RESEARCH PAPER

Silibinin prevents amyloid β peptide-induced memory impairment and oxidative stress in mice

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Background and purpose: Accumulated evidence suggests that oxidative stress is involved in amyloid β (Aβ)-induced cognitive dysfunction. Silibinin (silybin), a flavonoid derived from the herb milk thistle (Silybum marianum), has been shown to have antioxidative properties; however, it remains unclear whether silibinin improves Aβ-induced neurotoxicity. In the present study, we examined the effect of silibinin on the memory impairment and accumulation of oxidative stress induced by Aβ25–35 in mice.

Experimental approach: Aggregated Aβ25–35 (3 nmol) was intracerebroventricularly administered to mice. Treatment with silibinin (2, 20 and 200 mg·kg−1, once a day, p.o.) was started immediately after the injection of Aβ25–35. Locomotor activity was evaluated 6 days after the Aβ25–35 treatment, and cognitive function was evaluated in a Y-maze and novel object recognition tests 6–11 days after the Aβ25–35 treatment. The levels of lipid peroxidation (malondialdehyde) and antioxidant (glutathione) in the hippocampus were measured 7 days after the Aβ25–35 injection.

Key results: Silibinin prevented the memory impairment induced by Aβ25–35 in the Y-maze and novel object recognition tests. Repeated treatment with silibinin attenuated the Aβ25–35-induced accumulation of malondialdehyde and depletion of glutathione in the hippocampus.

Conclusions and implications: Silibinin prevents memory impairment and oxidative damage induced by Aβ25–35 and may be a potential therapeutic agent for Alzheimer’s disease.

Keywords: amyloid β; silibinin; memory deficits; oxidative stress

Abbreviations: Aβ, amyloid β; AD, Alzheimer’s disease; CMC, carboxymethylcellulose; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GSH, glutathione; MDA, malondialdehyde; MES buffer, 0.2 M 2-(N-morpholino)ethanesulphonic acid buffer; TBARS, thiobarbituric acid-reactive substance

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function (Palmer, 2002). Common pathological features of AD are senile plaques, neurofibrillary tangles and neuronal loss in the medial temporal lobe structures and cortical areas of the brain (Blennow et al., 2006). The deposition of amyloid β (Aβ) peptide in neuritic plaques is the defining feature for diagnosis of AD, and the level of Aβ peptide correlates well with the extent of cognitive impairment (Naslund et al., 2000). Aβ, a spontaneously aggregating peptide of 39–43 amino acids, is the primary protein component of senile plaques, a pathological hallmark of AD in the brain (Shen et al., 2002). Several studies have demonstrated that oxidative stress is involved in Aβ-induced neurotoxicity and the progression of AD (Schubert et al., 1995; Yankner, 1996). In particular, Aβ fragment 25–35 (Aβ25–35) seems to be responsible for toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation (Trubetskaya et al., 2003), protein nitration, induction of inducible nitric oxide synthase (Tran et al., 2001; Alkam et al., 2008) and protein oxidation in fibroblasts derived from AD patients (Choi et al., 2003).
In animal experiments, intrahippocampal or i.c.v. injections of Aβ25–35 induced histological and biochemical changes, learning deficits (Maurice et al., 1996; Meunier et al., 2006; Alkam et al., 2008) and dysfunction of the cholinergic system, which plays an important role in the cognitive deficits associated with aging and neurodegenerative diseases (Tran et al., 2001). Thus, Aβ25–35-injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD (Maurice et al., 1996).

Several synthetic antioxidants are available, but there is a growing trend towards the use of natural products (polyphenols, flavonoids, vitamins, carotenes and lycopenes) as antioxidants. Of these, bioflavonoids are a ubiquitous group of polyphenolic substances present in most plants (Nijveldt et al., 2001). Silibinin (silybin) is a flavonoid derived from the herb milk thistle (Silybum marianum) (Kren and Walterová, 2005; Figure 1A) and used as a hepatoprotectant in the clinical treatment of liver disease (Kren and Walterová, 2005). Silibinin seems to protect against oxidative stress as it has been reported to decrease lipid peroxidation, a sensitive marker of oxidative lipids, in liver microsomes and isolated hepatocytes (Bosisio et al., 1992). Furthermore, it has been demonstrated that the antioxidative activity of silibinin is related to the scavenging of free radicals (De Groot and Rauen, 1998; Trouillas et al., 2008) and activation of antioxidative defenses: increases in cellular glutathione (GSH) content (Valenzuela et al., 1989) and superoxide dismutase levels (Mázes et al., 1991). These findings suggest that silibinin could attenuate oxidative stress-induced brain dysfunction. In fact, there are a few reports that silymarin, a mixture of flavonoids present in milk thistle and whose main component is silibinin, has protective effects on the central nervous system against ethanol-induced brain injury (La Grange et al., 1999) and lipopolysaccharide-induced neurotoxicity (Wang et al., 2002). However, it remains unclear whether silibinin protects against Aβ-induced neurotoxicity.

In the present study, to confirm the usefulness of silibinin against Aβ-induced neurotoxicity, we investigated whether silibinin prevents memory impairment in an Aβ25–35-injected animal model of AD. Moreover, we examined the protective effect of silibinin on oxidative events indicated by an increase in malondialdehyde (MDA), the end product of lipid peroxidation, and decrease in GSH, an endogenous antioxidant, in the hippocampus of Aβ25–35-injected mice.

**Methods**

**Animals**

Male ICR mice, 5 weeks old at the beginning of experiments, 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 08.00 h to 20.00 h). The mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum. Behavioural experiments were carried out in a sound-proof 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 Meijo University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2006). The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**I.c.v. injection**

Aβ25–35 was dissolved in distilled water at a concentration of 1 mM as a stock solution and stored at −20°C. In the present study, the concentration and dose were selected according to a previous study (Maurice et al., 1996). It has been shown that
i.c.v. administration of Aβ25–35 at the dose of 9 nmol leads to memory impairment with severe neurotoxicity in mice, and at the dose of 3 nmol produces mild brain dysfunction, whereas it fails to cause cognitive deficits at the dose of 1 nmol (Maurice et al., 1996). The Aβ25–35 (3 nmol = 3 µg) is greatly diluted by cerebrospinal fluid and interstitial fluid to a level of about 6000 ng·g⁻¹ tissue, which is in the range of tissue levels (21.0–89.1 ng·g⁻¹ tissue: water-soluble Aβ; 377.3–3000.0 ng·g⁻¹ tissue: water-insoluble Aβ) found in brains of AD patients (Kuo et al., 1996), when it diffuses throughout the whole brain (average weight is about 0.5 g). Moreover, a certain amount of Aβ25–35 is cleared or moved out from brain, which further decreases its concentration. Therefore, Aβ25–35 at the dose of 3 nmol was used in this study. The Aβ25–35 was aggregated, or ‘aged’, by incubating it in distilled water at 37°C for 4 days. Aggregated Aβ25–35 (3 nmol·(3 µL)⁻¹) or distilled water (3 µL) was injected i.c.v., as described previously (Maurice et al., 1996). Briefly, a microsyringe with a specially made 28-gauge stainless steel needle, 3 mm in length, was used for microinjections. Mice were anaesthetized 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 (A, ~0.22 mm; L, 1 mm from the bregma; V, 2.5 mm from the skull). Aβ25–35 (3 nmol·(3 µL)⁻¹) or distilled water (3 µL) was delivered gradually within ~3 s. Mice exhibited normal behaviour within 1 min after injection. In preliminary experiments the injection site was confirmed by injecting Indian ink. Neither insertion of the needle nor injection of the distilled water had a significant influence on survival, behavioural responses or cognitive functions.

**Drug treatment**
Silibinin was suspended in a 0.3% carboxymethylcellulose solution. Mice were administered silibinin (2, 20 and 200 mg·kg⁻¹·p.o.) or the 0.3% carboxymethylcellulose solution by oral gavage 60 min before the Y-maze test and the training session of the novel object test. All compounds were administered systemically in a volume of 0.01 mL·g⁻¹ body weight (Figure 1B).

**Behavioural procedures**
Previously it has been shown that acute exposure to aged Aβ25–35 induces apoptosis-mediated neuronal toxicity during 6 days of incubation in hippocampal cultures, and cognitive dysfunction in several learning and memory tests in mice (Maurice et al., 1996). The behavioural tests were started 6 days after the Aβ25–35 injection and were carried out sequentially according to the experimental schedule shown in Figure 1B. The present study was conducted in a blind manner.

**Measurement of locomotor activity**
The measurement of locomotor activity in a novel environment was carried out on day 6 after the start of silibinin administration. Spontaneous locomotor activity was measured as previously reported with a minor modification (Miyamoto et al., 2001). Mice were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (45 × 26 × 40 cm) for 90 min, and locomotor activity was measured in 5 min intervals by using digital counters with infra-red sensors. The system was equipped with photosensor frames in the side walls. Locomotor activity was defined as the total number of beam cuts due to horizontal movement measured by the photosensors. The acrylic cage was wiped with a paper towel between uses and kept clean.

**Spontaneous alternation in a Y-maze test**
This behavioural test was performed 6 days after the Aβ25–35 injection, according to a previous study (Mouri et al., 2007), with minor modifications. The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the centre of the apparatus and allowed to move freely through the maze for 8 min. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternation behaviour (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two) multiplied by 100.

**Novel object recognition test**
The novel object recognition test was performed 7–11 days after the Aβ25–35 injection, according to a previous study (Mouri et al., 2007), with minor modifications. The task consisted of three sessions: habituation, training and retention. Each mouse was individually habituated to the box (30 × 30 × 30 high cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the middle of the box. A mouse was then placed midway at the front of the box, and total time spent exploring the two objects was recorded for 10 min. During the retention session, animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function.

**Determination of lipid peroxidation level**
Malondialdehyde was measured with a thiobarbituric acid-reactive substance (TBARS) assay kit. Briefly, the hippocampus was isolated and homogenized in cooled RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSE, 1 mM Na₃VO₄, 1 mM NaF and protease inhibitor cocktail). Tissue homogenate from the
hippocampus was incubated with 8.1% sodium dodecylsulphate for 10 min followed by the addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% thiobarbituric acid at 99°C for 1 h. After being cooled, the mixture was centrifuged at 1600×g for 10 min at 4°C. The absorbance was measured by using a plate reader (Bio-Rad 550) at 540 nm. MDA content was expressed as μmol·g⁻¹ tissue.

**Determination of glutathione content**
A GSH assay kit was used for detecting GSH in the hippocampus. Briefly, the hippocampal tissues were isolated and homogenized in cold buffer (0.05 M phosphate, pH 6–7, containing 1 mM EDTA). The homogenates were centrifuged at 10 000×g for 15 min at 4°C, and the supernatant was subsequently incubated with MES buffer (0.2 M 2-(N-morpholino)ethanesulphonic acid buffer, 0.1 M phosphate and 2 mM EDTA, pH 6.0), cofactor mixture, enzyme mixture and DTNB [5,5′-dithiobis (2-nitrobenzoic acid)] in the dark on the orbital shaker at room temperature for 30 min. Absorbance at 405 nm was measured by using a plate reader (Bio-Rad 550), and GSH content was calculated as μmol·g⁻¹ tissue.

**Statistical analysis**
All data are expressed as the means ± s.e.mean. Statistical differences among the experimental groups were tested by using a one- or two-way analysis of variance (ANOVA) for behavioural tests, and Tukey’s post hoc test was employed for multiple comparisons. *P*-values less than 0.05 were accepted as significant.

**Materials**
Aβ25–35 was purchased from Bachem (Bubendorf, Switzerland); silibinin from Panjin Green Biological Development Co., Ltd. (Panjin, China); digital counters with infrared sensors Scanet SV-10 (Melquest Ltd., Toyama, Japan); TBARS assay kit and GSH assay kit, Cayman (Ann Arbor, MI, USA).

**Results**

**Effect of silibinin on locomotor activity**
The counts of spontaneous locomotor activity of mice were measured on day 6 after Aβ25–35 injection. There were no significant differences in the time course of locomotor activity [F(_group_, 5, 756) = 1.556, _P_ = 0.170; _F_(time, 17, 756) = 108.92, _P_ < 0.01; _F_(_group_ × _time_, 85, 756) = 0.347, _P_ = 0.999] and total locomotor activity [F(5, 47) = 0.202, _P_ = 0.96] among the groups (Figure 2A).

**Effect of silibinin on short-term memory impairment induced by Aβ25–35 in Y-maze test**
We evaluated the effects of silibinin on impairment of short-term memory 6 days after the Aβ25–35 injection in a Y-maze test. Aβ25–35-injected mice showed significantly reduced spontaneous alternation behaviour compared with vehicle-injected mice (_P_ < 0.05, Figure 2B). Treatment with silibinin (2, 20 and 200 mg·kg⁻¹) dose-dependently attenuated the impairment of spontaneous alternation behaviour in Aβ25–35 injected mice [F(5, 66) = 6.325, _P_ < 0.05, Figure 3]. Silibinin doses of 20 and 200 mg·kg⁻¹ significantly prevented Aβ25–35-induced memory impairment (_P_ < 0.05, Figure 3). There was no significant difference in the number of arm entries among the groups [F(4, 66) = 0.463, _P_ = 0.763; data not shown]. Silibinin did not affect spontaneous alternation (Figure 2B) and the number of arm entries (data not shown) in mice injected with distilled water.

**Figure 2** Effects of silibinin on locomotor activity (A) and impairment of short-term memory in Y-maze test (B) in amyloid β (Aβ25–35)-injected mice. Results are expressed as the means ± s.e.mean (A: _n_ = 8, B: _n_ = 13–15) and were analysed by a one or two-way ANOVA, followed by Tukey’s test for multiple comparisons. #_P_ < 0.05 versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *_P_ < 0.05 versus CMC-treated, Aβ25–35-injected mice.
Effect of silibinin on recognition memory impairment induced by \(\text{A}^\beta_{25-35}\) in the novel object recognition test

We evaluated the effect of silibinin on impairment of visual recognition memory 7 days after the \(\text{A}^\beta_{25-35}\) injection in a novel object recognition test. In the training session, mice injected with \(\text{A}^\beta_{25-35}\) or distilled water spent equal amounts of time exploring either of the two objects (Figure 3A), showing there was no biased exploratory preference in either group of animals. In addition, total time spent in the exploration of objects in the training and retention sessions did not differ between the groups injected with \(\text{A}^\beta_{25-35}\) or distilled water (Figure 3B and D).

When the retention session was performed 24 h after the training session, the level of exploratory preference for the novel objects in the \(\text{A}^\beta_{25-35}\)-injected mice was significantly decreased compared with that in the distilled water-injected mice (\(P<0.05\), Figure 3C). Treatment with silibinin (2, 20 and 200 mg·kg\(^{-1}\)) dose-dependently and significantly reversed the decreased level of exploration preference in the retention session in \(\text{A}^\beta_{25-35}\)-injected mice \([F(5, 66) = 16.195, P<0.05,\) Figure 3C]. Tukey’s post hoc analysis revealed that silibinin at 200 mg·kg\(^{-1}\) significantly prevented the memory impairment in \(\text{A}^\beta_{25-35}\)-injected mice (\(P<0.05\), Figure 3C). Treatment with silibinin did not affect either the level of exploratory preference for the objects or the total exploration time in either the training or retention sessions for the mice injected with \(\text{A}^\beta_{25-35}\) or those injected with distilled water (Figure 3A–D).

Effect of silibinin on the MDA and GSH levels in the hippocampus of \(\text{A}^\beta_{25-35}\)-injected mice

To determine whether lipid peroxidation is involved in the ameliorating effect of silibinin in \(\text{A}^\beta_{25-35}\)-injected mice, we...
examined the effect of silibinin on the levels of MDA in the hippocampus 7 days after the Aβ25–35 injection. A significant increase in the level of MDA was observed in the hippocampus of Aβ25–35-treated mice as compared with the control mice. Treatment with silibinin (200 mg·kg−1) significantly prevented the increase of MDA levels in the hippocampus of Aβ25–35-treated mice (P < 0.05, Figure 4A). In contrast, Aβ25–35-injected mice showed a significant decrease of GSH levels in the hippocampus compared with the control mice. Treatment with silibinin (2, 20 and 200 mg·kg−1) dose-dependently prevented the decrease in GSH levels in the hippocampus of Aβ25–35-injected mice [F(5, 34) = 9.351, P < 0.05, Figure 4B]. 

Figure 4 Effects of silibinin on amyloid β (Aβ25–35)-induced increase in malondialdehyde (MDA) levels and decrease in glutathione (GSH) levels in the hippocampus. (A) MDA levels. (B) GSH levels. Results are expressed as the means ± S.E.M. (n = 6–7) and analysed by a one-way ANOVA, followed by Tukey’s test for multiple comparisons. #P < 0.05 versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *P < 0.05 versus CMC-treated, Aβ25–35-injected mice.

Discussion and conclusions

In the present study, we examined the effect of silibinin on the memory impairment induced by Aβ25–35 in mice. Silibinin prevented Aβ25–35-induced impairment of short-term and recognition memory in the Y-maze and novel object recognition tests respectively. Furthermore, silibinin prevented the accumulation of lipid peroxide (MDA) and decrease of antioxidant (GSH) in the hippocampus after the Aβ25–35 treatment. To our knowledge, this is the first study to show that silibinin protects against Aβ-injected neurotoxicity by regulating oxidative stress in the brain.

The accumulation of Aβ proteins is highly toxic to primary and other cell lines (Kim et al., 2007; Nie et al., 2008). Aβ25–35 is most toxic Aβ fragment that has been detected in the brain of AD patients (Pike et al., 1995; Kubo et al., 2002; Zameer et al., 2006). Aβ25–35 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 properties such as learning and memory impairment, morphological alterations and cholinergic dysfunction (Pike et al., 1995; Tran et al., 2001; Kubo et al., 2002; Alkam et al., 2008). Therefore, the effect of silibinin on Aβ-induced neurotoxicity was evaluated in Aβ25–35-injected mice. In the present study, Aβ25–35-injected mice showed memory impairment in both the Y-maze and the novel object recognition tests. These results are consistent with our previous findings that Aβ25–35 induces cognitive impairment in mice (Alkam et al., 2007; 2008; Tsunekawa et al., 2008).

Silibinin dose-dependently and significantly prevented the impairment of short-term and recognition memory induced by Aβ25–35. It is unlikely that the protective effect of silibinin is due to changes in motivation or sensorimotor function, as various motivations are involved in these behavioural tasks, and different skills are required for better performance in each task. Actually, silibinin had no effect on locomotor activity, number of arm entries in the Y-maze test and total time spent exploring objects in the novel object test. These results suggest that silibinin attenuates cognitive impairments in the Aβ25–35-injected mice without affecting motor function, motivation, and exploratory activity.

It has been proposed that oxidative stress plays a critical role in the development of AD (Smith et al., 1996). Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages membranes and generates a number of secondary products including aldehydes, such as MDA, and 4-hydroxy-2-nonenal, ketones, etc. (Slater, 1984). Analysis of AD brains demonstrates an increase in lipid peroxidation products in the amygdala, hippocampus and parahippocampal gyrus of the AD brain compared with
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As another possible mechanism, silibinin may directly suppress aggregation of Aβ or stability of aggregated Aβ, so affecting Aβ conformation in the brain. Polyphenols, such as silibinin, have shown to inhibit aggregation of Aβ and exhibit significant destabilizing activity on aggregated Aβ (Ono et al., 2003; Porat et al., 2006; Shoval et al., 2007). Because aggregated Aβ25–35 was used in this study, it is possible that silibinin may affect the degradation of aggregated Aβ. However, further studies are needed to clarify the effect of silibinin on the conformation and levels of Aβ.

The results from the present study confirm, for the first time, that silibinin could alleviate the memory deficits induced by Aβ25–35 in mice. The effect of silibinin may be attributed to the prevention of oxidative damage in the hippocampus, measured in terms of the amount of peroxidized lipid and the level of GSH. As a therapeutic agent, silibinin is well tolerated and largely free of adverse effects and has few negative drug interactions (Jacobs et al., 2002). Therefore, silibinin is a potential candidate for further preclinical study aimed at the treatment of cognitive deficits in AD.

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