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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

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2. 研究テーマ

アルツハイマー病動物モデルを用いた silibinin の薬効評価

3. 成果の概要

アミロイドβペプチド₂₅₋₃₅(Aβ₂₅₋₃₅)処置による恐怖条件付け記憶障害に対して、中国由来の植物、水飛薊(マリアアザミ)から抽出された silibinin を連続投与するとその障害が緩解されることを発見しました。また、silibinin のその効果には海馬と扁桃体におけるニトロ化チロシン、TNFα および iNOS の発現変化が関与していることが明らかとなりました。このことは silibinin がアルツハイマー病治療の候補物質となりうる可能性を示しております。

4. 研究業績

(1)学会における発表 無・有(学会名・演題)

The 6 th Nagoya/Nanjing/Shenyang Symposium of Pharmaceutical Sciences.
Prenatal psychological stress produces anxiety-like behavior in offspring.

The 115 th Kinki Branch Meeting of Japanese Pharmacological Society. Abnormal behavior and NMDA receptor in the postpubertal mice after prenatal exposure to phencyclidine.

The 2 th International Seminar of Meiji Pharmaceutical University Asia/Africa Center for Drug Discovery. Long-lasting effects of prenatal exposure to NMDA receptor antagonist, phencyclidine on behavior in mice.

(2)発表した論文 無・ (雑誌名・題名)

1. Lu P, Mamiya T, Lu L, Mouri A, Niwa M, Kim HC, Zou LB, Nagai T, Yamada K, Ikejima T,

- Nabeshima T. Silibinin attenuates cognitive deficits and decreases of dopamine and serotonin induced by repeated methamphetamine treatment. **Behav Brain Res.** 2010; 207(2):387-93.
2. Lu P, Mamiya T, Lu LL, Mouri A, Niwa M, Hiramatsu M, Zou LB, Nagai T, Ikejima T, Nabeshima T. Silibinin attenuates amyloid beta (25-35) peptide-induced memory impairments: implication of inducible nitric-oxide synthase and tumor necrosis factor-alpha in mice. **J Pharmacol Exp Ther.** 2009; 331(1):319-326.
 3. Lu P, Mamiya T, Lu LL, Mouri A, Zou L, Nagai T, Hiramatsu M, Ikejima T, Nabeshima T. Silibinin prevents amyloid beta peptide-induced memory impairment and oxidative stress in mice. **Br J Pharmacol.** 2009; 157(7):1270-1277.

アルツハイマー病動物モデルを用いた silibinin の薬効評価

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Abstract:

In this study, we examined the effect of silibinin on the fear-conditioning memory deficits, inflammatory response and oxidative stress induced by the intracerebroventricularly (i.c.v.) injection of A β peptide₂₅₋₃₅ (A β ₂₅₋₃₅) in mice. Mice were treated with silibinin from the day of the A β 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 α (TNF α) in the hippocampus and amygdala was measured 2 hours after the A β ₂₅₋₃₅ injection. We found that silibinin significantly attenuated memory deficits caused by A β 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 β ₂₅₋₃₅. Moreover, real-time RT-PCR revealed that silibinin inhibited the overexpression of iNOS and TNF α mRNA in the hippocampus and amygdala induced by A β ₂₅₋₃₅. These findings suggest that silibinin (i) attenuates memory impairment through amelioration of oxidative stress and inflammatory response induced by A β ₂₅₋₃₅ and (ii) may be a potential candidate for an AD medication.

Key Words A β , memory, silibinin, TNF α , iNOS

Introduction:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by extraneuronal deposits of amyloid β (A β) peptide. The deposition A β is invariably associated with oxidative stress and inflammatory response (Butterfield et al., 2007). TNF α , a pro-inflammatory cytokine, has been shown to increase in AD patients (Perry et al., 2001). A β -induced expression of TNF α leads to overexpression of inducible nitric oxide synthase (iNOS) in experimental animals. 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 (Tran et al., 2003). Interestingly, the accumulation of nitrotyrosine correlated with increased levels of cerebral A β and the severity of cognitive impairment (Tran et al., 2003).

A β ₂₅₋₃₅ is the core fragment of full-length A β and possesses many of the characteristics of the full-length A β peptide, including aggregative ability and neurotoxic property. There are reports that the i.c.v. administration of A β ₂₅₋₃₅ peptide into rodent brain is useful animal model for screening new candidates for AD therapy worldwide.

Silibinin is a flavonoid derived from the herb milk thistle, and has been reported to have anti-inflammatory and antioxidative effects. Recently, we have reported that silibinin ameliorates A β 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. In this study, we investigated the effect of silibinin on memory impairment induced by A β 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 brain of mice.

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 ($23 \pm 0.5^\circ\text{C}$, $50 \pm 5\%$ humidity) with a 12/12-h light/dark cycle (lights on from 08:00 to 20:00). The mice received food and water ad libitum.

Treatment.

$\text{A}\beta_{25-35}$ (1 mg/ml) was aggregated, by incubating it in distilled water at 37°C for 4 days before the injection. $\text{A}\beta_{25-35}$ was injected i.c.v. in a volume of 3 μl (3 nmol/mouse) on day 0 as in our previous report (Fig.1, Lu et al., 2009). Mice were administered orally (p.o.) silibinin (2, 20 or 200 mg/kg/day) or the 0.3% CMC solution by gavage for 8 days after the treatment with $\text{A}\beta_{25-35}$.

Cued and contextual fear-conditioning tests.

For conditioning, mice were placed in the conditioning cage, and then a 15-sec tone (80 dB). During the last 5 sec of the tone stimulus, a foot shock of 0.6 mA was delivered. This procedure was repeated four times with 15-sec intervals. Cued and contextual tests were carried out 24 hours 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.

Western blotting.

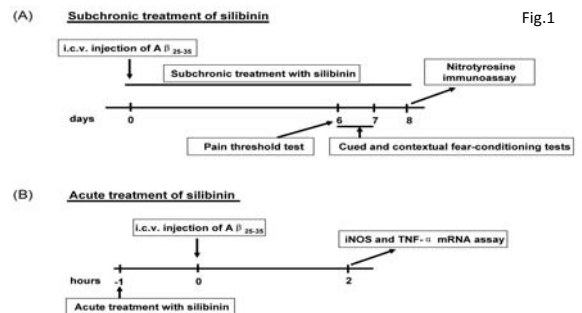
The hippocampus and amygdala were homogenized in an ice-cold extraction buffer. Equal amounts of protein (20 μg), were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically to a polyvinylidene difluoride membrane. It was then incubated in 5% skim milk in a TBS-T washing buffer for 2 hours at room temperature. Then the membranes were incubated with mouse anti-nitrotyrosine clone1A6 (1:1000) or mouse anti-actin primary antibody (1:1000) at 4°C overnight. The membrane was incubated with horseradish peroxidase-labeled anti-mouse IgG (1:1000). Immunoreactive complexes on the membrane were detected using Western blotting detection reagents and exposed to X-ray film.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR).

The hippocampus and amygdala were homogenized and total RNA was extracted using an RNeasy total RNA isolation kit. The primers used were as follows: For iNOS, forward primer: 5'-GGGCAGCCTGTGAGACCTT-3'; reverse primer: 5'-GCATTGGAAGTGAAGCGTTTC-3'; TaqMan probe: TGTCGAAGCAAACATCACATTCAGATCC; For TNF α , forward primer: 5'-CTTTCGGTTGCTCTTTGGTTGAG-3'; reverse primer: 5'-GCAGCTCTGTCTGTTGGATCAG-3'. PCRs were performed using the One Step SYBR[®] PrimeScript[™] RT-PCR Kit. 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 sec, annealing at 60°C for 34 sec, and extension at 72°C for 1 min, with a final extension reaction carried out at 72°C for 10 min.

Statistical analyses.

The results are expressed as the mean \pm 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.

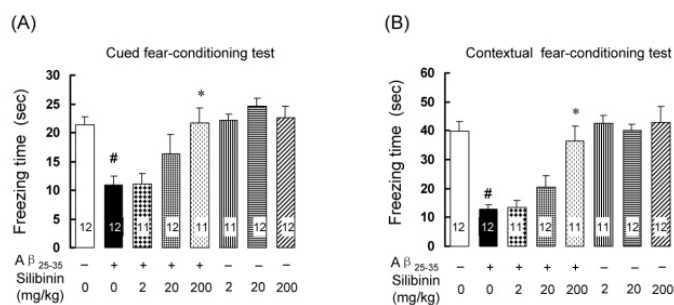


Results:

Effect of silibinin on memory impairment induced by A β_{25-35} in fear-conditioning tests.

A β_{25-35} -injected mice exhibited less of a cued or contextual-dependent freezing response than distilled water-injected mice ($p < 0.05$, Fig. 2A; $p < 0.05$, Fig. 2B), indicating an impairment of associative memory. Silibinin dose-dependently attenuated the impairment of cued and contextual freezing responses ($p < 0.001$, Fig. 2A; $p < 0.001$, Fig. 2B).

Fig. 2



Effect of silibinin on the level of nitrotyrosine.

Silibinin significantly attenuated the increase in nitrotyrosine levels induced by A β_{25-35} ($p < 0.05$, Fig. 3A; $p < 0.05$, Fig. 3B). In addition, nitrotyrosine levels in the hippocampus and amygdala negatively correlated with contextual freezing responses ($r = -0.468$, $p < 0.05$; $r = -0.489$, $p < 0.05$, data not shown), although the negative correlation between nitrotyrosine level and cued freezing response was observed in the amygdala, but not in the hippocampus ($r = -0.305$, $p = 0.136$; $r = -0.565$, $p < 0.05$, data not shown). We also found that the increase in nitrotyrosine immunoreactivity in the hippocampus induced by A β_{25-35} correlates with that in the amygdala ($r = -0.564$, $p < 0.05$, data not shown).

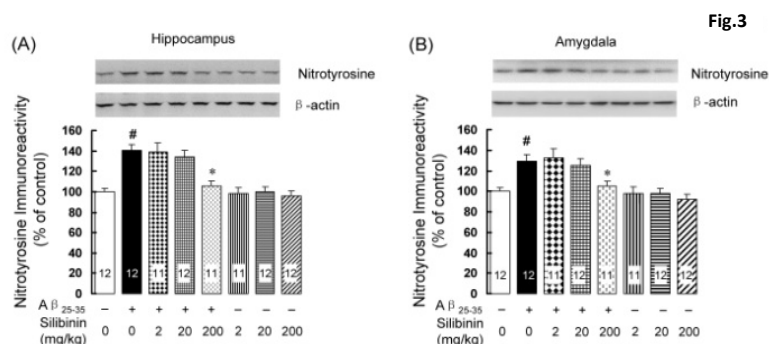


Fig.3

Effect of silibinin on iNOS mRNA expression.

Silibinin significantly attenuated the increase induced by A β_{25-35} in the hippocampus and amygdala ($p < 0.001$, Fig. 4A; $p < 0.001$, 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).

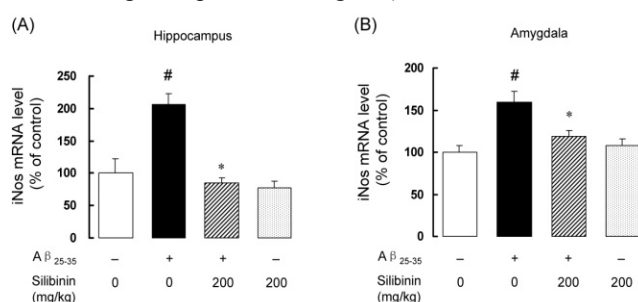
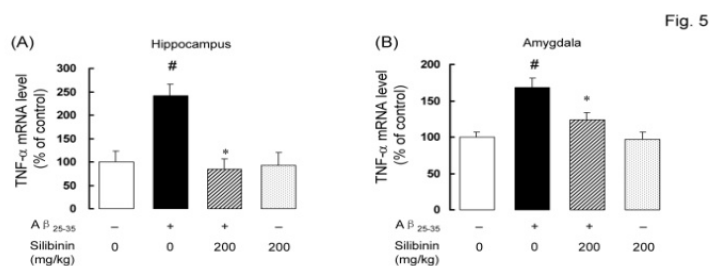


Fig.4

Effect of silibinin on TNF α mRNA expression.

Silibinin significantly attenuated the increase in the TNF α mRNA induced by A β ₂₅₋₃₅ in both the hippocampus and amygdala ($p < 0.05$, Fig. 5A; $p < 0.001$, Fig. 5B). In addition, iNOS mRNA expression correlated with TNF α mRNA expression in the hippocampus and amygdala ($r = 0.416$, $p < 0.05$; $r = 0.429$, $p < 0.05$, data not shown).



Discussion:

In this study, A β ₂₅₋₃₅ caused memory impairment in both cued and contextual fear conditioning tests. Repeated silibinin treatment significantly attenuated the memory impairment induced by A β ₂₅₋₃₅ without affecting the responses to electrical foot shock. It has been confirmed that peroxynitrite-mediated damage contributes to A β -induced neuronal toxicity and cognitive deficits (Tran et al., 2003) and is widespread in the brain of AD patients. In the present study, we found that nitrotyrosine levels in the hippocampus and amygdala negatively correlated with contextual freezing responses. Moreover, silibinin significantly attenuated the elevation of nitrotyrosine in the hippocampus and amygdala induced by A β ₂₅₋₃₅. These findings suggest that protection from peroxynitrite may be involved in the ameliorating effects of silibinin on cognitive deficits.

It has been demonstrated in vitro that the stimulation of neuronal cell lines with TNF α leads to increased expression of iNOS which catalyzes a high-output pathway of NO production and is capable of causing neuronal peroxynitrite-mediated dysfunction (Tran et al., 2003). In the present study, silibinin significantly inhibited the increase in iNOS and TNF α mRNA in the hippocampus and amygdala induced by A β ₂₅₋₃₅. It is possible that silibinin prevents A β ₂₅₋₃₅-induced peroxynitrite-mediated damage by downregulation of TNF α which inhibits iNOS expression.

In conclusion, the present study confirmed that silibinin could ameliorate memory impairment induced by A β ₂₅₋₃₅. The effect of silibinin may be attributed to the blocking of inflammatory responses and oxidative stress in the hippocampus and amygdala.

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