0022-3565/09/3311-319–326$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 331, No. 1
155069/3519288
Printed in U.S.A.

JPET 331:319–326, 2009



# Silibinin Attenuates Amyloid $\beta_{25–35}$ Peptide-Induced Memory Impairments: Implication of Inducible Nitric-Oxide Synthase and Tumor Necrosis Factor-$\alpha$ in Mice

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Received April 15, 2009; accepted July 27, 2009

## ABSTRACT

In Alzheimer's disease (AD), the deposition of amyloid peptides is invariably associated with oxidative stress and inflammatory responses. Silibinin (silybin), a flavonoid derived from the herb milk thistle, has potent anti-inflammatory and antioxidant activities. However, it remains unclear whether silibinin improves amyloid $\beta$ (A$\beta$) peptide-induced neurotoxicity. In this study, we examined the effect of silibinin on the fear-conditioning memory deficits, inflammatory response, and oxidative stress induced by the intracerebroventricular injection of A$\beta$ peptide$_{25–35}$ (A$\beta_{25–35}$) in mice. Mice were treated with silibinin (2, 20, and 200 mg/kg p.o., once a day for 8 days) from the day of the A$\beta_{25–35}$ injection (day 0). Memory function was evaluated in cued and contextual fear-conditioning tests (day 6). Nitrotyrosine levels in the hippocampus and amygdala were examined (day 8). The mRNA expression of inducible nitric-oxide synthase (iNOS) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) in the hippocampus and amygdala was measured 2 h after the A$\beta_{25–35}$ injection. We found that silibinin significantly attenuated memory deficits caused by A$\beta_{25–35}$ in the cued and contextual fear-conditioning test. Silibinin significantly inhibited the increase in nitrotyrosine levels in the hippocampus and amygdala induced by A$\beta_{25–35}$. Nitrotyrosine levels in these regions were negatively correlated with memory performance. Moreover, real-time RT-PCR revealed that silibinin inhibited the overexpression of iNOS and TNF-$\alpha$ mRNA in the hippocampus and amygdala induced by A$\beta_{25–35}$. These findings suggest that silibinin (i) attenuates memory impairment through amelioration of oxidative stress and inflammatory response induced by A$\beta_{25–35}$ and (ii) may be a potential candidate for an AD medication.



Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extraneuronal deposits of amyloid β (Aβ) peptide and the intraneuronal accumulations of hyperphosphorylated τ (Blennow et al., 2006). The deposition of Aβ in the brain is assumed to initiate a pathological cascade that results in synaptic dysfunction, synaptic loss, neuronal death, and cognitive dysfunction (Walsh and Selkoe, 2004).

The deposition of Aβ is invariably associated with oxidative stress and inflammatory response, which may contribute to neuronal dysfunction or death (Butterfield et al., 2007; Farfara et al., 2008). Abundant reactive microglia and astrocytes surround the Aβ plaques in the AD brain (Miyazono et al., 1991; McGeer et al., 2006). The activated microglia or

This work was supported in part by grants-in-aid for "Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Grant-in-Aid for Scientific Research (B) from MEXT of Japan [Grant 20390073]; Grant-in-Aid for Exploratory Research from Sprout Society for the Promotion of Science [Grant 19659017]; grants-in-aid for Regional Joint Research Program for Private Universities to cover current expenses from MEXT; Research Science of Pharmaceutical and Medical Devices and Research on the Risk of Chemical Substances from the Ministry of Health, Labor and Welfare (MHLW) of Japan; Research on Regulatory Science of Pharmaceutical and Medical Devices from MHLW, International Research Project Supported by the Meijo Asian Research Center; a research grant from Japan China Medical Association, Japan; and research grants from the Takeda Science Foundation, from the Uehara Memorial Foundation, and from the Nagai Foundation Tokyo.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.109.155069.

**ABBREVIATIONS:** AD, Alzheimer's disease; NO, nitric oxide; A$\beta$, amyloid $\beta$ peptide; CMC, carboxymethylcellulose; $ONOO^-$, peroxynitrite; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; silibinin, (2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl]chroman-4-one; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

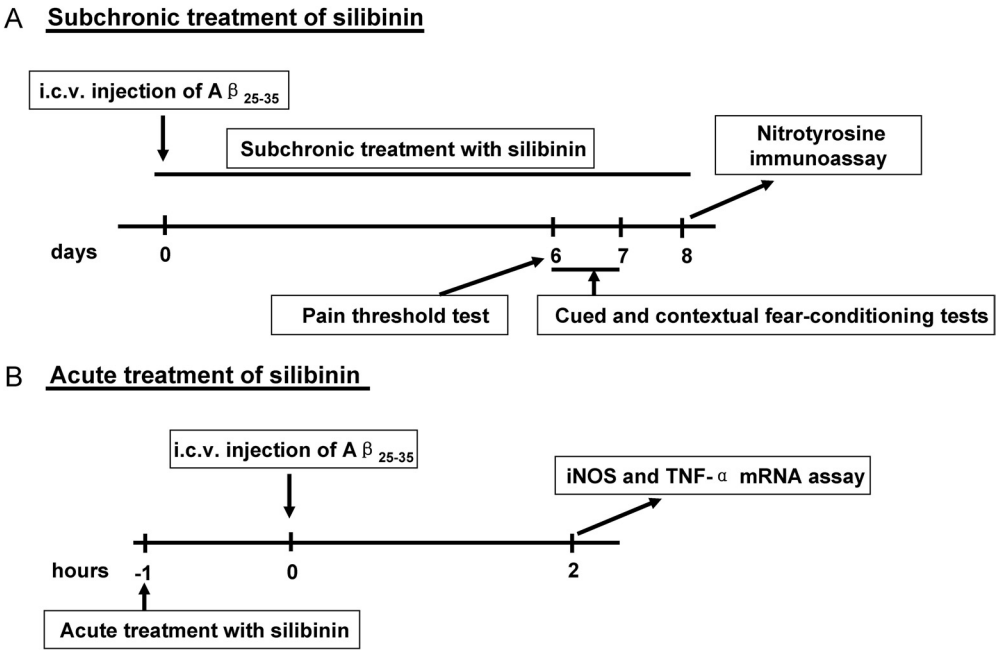



**Fig. 1.** The experimental design of the study showing subchronic treatment of silibinin (A) and acute treatment of a silibinin (B).

astrocytes release reactive oxygen species and proinflammatory molecules that act to exacerbate the disease process and contribute to neuronal death (Combs et al., 2001). TNF-α, a pro-inflammatory cytokine, has been shown to increase in AD patients (Fillit et al., 1991; Perry et al., 2001). Aβ-induced expression of TNF-α leads to overexpression of inducible nitric-oxide synthase (iNOS) in experimental animals (Akama and Van Eldik, 2000; Combs et al., 2001; Alkam et al., 2008). Peroxynitrite ($ONOO^-$) is one of the products formed from nitric oxide and superoxide and has a variety of chemical reactions producing compounds such as nitrotyrosine (Reiter et al., 2000; Tran et al., 2003). Interestingly, the accumulation of nitrotyrosine correlated with increased levels of cerebral Aβ and the severity of cognitive impairment (Smith et al., 1997; Ishii et al., 2000; Tran et al., 2003).

$A\beta_{25–35}$ is the core fragment of full-length Aβ and possesses many of the characteristics of the full-length Aβ peptide, including aggregative ability and neurotoxic property and is detected in the brain of AD patients (Pike et al., 1995; Kubo et al., 2002). There are reports that the intracerebroventricular administration of $A\beta_{25–35}$ peptide into rodent brain induces histological and biochemical changes, memory deficits, oxidative damage, and inflammatory responses within 1 or 2 weeks (Maurice et al., 1996; Alkam et al., 2008). Therefore, this animal model is used for screening new candidates for AD therapy worldwide.

Silibinin [(2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxy-methyl)-2,3-dihydrobenzo[*b*][1,4] dioxin-6-yl]chroman-4-one] is a flavonoid derived from the herb milk thistle (*Silybum marianum*) and has been reported to have anti-inflammatory and antioxidative effects (Kren and Walterová, 2005). For instance, silymarin, a mixture of flavonoids present in milk thistle, has protective effects against ethanol-induced brain injury (La Grange et al., 1999) and lipopolysaccharide (LPS)-induced neurotoxicity (Wang et al., 2002). We have reported recently that silibinin ameliorates $A\beta_{25–35}$-induced recognition memory impairment in mice (Lu et al., 2009). However, it is unclear whether silibinin ameliorates impairments of other types of memory such as fear memory and whether the inflammatory system is involved in the ameliorative effect of silibinin on Aβ-induced memory impairment. In this study, we investigated the effect of silibinin on memory impairment induced by $A\beta_{25–35}$ in cued and contextual fear-conditioning tests. We also examined its effect on changes in nitrotyrosine levels as well as TNF-α and iNOS mRNA expression in the brains of mice.

## Materials and Methods

**Animals.** Male ICR mice (5 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan). They were housed in plastic cages and kept in a regulated environment (23 ± 0.5°C, 50 ± 5% humidity) with a 12/12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). The mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum. Behavioral experiments were carried out in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences of Meijo University and the *Guiding Principles for the Care and Use of Laboratory Animals*. The procedures involving animals and their care conformed to the international guidelines set out in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

**Treatment.** Silibinin was purchased from Panjin Green Biological Development Co., Ltd. (Panjin, Liaoning, China) and suspended in a 0.3% carboxymethylcellulose (CMC) solution. $A\beta_{25–35}$ (Bachem, Bubendorf, Switzerland) was dissolved in double-distilled water at a concentration of 1 mg/ml and stored at −20°C. $A\beta_{25–35}$ was aggregated or "aged," by incubating it in distilled water at 37°C for 4 days before the injection. $A\beta_{25–35}$ was injected intracerebroventricularly in a volume of 3 μl (3 nmol/mouse) on day 0 as in our previous report (Lu et al., 2009) (Fig. 1). Mice were anesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye at an equal distance between the eyes and the ears and perpendicular to the plane of the skull (anteroposterior, −0.22 mm from the bregma; lateral, 1 mm from the bregma; ventral, −2.5 mm from the skull). Mice were administered silibinin (2, 20, or 200 mg/kg/day p.o.) or the 0.3% CMC

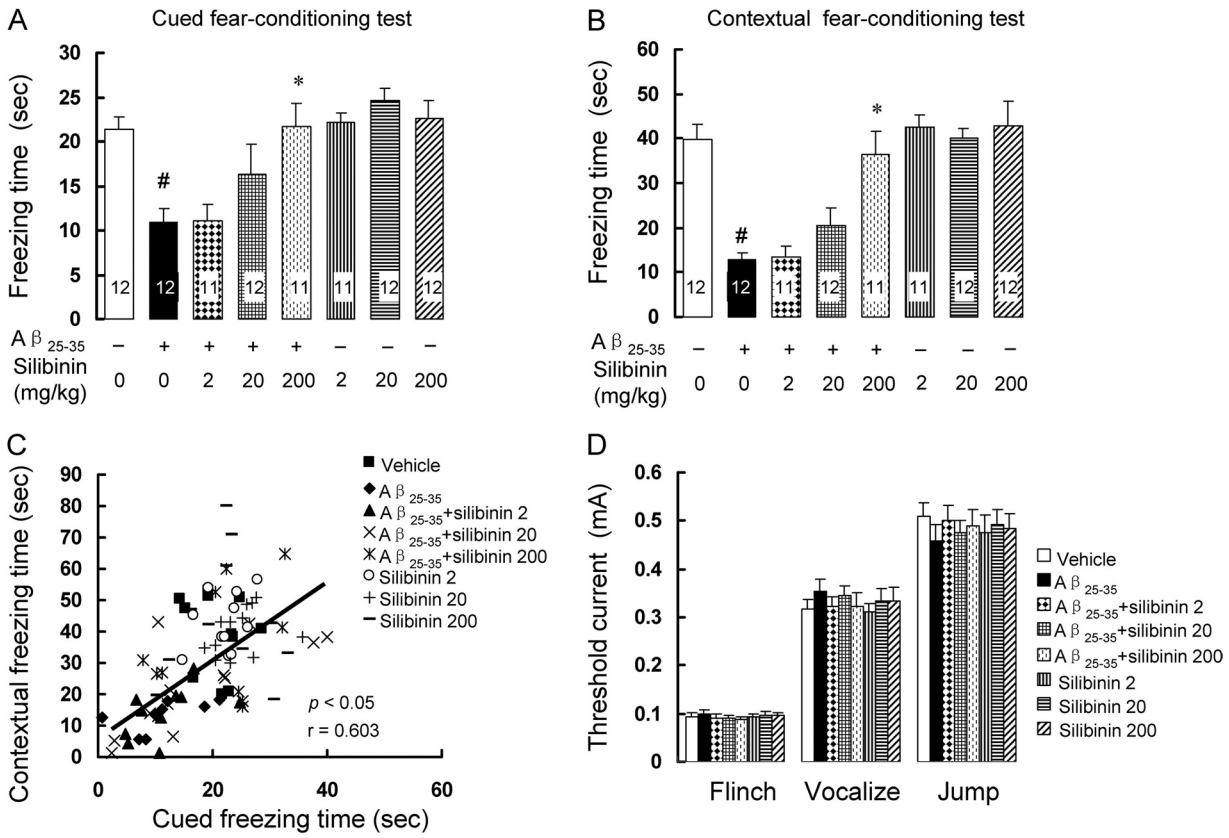


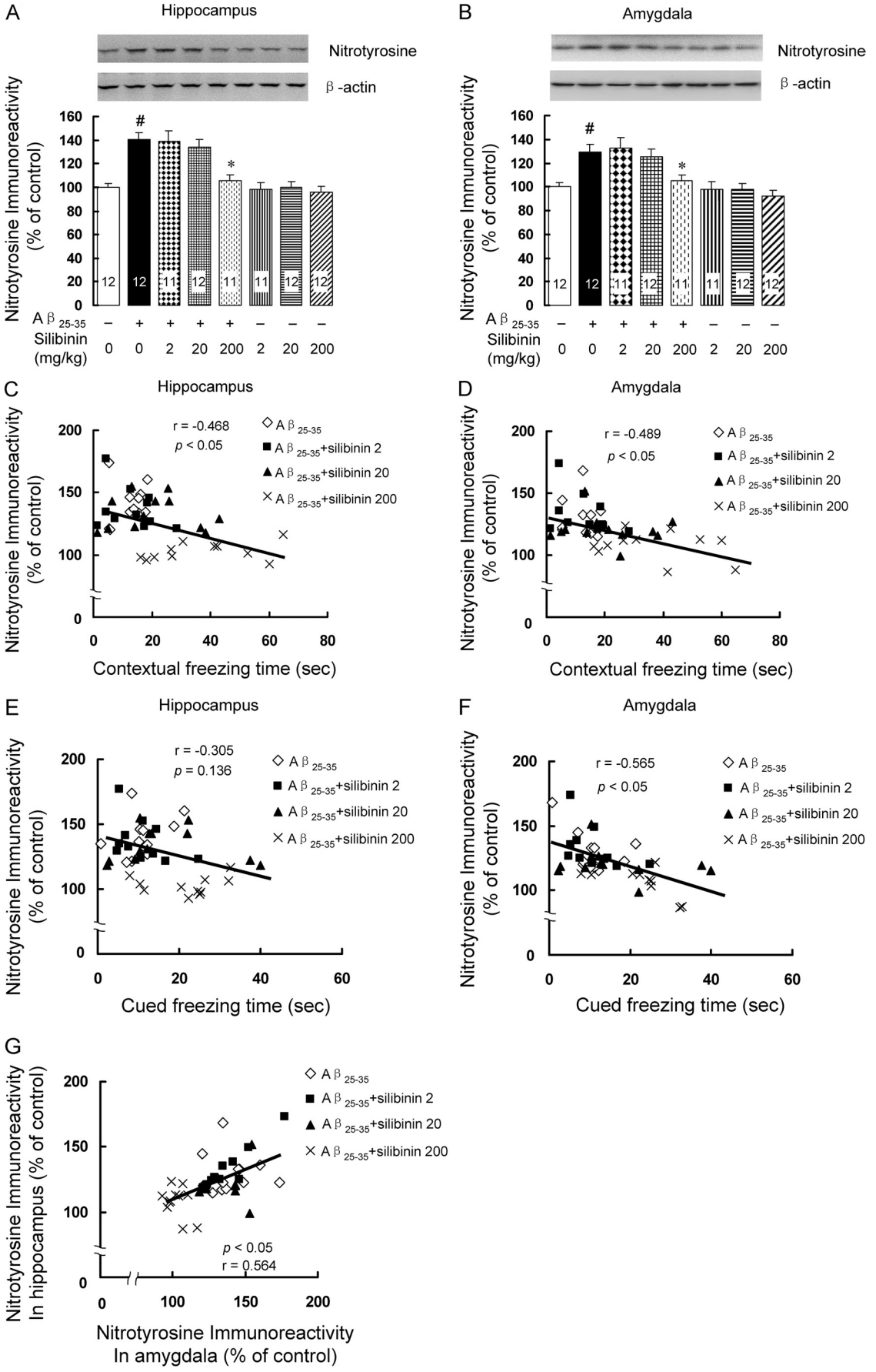

**Fig. 2.** Effect of silibinin on memory impairment induced by $A\beta_{25-35}$ in cued and contextual fear-conditioning tests. A, cued fear-conditioning test. B, contextual fear-conditioning test. C, correlation between cued and contextual freezing time. D, pain threshold test. Results were expressed as the mean ± S.E.M. ($n$ = 11 or 12 and is shown in each column) and analyzed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #, $p < 0.05$ compared with CMC-treated, distilled water-injected mice; ∗, $p < 0.05$ compared with CMC-treated $A\beta_{25-35}$-injected mice.

solution by gavage for 8 days after the treatment with $A\beta_{25-35}$. All compounds were systemically administered in a volume of 0. 1 ml/10 g body weight.

**Cued and Contextual Fear-Conditioning Tests.** Cued and contextual fear-conditioning tests were carried out on days 6 and 7 (Fig. 1A) after the injection of $A\beta_{25-35}$ according to a previous report (Wang et al., 2007). For measuring basal levels of the freezing response (preconditioning phase), mice were individually placed in a neutral (uncontexual) cage (width × length × height; 23 × 23 × 12 cm) for 1 min and then in the conditioning cage (25 × 31 × 11 cm) for 2 min before the conditioning phase. For conditioning, mice were placed in the conditioning cage, and then a 15-s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.6 mA was delivered as an unconditioned stimulus through a shock generator (Brainscience Idea Co. Ltd, Osaka, Japan). This procedure was repeated four times with 15-s intervals. Cued and contextual tests were carried out 24 h after the fear-conditioning phase on day 7. For the cued fear-conditioning test, the freezing response was measured in a neutral cage for 1 min in the presence of a continuous tone stimulus identical to the conditioned stimulus. For the contextual fear-conditioning test, mice were placed in the conditioning cage, and the freezing response was measured for 2 min without tone and the unconditioned stimulus. A freezing response was defined as four paws of a mouse staying still and the animal stooped down.

**Pain Threshold.** This test was carried out on day 6 after $A\beta_{25-35}$ injection. The dark compartment of the passive avoidance apparatus was used to determine the threshold of pain from electrical stimuli. Mice were habituated to apparatus for 15 min before a series of inescapable shocks was delivered. Each series consisted of 11 shocks at the following intensities: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8 mA. The duration of each shock was 2 s, and the shocks were delivered at 30-s intervals. Thresholds for flinch (forepaws off of the grid floor), jump (all four paws off of the grid floor), and vocalization were measured.

**Western Blotting.** The mice received the final administration of silibinin 1 h before decapitation on day 8 after Aβ treatment (Fig. 1A). The hippocampus and amygdala were removed on an ice-cold glass plate and stored at −80°C. Tissues were homogenized in 150 μl of ice-cold extraction buffer [20 mM Tris-HCl buffer, pH 7.6, 150 mM NaCl, 2 mM EDTA·2Na, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 1 mg/ml leupeptin]. The protein concentration of lysate was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). It was then incubated in 5% skim milk in a washing buffer [Tris-buffered saline containing 0.05% (v/v) Tween 20] for 2 h at room temperature. The membranes were incubated with mouse anti-nitrotyrosine clone 1A6 (1:1000) (Upstate Cell Signaling, Lake Placid, NY) or mouse anti-actin primary antibody (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight. The membrane was incubated with horseradish peroxidase-labeled anti-mouse IgG (1:1000) (KPL, Gaithersburg, MD). Immunoreactive complexes on the membrane were detected using Western blotting detection reagents (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions and exposed to X-ray film. The intensity of each protein band on the film was analyzed with the Atto Densitograph 4.1 system (Atto, Tokyo, Japan) and corrected with the corresponding β-actin level. The results were expressed as a percentage of the control.


A
Hippocampus
Nitrotyrosine
β -actin
Nitrotyrosine Immunoreactivity (% of control)
Aβ 25-35
Silibinin (mg/kg)
B
Amygdala
C
Hippocampus
r = -0.468
p < 0.05
Aβ 25-35
Aβ 25-35+silibinin 2
Aβ 25-35+silibinin 20
Aβ 25-35+silibinin 200
Contextual freezing time (sec)
D
Amygdala
r = -0.489
p < 0.05
E
Hippocampus
r = -0.305
p = 0.136
Cued freezing time (sec)
F
Amygdala
r = -0.565
p < 0.05
G
Nitrotyrosine Immunoreactivity In hippocampus (% of control)
p < 0.05
r = 0.564
Nitrotyrosine Immunoreactivity In amygdala (% of control)

**Real-Time Reverse Transcription-Polymerase Chain Reaction.** Mice were treated with silibinin and $A\beta_{25-35}$ 3 and 2 h before the decapitation, respectively (Fig. 1B) because our previous report demonstrated that levels of TNF-α and iNOS mRNA peaked 2 h after $A\beta_{25-35}$ injection (Alkam et al., 2008). The hippocampus and amygdala were removed on an ice-cold glass plate and stored at −80°C. Tissues were homogenized, and total RNA was extracted using an RNeasy total RNA isolation kit (Qiagen, Valencia, CA). The primers used were as follows: for iNOS (GenBank accession number NM 010927), forward primer, 5′-GGGCAGCCTGTGAGACCTT-3′, and reverse primer, 5′-GCATTGGAAGTGAAGCGTTTC-3′; TaqMan probe, 5′-TGCGACAGCACAAGTCACAGCCCC-3′; and for TNF-α (GenBank accession number NM 023517), forward primer, 5′-CTTTCGGTTGCTCTTTGGTTGAG-3′, and reverse primer, 5′-GCAGCTCTGTCTGTTGGATCAG-3′; PCRs were performed using the One-Step SYBR PrimeScript RT-PCR kit (Takara, Kyoto, Japan). The reaction profile consisted of a first round at 95°C for 3 min and then 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 34 s, and extension at 72°C for 1 min, with a final extension reaction carried out at 72°C for 10 min. RT-PCR was carried out with a Bio-Rad iCycler iQTM real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Expression levels were calculated as described previously (Wada et al., 2000).

To standardize the quantification, β-actin was amplified simultaneously. The threshold cycle of each gene was determined as the PCR cycle at which there was an increase in reporter fluorescence above a baseline signals. The difference in threshold cycles between the target gene and β-actin gives the standardized expression level (delta threshold cycle, dCt). Subtraction of the dCt of distilled water-injected and CMC-treated mice from each group gives the delta delta threshold cycle (ddCt) values that were used to calculate relative expression levels in each group with the formula $2^{-ddCt}$. The expression levels of each gene were expressed as the fold increase in each group compared with distilled water-injected and CMC-treated mice.

**Statistical Analyses.** The results are expressed as the mean ± S.E.M. Statistical significance was determined with the one-way ANOVA followed by Tukey's multiple comparisons test. A Pearson correlation analysis was performed to elucidate the relationships. $p < 0.05$ was taken as a significant level of difference.

## Results

**Effect of Silibinin on Memory Impairment Induced by $A\beta_{25-35}$ in Fear-Conditioning Tests.** Cued and contextual fear-conditioning tests were carried out on day 6 after the injection of $A\beta_{25-35}$. In the preconditioning phase (training), mice showed less of a freezing response. There were no differences in the basal levels of freezing response in the groups [$F(7,92) = 0.763$, $p = 0.619$ in a neutral cage; $F(7,92) = 0.120$, $p = 0.997$ in the conditioning cage; data not shown]. The contextual and cued-dependent tests were performed 24 h after conditioning. $A\beta_{25-35}$-injected mice exhibited less of a cued or contextual-dependent freezing response than distilled water-injected mice (cued freezing response: $p < 0.05$, Fig. 2A; contextual freezing response: $p < 0.05$, Fig. 2B), indicating an impairment of associative memory. Silibinin dose-dependently attenuated the impairment of cued and contextual freezing responses in $A\beta_{25-35}$-injected mice [$F(7,92) = 6.799$, $p < 0.001$, Fig. 2A; $F(7,92) = 13.09$, $p < 0.001$, Fig. 2B]. Tukey's post hoc analysis revealed that silibinin at 200 mg/kg significantly attenuated the memory impairment in $A\beta_{25-35}$-injected mice ($p < 0.05$, Fig. 2A; $p < 0.05$, Fig. 2B). In addition, silibinin itself had no significant effect on either freezing response in distilled water-injected mice [$F(3,46) = 0.799$, $p = 0.501$, Fig. 2A; $F(3,46) = 0.200$, $p = 0.896$, Fig. 2B]. We also observed a correlation between cued and contextual freezing responses ($r = 0.603$, $p < 0.05$, Fig. 2C).

As shown in Fig. 2D, there were no differences among the groups in the levels of electric current required to elicit flinching [$F(7,92) = 0.309$, $p = 0.948$], vocalization [$F(7,92) = 0.369$, $p = 0.918$], and jumping [$F(7,92) = 0.243$, $p = 0.973$].

**Effect of Silibinin on the Level of Nitrotyrosine.** $A\beta_{25-35}$-injected mice showed a significant increase of nitrotyrosine levels in the hippocampus and amygdala compared with distilled water-injected mice [hippocampus: $F(7,92) = 15.33$, $p < 0.001$, post hoc, $p < 0.05$, Fig. 3A; amygdala: $F(7,92) = 9.165$, $p < 0.001$, post hoc, $p < 0.05$, Fig. 3B]. Silibinin significantly attenuated the increase in nitrotyrosine levels induced by $A\beta_{25-35}$ ($p < 0.05$, Fig. 3A; $p < 0.05$, Fig. 3B). Silibinin did not affect nitrotyrosine levels in the hippocampus or amygdala of distilled water-injected mice [$F(3,46) = 0.180$, $p = 0.909$, Fig. 3A; $F(3,46) = 0.328$, $p = 0.805$, Fig. 3B]. In addition, nitrotyrosine levels in the hippocampus and amygdala negatively correlated with contextual freezing responses (correlation between nitrotyrosine levels in the hippocampus and contextual freezing response: $r = -0.468$, $p < 0.05$, Fig. 3C; correlation between nitrotyrosine levels in the amygdala and contextual freezing response: $r = -0.489$, $p < 0.05$, Fig. 3D), although the negative correlation between nitrotyrosine level and cued freezing response was observed in the amygdala but not in the hippocampus (correlation between nitrotyrosine levels in the hippocampus and cued freezing response: $r = -0.305$, $p = 0.136$, Fig. 3E; correlation between nitrotyrosine levels in the amygdala and cued freezing response: $r = -0.565$, $p < 0.05$, Fig. 3F). We also found that the increase in nitrotyrosine immunoreactivity in the hippocampus induced by $A\beta_{25-35}$ correlates with that in the amygdala ($r = -0.564$, $p < 0.05$, Fig. 3G).

**Effect of Silibinin on iNOS mRNA Expression.** A significant increase of iNOS mRNA expression was observed in the hippocampus and amygdala of $A\beta_{25-35}$-injected mice compared with distilled water-injected mice (hippocampus: $p < 0.05$, Fig. 4A; amygdala: $p < 0.001$, Fig. 4B). Silibinin significantly attenuated the increase induced by $A\beta_{25-35}$ in the hippocampus and amygdala [$F(3,31) = 10.846$, $p < 0.001$, post hoc, $p < 0.05$, Fig. 4A; $F(3,31) = 8.345$, $p < 0.001$, post hoc, $p < 0.05$, Fig. 4B]. Silibinin did not affect iNOS mRNA expression in the hippocampus or amygdala of distilled water-injected mice ($p = 0.534$, Fig. 4A; $p = 0.864$, Fig. 4B).

**Effect of Silibinin on TNF-α mRNA Expression.** A significant increase of TNF-α mRNA expression was observed in the hippocampus and amygdala of $A\beta_{25-35}$-injected mice compared with distilled water-injected mice (hippocampus: $p < 0.05$, Fig. 5A; amygdala: $p < 0.05$, Fig. 5B). Silibinin

**Fig. 3.** Effect of silibinin on nitrotyrosine levels in the hippocampus and amygdala of $A\beta_{25-35}$-injected mice. A and B, nitrotyrosine levels in the hippocampus (A) and amygdala (B). C to F, correlation of contextual freezing time with nitrotyrosine-immunoreactivity in the hippocampus (C) or amygdala (D) and correlation of cued freezing time with nitrotyrosine immunoreactivity in the hippocampus (E) or amygdala (F). G, correlation of the nitrotyrosine immunoreactivity in the hippocampus with that in the amygdala. Results were expressed as the mean ± S.E.M. ($n = 11$ or 12 and was shown in each column) and analyzed by a one-way ANOVA, followed by Tukey's test for multiple comparisons in A and B, by Pearson correlation analysis (C to G). #, $p < 0.05$ compared with CMC-treated, distilled water-injected mice; ∗, $p < 0.05$ compared with CMC-treated, $A\beta_{25-35}$-injected mice.

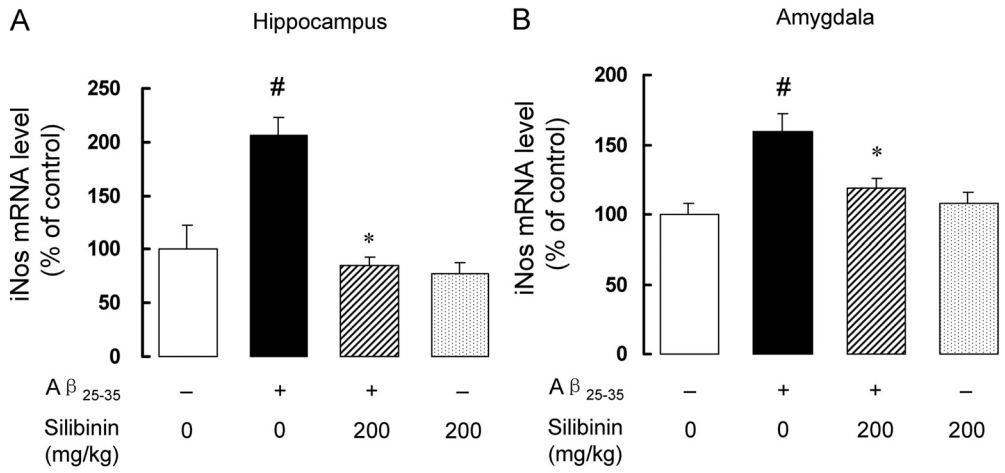



**Fig. 4.** Effect of silibinin on iNOS mRNA expression in the hippocampus and amygdala of $A\beta_{25–35}$-injected mice. A, hippocampus. B, amygdala. Results were expressed as the mean ± S.E.M. ($n = 8$), and analyzed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #, $p < 0.05$ compared with CMC-treated, distilled water-injected mice; ∗, $p < 0.05$ compared with CMC-treated, $A\beta_{25–35}$-injected mice.

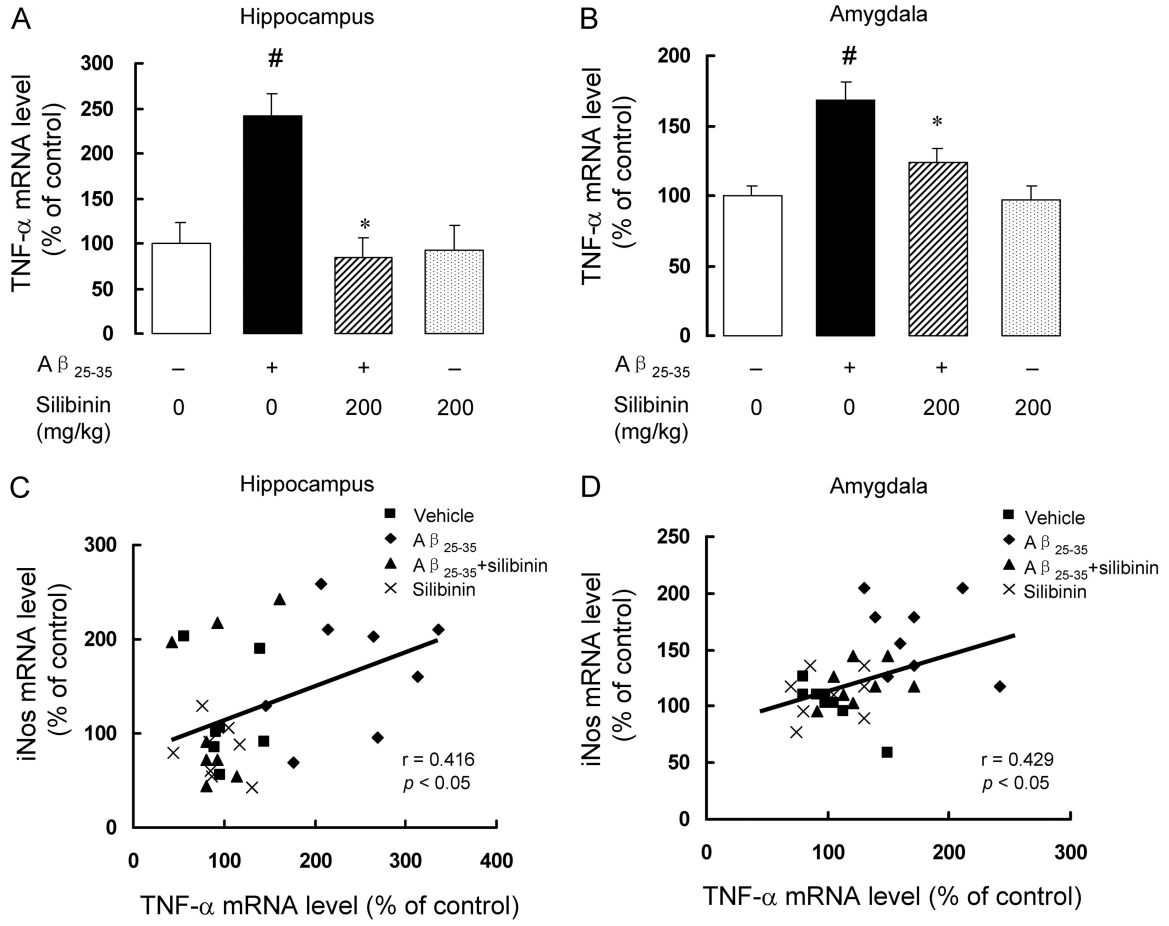



**Fig. 5.** Effect of silibinin on TNF-α mRNA expression in the hippocampus and amygdala of $A\beta_{25–35}$-injected mice. A and B, TNF-α mRNA expression in the hippocampus (A) and amygdala (B). C and D, correlation of TNF-α mRNA levels with iNOS mRNA levels in the hippocampus (C) and amygdala (D). Results were expressed as the mean ± S.E.M. ($n = 8$) and analyzed by a one-way ANOVA, followed by Tukey's test for multiple comparisons (A and B) or by a Pearson correlation analysis (C and D). #, $p < 0.05$ compared with CMC-treated, distilled water-injected mice; ∗, $p < 0.05$ compared with CMC-treated, $A\beta_{25–35}$-injected mice.

significantly attenuated the increase induced by $A\beta_{25–35}$ in both the hippocampus and amygdala [$F(3,31) = 5.55$, $p < 0.05$, post hoc, $p < 0.05$, Fig. 5A; $F(3,31) = 10.497$, $p < 0.001$, post hoc, $p < 0.05$, Fig. 5B]. Silibinin did not affect TNF-α expression in the hippocampus or amygdala of distilled water-injected mice ($p = 0.994$, Fig. 5A; $p = 0.995$, Fig. 5B). In addition, iNOS mRNA expression correlated with TNF-α mRNA expression in the hippocampus and amygdala (hippocampus: $r = 0.416$, $p < 0.05$, Fig. 5C; amygdala: $r = 0.429$, $p < 0.05$, Fig. 5D).

## Discussion

In this study, we demonstrated that silibinin attenuated $A\beta_{25–35}$-induced memory impairment in the cued and contextual fear-conditioning tests and the accumulation of nitroty-

rosine and overexpression of TNF-α and iNOS mRNA in the hippocampus and amygdala. Fear conditioning is a form of memory in which fear is associated with a particular neutral context (e.g., a room) or neutral stimulus (e.g., a tone). Impairment of this type of memory has been found in AD patients (Hamann et al., 2002). Cued and contextual fear-conditioning tests are widely used in experimental animals and have well confirmed that the cued fear response is mainly dependent on the amygdala, whereas the contextual fear response is dependent on the hippocampus and amygdala (Phillips and LeDoux, 1992). In this study, $A\beta_{25-35}$ caused memory impairment in both cued and contextual fear-conditioning tests, and the result consisted with our previous report (Wang et al., 2007). Repeated silibinin treatment significantly attenuated the memory impairment induced by $A\beta_{25-35}$ without affecting the responses to electrical foot shock (flinching, jumping, and vocalization). It is unlikely that the effect of silibinin is due to changes of pain threshold. Furthermore, silibinin itself affected neither motivation nor motor function because our previous study has demonstrated that silibinin had no effect on locomotor activity and exploratory activity (Lu et al., 2009). Taken together, these results suggest that repeated administration of silibinin attenuates the deficit of fear-associative memory induced by $A\beta_{25-35}$.

It has been confirmed that peroxynitrite-mediated damage contributes to Aβ-induced neuronal toxicity and cognitive deficits (Tran et al., 2003; Alkam et al., 2008) and is widespread in the brain of AD patients (Smith et al., 1997). Tyrosine residues are important for redox and cell signaling (Schopfer et al., 2003). Aβ-induced tyrosine nitration, which inhibits the phosphorylation and conformational change of protein, results in memory deficits (Tran et al., 2003; Butterfield et al., 2007). The level of nitrotyrosine has been used as a marker of nitrosative stress and negatively correlated with the level of Aβ in the cerebrum and cognitive function (Smith et al., 1997; Ishii et al., 2000; Tran et al., 2003). In the present study, we found that nitrotyrosine levels in the hippocampus and amygdala negatively correlated with contextual freezing responses. Furthermore, nitrotyrosine level in the amygdala, but not the hippocampus, negatively correlated with the performance in cued conditioning. The result is consistent with previous report that the cued fear response is mainly dependent on the amygdala, whereas the contextual fear response is dependent on both hippocampus and amygdala (Phillips and LeDoux, 1992). It also suggested that the damage of both hippocampus and amygdala contributes to the cognitive deficits induced by Aβ. Moreover, silibinin significantly attenuated the elevation of nitrotyrosine in the hippocampus and amygdala induced by $A\beta_{25-35}$. Our group has demonstrated previously that the activation of iNOS and damage from peroxynitrite contributed to Aβ-induced cognitive deficits in a water-maze test and a novel object recognition test (Tran et al., 2001, 2003; Alkam et al., 2008). These findings suggest that protection from peroxynitrite may be involved in the ameliorating effects of silibinin on cognitive deficits.

Peroxynitrite is produced by superoxides and large amounts of NO synthesized by iNOS under pathological conditions (Reiter et al., 2000). iNOS plays critical roles in Aβ-induced neurotoxicity (Tran et al., 2001, 2003; Alkam et al., 2008) and is up-regulated in the brain of AD patients (Lee et al., 1999). Although the NO synthesized by neuronal NOS facilitates the formation of memories under physiological conditions (Yamada et al., 1995), NO production from iNOS is deleterious under pathological conditions. Because of its independence of elevated intracellular $Ca^{2+}$ levels (Cho et al., 1992), iNOS catalyzes a high-output pathway of NO production (Xie et al., 1999) that is capable of causing neuronal peroxynitrite-mediated damage and dysfunction (Tran et al., 2001, 2003). In the present study, silibinin significantly inhibited the increase in iNOS mRNA in the hippocampus and amygdala induced by $A\beta_{25-35}$. It has been reported that silibinin suppressed the expression or activation of iNOS in several tissues and cell lines, including a glial cell line (Wang et al., 2002). Taken together, it is possible that silibinin prevents $A\beta_{25-35}$-induced peroxynitrite-mediated damage by inhibiting iNOS expression.

The molecular analysis of the iNOS gene has shown the presence of binding sites for nuclear factor-κB and TNF-α-response element in its promoter region (Eberhardt et al., 1996). In this study, we found that silibinin significantly inhibited the increase of TNF-α mRNA in the hippocampus and amygdala induced by $A\beta_{25-35}$. In addition, iNOS mRNA expression correlated with TNF-α mRNA expression in the hippocampus and amygdala. Furthermore, it has been demonstrated in vitro that the stimulation of neuronal cell lines with TNF-α leads to increased expression of inducible nitric-oxide synthase and subsequent apoptosis (Heneka et al., 1998). A specific TNF-α antibody blocked iNOS expression evoked by $A\beta_{1-40}$ in mice (Medeiros et al., 2007). Genetic deletion or pharmacological inhibition of TNF-α suppresses iNOS mRNA expression in the hippocampus and the cognitive deficit induced by $A\beta_{25-35}$ in mice (Alkam et al., 2008). These findings suggested that silibinin inhibits the iNOS expression possibly via down-regulation of TNF-α expression.

Several studies has confirmed that overactivation of glial cells induces TNF-α expression (Combs et al., 2001). Morphological studies have demonstrated that many reactive microglia and astrocytes surround Aβ plaques in the AD brain (Miyazono et al., 1991; McGeer et al., 2006). It is now well documented that fibrillar forms of Aβ serve as a stimulus for glial cell line (Combs et al., 2001; McGeer et al., 2006). It has been reported that silibinin inhibits the microglia activation and overexpression of TNF-α and iNOS evoked by LPS in vitro (Wang et al., 2002). However, whether silibinin regulates the activation of microglia or astrocytes induced by $A\beta_{25-35}$ and the exact molecular targets of silibinin responsible for its antioxidative and anti-inflammatory properties remain unknown.

In addition, silibinin improves antioxidative system (Kren and Walterová, 2005), which may also contribute to its alleviative effect on oxidative damage and cognitive deficits. The delicate balance between oxidative species and antioxidative defenses is disturbed in some pathological conditions such as AD (Guidi et al., 2006). Glutathione (GSH) is an important intracellular antioxidant and responsible for removing oxygen free radical, which is necessary for the formation of peroxynitrite. Previously, we found that silibinin alleviated a reduction in GSH levels induced by $A\beta_{25-35}$ in the hippocampus in this model (Lu et al., 2009). Indeed, silibinin has been reported to increase cellular glutathione content (Valenzuela et al., 1989) and superoxide dismutase levels (Müzes et al., 1991). Therefore, we cannot rule out the possibility that antioxidative systems are involved in alleviative effect of

silibinin on oxidative damage and cognitive deficits induced by $A\beta_{25-35}$.

In conclusion, the present study confirmed that silibinin could ameliorate memory impairment induced by $A\beta_{25-35}$. The effect of silibinin may be attributed to the blocking of inflammatory responses and oxidative stress in the hippocampus and amygdala. As a therapeutic agent, silibinin is well tolerated and largely free of adverse effects, with few negative drug interactions (Jacobs et al., 2002). Therefore, silibinin may be a potential candidate for an AD medication.

**Acknowledgments**

We thank all of the members in the department of Chemical Pharmacology (Meijo University).

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