separate experiments performed with different samples, respectively.

days per week for 9 months. Prominent staining of p-eIF2α in these 12 month-old Tg2576 mice appeared in CA1 and CA3, compared to the same-aged wt mice (Figure 4c and d). Minocycline administered Tg2576 mice showed significantly low level of the p-eIF2α immunoreactivities in the same regions (Figure 4c and d). We confirmed that the p-eIF2α in Tg2576 mice was significantly upregulated compared to age-matched wt mice (Figure 4c and d), whereas no significant difference in eIF2α level was observed between Tg2576 and wt mice (data not shown).

p-eIF2α Immunoreactivities were Elevated in CA1, CA3, and Dentate Gyrus from Human AD Brains

To seek after a physiological significance for the increased p-eIF2α levels in the brains of $A\beta_{1-42}$ infused rats or Tg2576 mice, we examined the changes in p-eIF2α in human AD brains compared to age-matched control brains using immunohistochemical analysis. AD brain sections revealed stronger immunoreactivity for p-eIF2α in CA1 (Figure 5b), CA3, (Figure 5d) and dentate gyrus (Figure 5f), compared to the same regions of age-matched control brains (Figure 5a, c and e), consistent with a previous finding (Chang *et al*, 2002a).

DISCUSSION

Minocycline: Generalities and Therapeutic, Experimental Doses

Minocycline, a broad-spectrum, semi-synthetic antibiotic belonging to the tetracycline family is being used for acne vulgaris, some sexually transmitted diseases and rheumatoid arthritis (Blum *et al*, 2004; Good and Hussey, 2003). The therapeutic doses of minocycline, approved by the FDA, for the diseases described above are 100–200 mg/day (Blum *et al*, 2004). In animals, minocycline is lethal at very high doses (LD_{50}:3600 mg/kg; Blum *et al*, 2004; Smith *et al*, 2003). In humans, long-term treatment with minocycline up to 200 mg/day is generally safe and well tolerated as

demonstrated by tolerability tests and clinical trials in rheumatoid arthritis, acne vulgaris, and HD (Blum *et al*, 2004; Bonelli *et al*, 2003).

In *in vitro* experiments recently reported on the neuroprotective effects exerted by minocycline, 2–100 μM concentrations of minocycline were used in various neuronal cell experimental models (Wang *et al*, 2003, 2004; Zhu *et al*, 2002). In the present study, we used 10 μM concentration of minocycline in *in vitro* experiments based on dose-dependent study (Figure 1b) and the study by others (Suk, 2004; Zhu *et al*, 2002). In *in vivo* study, a large range of doses were used for testing the effects of minocycline in various animal models, that is 5 mg/kg/day i.p. in R6/2 mice, transgenic mice for HD (Chen *et al*, 2000), 10–50 mg/kg/day i.p. in SOD^{G93A} mutant mice (Van Den *et al*, 2002; Zhu *et al*, 2002), 10 mg/kg/day subcutaneous release pellet in Ts65Dn mice, animal model for Down's syndrome (Hunter *et al*, 2004b), 45 mg/kg/day i.p. in C57BL/6-p75-SAP mice (Hunter *et al*, 2004a) and 60, 90, 90 mg/kg/day p.o. in MPTP PD animal model (Du *et al*, 2001). We administrated 45 mg/kg/day dose of minocycline for 3 weeks into $A\beta_{1-42}$ infused rats and tested the effects of the drug treatment compared with PBS-administered groups.

Minocycline and Neurodegenerative Diseases

In recent years, minocycline has been reported to have neuroprotective effects in various experimental neurodegenerative disease models such as cerebral ischemia (Yrjanheikki *et al*, 1999), traumatic brain injury (Sanchez Mejia *et al*, 2001), ALS (Zhu *et al*, 2002), PD (Wu *et al*, 2002), and HD (Chen *et al*, 2000; Wang *et al*, 2003). At present, a few number of studies are focused on the therapeutical potential of minocycline in AD (Hunter *et al*, 2004a; Seabrook *et al*, 2006), where it suppressed microglial production of IL-1beta, IL-6, TNF, and NGF in *in vitro* as well as APP transgenic mice (Seabrook *et al*, 2006). In addition, minocycline attenuated cholinergic cell loss, glial activation, and transcription of downstream pro-inflammatory mediators and mitigated the cognitive impairment, induced by mu p75-saporin, a novel immunotoxin that mimics the selective loss of basal forebrain cholinergic neurons and induces cognitive impairment in mice. These reports showed that minocycline exerts neuroprotective effects based on its anti-inflammatory actions in AD experimental animal models.

We have now demonstrated that minocycline attenuates the increases in phosphorylation of eIF2α, thereby reducing neuronal cell death and improving cognitive impairment in *in vitro* and *in vivo* AD models.

Possible Relationship between Phosphorylation of eIF2α and Impaired Synaptic Plasticity in AD

Whereas some previous studies support the hypothesis that neuronal cell death induced by Aβ deposited in neuritic plaque is the causative factor for AD, another possibility may be that synaptic failure and impairment of cognitive function precede neuronal degeneration, especially in the early phase of AD (Selkoe, 2002).

A common strategy in the cellular response to stress signals is to shut down protein synthesis (Taylor *et al*, 2005). Translation of eukaryotic mRNAs is regulated primarily at the level of initiation (Costa-Mattioli *et al*, 2005). Binding of the initiator tRNA, Met-$tRNAi^{Met}$, to the 40S subunit is facilitated by the eIF2α which forms a ternary complex with GTP and Met-$tRNAi^{Met}$. Although the phosphorylation of eIF2α can inhibit general translation (Sonenberg and Dever, 2003), it stimulates the mRNA translation of the transcriptional modulator ATF4 (Harding *et al*, 2000) that inhibits CREB activity, thereby down-regulating its immediate early genes (*BDNF*, *c-fos*, *EGR-1*) (Costa-Mattioli *et al*, 2005).

ER stress has recently been believed to be involved in the pathogenesis of neurodegenerative diseases including AD (DeGracia and Montie, 2004; Katayama *et al*, 2004; Lindholm *et al*, 2006; Smith *et al*, 2005). e-IF2α is known to be phosphorylated by some kinases including PKR, PERK, HRI, and GCN2 under various stresses such as ER stress (Taylor *et al*, 2005).

A recent paper reported that the inhibition of phosphorylation of eIF-2α by knocking out GCN2 enhanced long-term potentiation (Costa-Mattioli *et al*, 2005).

The interesting finding of our study is that minocycline attenuates increased phosphorylation of eIF-2α in $A\beta_{1-42}$-treated or APP-CTs-transfected differentiated PC 12 cells and in the CA1 and CA3 of two animal models of AD; $A\beta_{1-42}$ infused rats and Tg2576 mice. Aβ has been reported to phosphorylate eIF-2α via PKR activation in neuronal cells (Chang *et al*, 2002b; Suen *et al*, 2003). We found that minocycline reduced PKR phosphorylation at threonine 446 and 451 induced by $A\beta_{1-42}$ treatment (Figure 1d). Previous reports demonstrated that activation of PKR and p-eIF2α are observed in the degenerating neurons of AD brains (Chang *et al*, 2002a) and in cultured neuronal cells treated with Aβ (Chang *et al*, 2002b). PKR is known to play a crucial role in mediating Aβ-induced neuronal death because primary cortical neurons from PKR KO mice and neuroblastoma SH-SY5Y cells stably transfected with dominant-negative PKR mutants are less susceptible to Aβ toxicity (Chang *et al*, 2002b). Activated PKR can phosphorylate eIF2α at serine 51 (Suen *et al*, 2003). Phosphorylation of eIF2α that could increase owing to many factors by $A\beta_{1-42}$, APP-CTs expression or Swedish APP transgenes in Tg2576 mice, might contribute to impairment in synaptic plasticity and cognitive function before causing neurodegeneration.

In addition, our Morris Water Maze and passive avoidance test results show that minocycline improves learning and memory impairment in $A\beta_{1-42}$ infused animal model. Taking these *in vitro* and *in vivo* results together, our results suggest that minocycline has a therapeutic potential for AD.

Minocycline: Proposed Modes of Action

Almost all the beneficial effects of minocycline are demonstrated to be related to an inhibitory activity on inflammation and/or apoptotic cell death, both phenomena being closely associated with neurodegeneration (Blum *et al*, 2004). Minocycline reduces the proliferation/activation of resting microglial cells as evidenced by CD11b/

OX42, MAC-2 or isolectine-B4 staining (Dommergues *et al*, 2003—45 mg/kg i.p.; He *et al*, 2001—45 mg/kg i.p.). Several reports showed that minocycline reduces the expression of inducible nitric oxide synthase and subsequent nitric oxide production as well as caspase-1 activity/expression and thereby prevents the formation of interleukin 1β (Chen *et al*, 2000—5 mg/kg/day i.p.; Du *et al*, 2001—60–90 mg/kg p.o.). Additionally, minocycline can modulate neuronal cell death through an interaction with the apoptotic machinery (Blum *et al*, 2004). It acts at the mitochondria to rescue the collapse of transmembrane potential and the alteration of permeability transition, which are responsible for the cytosolic release of apoptogenic factors such as cytochrome c, AIF or Smac/Diablo mediating caspase-dependent and independent cell death (Matsuki *et al*, 2003; Wang *et al*, 2003—5 mg/kg i.p.; Zhu *et al*, 2002—22.5–45 mg/kg i.p.).

In this study, we report a new aspect of minocycline in *in vitro* and *in vivo* AD models. Minocycline attenuates the increases in the phosphorylation of PKR, eIF-2α and caspase 12 activation induced by Aβ_{1-42} treatment in NGF-differentiated PC 12 cells, as well as in Aβ_{1-42} infused rats and Tg2576 mice. The eIF2α kinases are activated by various stresses to halt translation by phosphorylating, and thereby inhibiting eIF2α. PKR, which is known to be activated by Aβ, is activated in response to ER stress. The ER is an essential intracellular organelle involved in intracellular calcium homeostasis, in folding and processing of proteins, and in cell death activation (Baumann and Walz, 2001). Changes in ER Ca^{2+} homeostasis and ROS induce the accumulation of unfolded proteins and activate the ER-stress-induced apoptosis pathway (Kaufman, 1999; Pashen, 2001).

It was previously shown that caspase-12 knockout mice are resistant to ER stress and to death caused by Aβ protein (Nakagawa *et al*, 2000), strongly supporting that ER stress is involved in the neuronal death that occurs in AD. *In vitro* studies have suggested that ER Ca^{2+} dyshomeostasis and oxidative stress are synergistically related. Although ER function is sensitive to the presence of oxidants (Dreher *et al*, 1995; Hayashi *et al*, 2005; Racay *et al*, 1995; Viner *et al*, 1996), ROS can also be produced intracellularly from the stress-regulated release of calcium from the ER.

Although further research is needed, we speculate that minocycline attenuates ER stress by Aβ, thereby reducing phosphorylation of eIF-2α shown in cell cultured models as well as Aβ_{1-42} infused rats or Tg2576 mice.

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