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Curcuma longae Radix, *Phellinus linteus* 및 *Scutellariae* Radix 혼합추출물의 Aβ (25-35) 유도 배양신경세포독성 및 마우스기억손상 억제효과

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Ethanol Extract of Three Plants of *Curcuma longae* Radix, *Phellinus linteus*, and *Scutellariae* Radix Inhibits Amyloid β Protein (25-35)-Induced Neurotoxicity in Cultured Neurons and Memory Impairment in Mice

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ABSTRACT : The present study investigated an ethanol extract (HS0608) of a mixture of three medicinal plants of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix for possible neuroprotective effects on neurotoxicity induced by amyloid β protein (Aβ) (25-35) in cultured rat cortical neurons and antidementia activity in mice. Exposure of cultured cortical neurons to 10 μM Aβ (25-35) for 36 h induced neuronal apoptotic death. At 1-50 ㎍/㎖, HS0608 inhibited neuronal death, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), and generation of reactive oxygen species (ROS) induced by Aβ (25-35) in primary cultures of rat cortical neurons. Memory loss induced by intracerebroventricular injection of ICR mice with 15 nmol Aβ (25-35) was inhibited by chronic treatment with HS0608 (25, 50 and 100 ㎎/㎏, p.o. for 7 days) as measured by a passive avoidance test. From these results, we suggest that the antidementia effect of HS0608 is due to its neuroprotective effect against Aβ (25-35)-induced neurotoxicity and that HS0608 may have a therapeutic role in preventing the progression of Alzheimer's disease.

Key Words **:** *Curcuma longae* Radix, *Phellinus linteus*, *Scutellariae* Radix, Neuroprotection, Amyloid β protein, Cultured Neurons, Memory Impairment

INTRODUCTION

Curcuma longae radix, the root of *Curcuma longa*, has been extensively studied for its biological activities, such as anti-inflammatory (Guo *et al*., 2008), anti-platelet (Srivastava *et al*., 1995), hypoglycemic (Sharma *et al*., 2006), anti-oxidant (Adaramoye, 2002) and neuroprotective effects (Rajakrishnan *et al*., 1999). Phytochemical screenings have shown that the main constituents of *Curcuma longae* radix are curcumins, curcuminoids, zingiberine, phelandreen, and essential oils (Kapoor, 1990; Srinivasan, 1953). *Phellinus linteus* has been used for its anti-cancer, anti-diabetes and anti-oxidant activities (Ajith and Janardhanan, 2002; Sliva *et al*., 2008). Recently, it was defined that *Phellinus linteus* and its active component, hispolon, shows anti-inflammation and analgesic effects via inhibition of nitric oxide (NO) and prostaglandin (PG) E_2 production and antioxidative activity (Chang *et al*., 2009; Kim *et al*., 2007). *Scutellariae* radix from *Scutellaria baicalensis* Gergi (Labiatae) has long been used as a medicinal herb in Asia due to its antipyretic, antibacterial, and anti-inflammatory properties (Bensky, 1992; Huang, 1999). Several flavonoids such as baicalin, baicalein and wogonin have been demonstrated as active components of *Scutellariae* radix (Huang, 1999). Furthermore, *Scutellariae* radix and its flavonoids have shown neuroprotection against ischemic brain damage, 6-hydroxydopamine-induced parkin-

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sonism, and Aβ (25-35)-induced amnesia (Mu *et al*., 2009; Wang *et al*., 2004; Zhang *et al*., 2006).

Alzheimer's disease (AD) is characterized by neuronal loss and extracellular senile plaque, whose major constituent is amyloid β protein (Aβ), a 39-43 amino acid peptide derived from amyloid precursor protein (Ivins *et al*., 1999). Both *in vitro* and *in vivo* studies have reported the toxic effects of Aβ or Aβ peptide fragments suggesting an important role of Aβ in the pathogenesis of AD. In cultures, Aβ can directly induce neuronal cell death (Ueda *et al*., 1997) and can render neurons vulnerable to excitotoxicity (Koh *et al*., 1990) and oxidative insults (Chen *et al*., 2007). The mechanisms underlying Aβ-neurotoxicity are complex but may involve *N*-methyl-$_D$-aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, sustained elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and oxidative stresses (Ekinci *et al*., 2000; Gray and Patel, 1995; Ueda *et al*., 1997).

An experimental model that mimics the progression of AD was developed using an intracerebroventricular (i.c.v.) injection of Aβ in mice (Van Dam and De Deyn, 2006). A continuous infusion of full-length Aβ, Aβ (1-40), into the cerebral ventricle in rats resulted in learning and memory deficits (Nitta *et al*., 1997). The deposition of Aβ in the pathogenesis of AD is invariably associated with oxidative stress and inflammatory responses. (Butterfield and Lauderback, 2002; Butterfield *et al*., 2007). Aβ has been reported to produce hydrogen peroxide and lipid peroxide in neurons and superoxide and proinflammatory cytokines in astrocytes and microglial cells (Gitter *et al*., 1995; McDonald *et al*., 1997). Antioxidants such as α-tocopherol protect against cytotoxicity induced by Aβ *in vitro* as well as against learning and memory deficits *in vivo* (Yamada *et al*., 1999). Also, α-tocopherol and anti-inflammatory agents such as indomethacin reportedly slow the progression of AD (Rogers *et al*., 1993; Sano *et al*., 1997). Aβ (25-35), the core toxic fragment of Aβ (1-40), produces similar neurodegenerative properties as Aβ (1-40), including oxidative damage, inflammatory responses, and memory impairment (Pike *et al*., 1995; Richardson *et al*., 1996).

Since reactive oxygen species (ROS) and inflammation are part of the complex series of pathophysiological events that contribute to neurodegenerative diseases such as AD and stroke (Hoehn *et al*., 2005; Pitchumoni and Doraiswamy, 1998), free radical scavengers and anti-inflammatory agents have attracted considerable attention as potential neuroprotective agents. We hypothesized that the combination of three plants of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix might protect neurons against neurodegenerative diseases such as AD and ischemia on the basis of their antioxidant, anti-inflammatory, and neuroprotective activities. The aim of the present study was to determine whether an ethanol extract of a mixture of three herbs of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix, which was named as HS0608, has a protective effect against Aβ (25-35)-induced memory deficits in mice and Aβ (25-35)-induced neuronal damage in cultured rat cortical neurons.

MATERIALS AND METHODS

1. Plant materials and extraction

The dried *Curcuma longae* radix and *Scutellariae* radix were purchased at Kyungdong Folk Medicine market, Seoul, Korea, and fruiting bodies of cultured *Phellinus linteus* were supplied from Han Kook Shin Yak, Nonsan, Chungnam. These plants were identified by one of the authors, Dr. KiHwan Bae, Chungnam National University. Each 100 g of the three plants was mixed, refluxed in 3 L ethanol at room temperature for 3 h, filtered, and lyophilized to yield an ethanol extract (HS0608, 64 g), which was then stored at −20℃ until required.

2. Experimental animals

Pregnant Sprague-Dawley rats for primary neuronal culture and male ICR mice for the passive avoidance test were supplied by Daehan BioLink Co., Ltd. (Chungbuk, Korea) and housed in an environmentally controlled room at 22 ± 2 ℃, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the animal care guidelines of the National Institutes of Health and the animal ethics committee of Chungbuk National University.

3. Induction of neurotoxicity in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 to 16 Sprague-Dawley rat fetuses, as described previously (Ban *et al*., 2006). Neurotoxicity experiments were performed on neurons after 3-4 days in

culture. Cultured neurons were treated with 10 μM Aβ (25-35) (Bachem, Bubendorf, Switzerland) in serum-free Dulbecco's modified Eagle's medium (DMEM) (Sigma) at 37℃ for 36 h (unless otherwise indicated) to produce neurotoxicity. An Aβ (25-35) stock solution of 2 mM was prepared in sterile distilled water, stored at −20℃, and incubated for more than 2 days at 37℃ to aggregate before use. HS0608 was dissolved in dimethylsulfoxide (DMSO) at concentrations of 50 ㎎/㎖ and further diluted in experimental buffers. The final concentration of DMSO was ≤ 0.1%, which did not affect cell viability. For each experiment, HS0608 was applied 15 min prior to treatment with 10 μM Aβ (25-35). It was also present in the medium during Aβ (25-35) incubation.

4. Measurements of Aβ (25-35)-induced neuronal death and intracellular biochemical changes

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death 36 h after exposure of cultured neurons to 10 μM Aβ (25-35), as described previously (Ban *et al*., 2006). Changes in $[Ca^{2+}]_i$ were measured with Fluo-4 AM (Molecular Probes), a calcium-sensitive fluorescent dye, using a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg GmbH, Germany) with a 488-㎚ excitation argon laser and 515-㎚ longpass emission filters (Ban *et al*., 2006; Lee *et al*., 2007). The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes), and a laser scanning confocal microscope (TCS SP2 AOBS; Leica, Heidelberg GmbH, Germany) with 488-㎚ excitation and 510-㎚ emission filters were used to monitor the generation of ROS in neurons treated with 10 μM Aβ (25-35) for 36 h (Ban *et al*., 2006; Kim *et al*., 2008; Lee *et al*., 2007).

5. Examination of learning and memory; Passive avoidance task

On day 1, HS 0608 was administered to 5-week-old ICR mice and 30 min later, the aggregated form of Aβ (25-35) was administered i.c.v. using a microsyringe with a 27-gauge stainless-steel needle (Hamilton), the tip of which had been adjusted to a depth of 3 ㎜. The needle was inserted unilaterally 2 ㎜ 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 (Maurice *et al*., 1996)). The injection volume of Aβ (25-35) was 15 ㎕ (15 ㎚ol). Fifteen ㎕ of saline was injected i.c.v. in sham-operated mice. HS0608 (25, 50 and 100 ㎎/㎏, p.o.) suspended in distilled water was administered daily for 7 days. Sham-operated and Aβ (25-35)-injected control groups were administered distilled water as a vehicle. Thirty min after administration of HS0608 on day 7, mice were trained on a one-trail step-through passive avoidance task. The passive avoidance box was divided into two chambers separated by a guillotine door and equipped with a grid floor and a shock generator (Gemini Avoidance System, San Diego, CA, USA). During the acquisition trial, each mouse was placed into the start chamber and kept in the dark until it was illuminated. After 50 s, the start chamber was illuminated and the door was opened for mouse to move into the dark chamber freely. Immediately after the mouse entered the dark chamber, the door was closed and the mouse received an inescapable electric shock (0.3 mA, 2 s). In the retention trial given 24 h after the acquisition trial, the mouse was again placed in the chamber and illuminated 50 s later, and the time it took until it re-entered the dark chamber was measured (the step-through latency; maximum testing limit was 300 s). To test for any possible effect of Aβ (25-35) on motor function, the locomotor activity of the animals was measured using a photobeam monitoring system (AM1051, Benwick Electronics, Benwick, UK), and motor coordination was measured using a rota-rod apparatus (Daejong Inc., Seoul, Korea). Each mouse was placed in the center of the activity cage and the total number of beam interruptions was registered for 5 min. Then the mouse was put on the rota-rod for 2 min and number of falls from the rod was noted.

6. Statistical analysis

Data are expressed as mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. $P < 0.05$ was considered significant.

RESULTS

1. HS0608 inhibits Aβ (25-35)–induced neuronal cell death

The concentration of 10 μM of Aβ (25-35) was used for

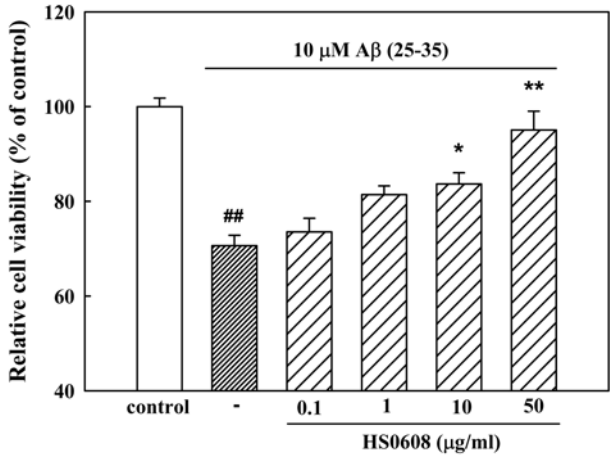

Fig. 1. Inhibitory effect of HS0608 on Aβ (25-35)-induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100%. Results are expressed as mean ± S.E.M. of data obtained from 5 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

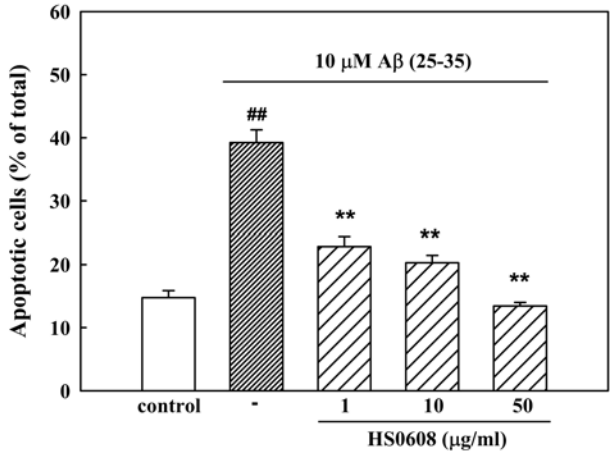

Fig. 2. Inhibitory effect of HS0608 on Aβ (25-35)-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. The values represent the apoptotic cells as a percentage of the total number of cells expressed as mean ± S.E.M. of data obtained from 3 independent experiments. $^{\#\#}P < 0.01$ vs control, $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

determining Aβ (25-35)-induced neuronal cell damage in the present experiments based on our previous result (Lee *et al*., 2005). When cortical neurons were exposed to 10 μM Aβ (25-35) for 36 h, absorbance in the MTT assay was 70.6 ± 2.2% of that of the untreated controls (Fig. 1), indicating that Aβ (25-35) caused neuronal cell death. Pretreatment of cortical neurons with 10 and 50 ㎍/㎖

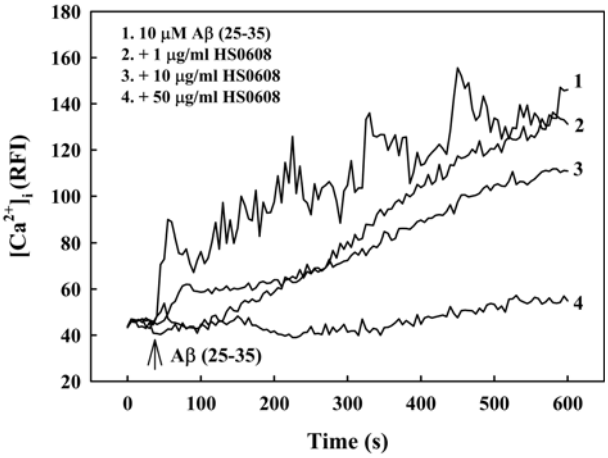

Fig. 3. Inhibitory effect of HS0608 on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least 3 independent experiments.

HS0608 reduced the neuronal death induced by 10 μM Aβ (25-35) (absorbance, 83.7 ± 2.4% and 95.1 ± 4.0% of control, respectively; Fig. 1).

An additional experiment was performed with Hoechst 33342 staining to detect condensed or fragmented DNA, which is indicative of Aβ (25-35)-induced neuronal apoptotic death. Treatment of neurons with 10 μM Aβ (25-35) produced apoptosis of 39.2 ± 2.0% of the total population of cultured cortical neurons, as compared with 14.8 ± 1.1% of apoptotic neurons in control cultures. The addition of HS0608 (1, 10 and 50 ㎍/㎖) significantly decreased the Aβ (25-35)-induced apoptotic cell death, showing 22.9 ± 1.6, 20.3 ± 1.1 and 13.4 ± 0.6%, respectively (Fig. 2).

2. HS0608 inhibits Aβ (25-35)–induced $[Ca^{2+}]_i$ elevation

Increases in $[Ca^{2+}]_i$ have been associated with Aβ-induced cell death. In our cell cultures, $[Ca^{2+}]_i$ gradually increased in response to treatment with 10 μM Aβ (25-35) with intermittent fluctuations over 10 min (Fig. 3). In contrast, pretreatment with HS0608 (1, 10 and 50 ㎍/㎖) showed inhibition of the increase of $[Ca^{2+}]_i$ induced by 10 μM Aβ (25-35) in a concentration-dependent manner. HS0608 did not affect basal $[Ca^{2+}]_i$.

3. HS0608 inhibits Aβ (25-35)–induced ROS generation

To clarify the involvement of oxidative stress in Aβ

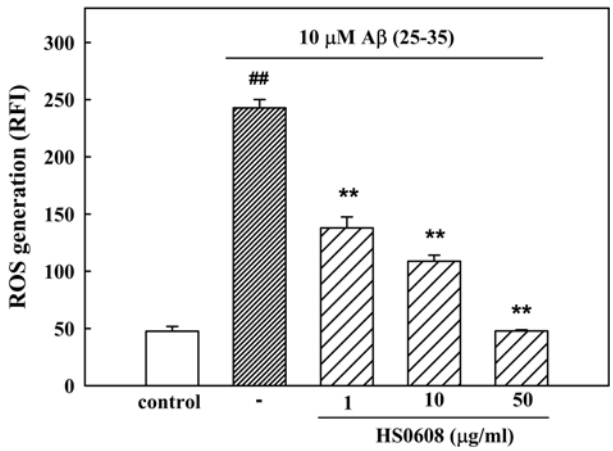

Fig. 4. Inhibitory effect of HS0608 on Aβ (25-35)-induced ROS generation in cultured cortical neurons. ROS was monitored using H_2DCF-DA dye and a confocal laser scanning microscope. Results are expressed as mean ± S.E.M. of RFI obtained from 4 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

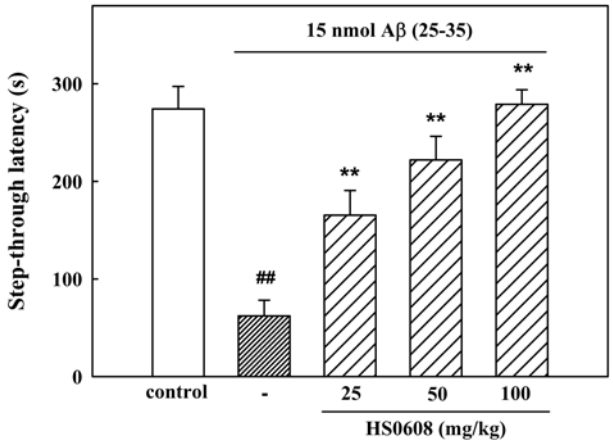

Fig. 5. Protective effect of HS0608 on Aβ (25-35)-induced memory impairment in mice. The learning and memory performance was assessed by the passive avoidance test. Values are expressed as mean ± S.E.M. of step-through latency (n = 8-12). $^{\#\#}P < 0.01$ vs sham-operated control; $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

neurotoxicity, we measured the accumulation of ROS after the exposure of the cells to Aβ (25-35) for 36 h. In H_2DCF-DA-loaded cerebral cortical neurons, 10 μM Aβ (25-35) increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with 10 μM Aβ (25-35), the relative fluorescence increased approximately 2.5-fold to 242.8 ± 7.3 compared with the value in control neurons (47.63 ± 4.2; Fig. 4). The Aβ (25-35)-induced increase in ROS generation was significantly inhibited by HS0608 (1, 10 and 50 ㎍/㎖).

4. HS0608 inhibits Aβ (25-35)–induced memory impairment

In the initial acquisition trial of the passive avoidance task, the step-through latency did not differ among the 5 groups (control, 15 nmol Aβ (25-35), Aβ (25-35) + 25 mg/kg HS0608, Aβ (25-35) + 50 mg/kg HS0608, and Aβ (25-35) + 100 mg/kg HS0608; data not shown). The step-through latency of the Aβ (25-35)-treated group in the retention trial significantly decreased to 62.2 ± 16.0 s, compared with 274.3 ± 23.1 s in the control group, indicating that Aβ (25-35) impaired memory in mice. Chronically administered HS0608 markedly protected against the memory impairment produced by Aβ (25-35). The step-through latency in groups administered HS0608 was 165.4 ± 25.3, 222.0 ± 24.2 and 279.0 ± 14.8 s at doses of 25, 50 and 100 mg/kg, respectively (Fig. 5). To elucidate whether HS0608 affects general motor functions, we measured spontaneous locomotor activity and motor coordination in the mice. Neither HS0608 nor Aβ (25-35) significantly affected locomotor and rota-rod activity (data not shown), indicating that the observed improvement of memory by HS0608 was not due to immobility.

DISCUSSION

The present study provides evidence that Aβ (25-35)-induced injury to rat cortical neurons can be prevented by HS0608, an ethanol extract of a mixture of *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix. HS0608 was able to reduce the Aβ (25-35)-induced $[Ca^{2+}]_i$ increase, ROS generation, and, in result, attenuate neuronal apoptotic death in primarily cultured rat cortical neurons. Furthermore, HS0608 prevented memory loss induced by i.c.v. injection of Aβ (25-35) in mice.

The involvement of the disruption of $[Ca^{2+}]_i$ homeostasis and ROS generation in Aβ-induced neurotoxicity has been demonstrated in various studies. Our previous studies confirmed that Aβ (25-35) caused neuronal cell death, which was blocked by treatment with MK-801, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-$_L$-arginine methyl ester ($_L$-NAME), a NO synthase (NOS) inhibitor (Ban and Seong, 2005; Lee *et al.*, 2005). This result implies the involvement of NMDA-glutamate receptor activation, an increase of Ca^{2+} influx and generation of ROS in Aβ (25-35)-induced neurotoxicity in cultured cortical

neurons, as evidenced in other studies (Ekinci *et al*., 2000; Gray and Patel, 1995; Ueda *et al*., 1997). The primary event following Aβ (25-35) treatment of cultured neurons has been suggested to be Ca^{2+} influx, apparently via L-type voltage-dependent Ca^{2+} channel (L-VDCC), since blockage of this channel and/or Ca^{2+} chelation prevents all other consequences (Ekinci *et al*., 1999; Ueda *et al*., 1997). In the present study, Aβ (25-35) elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by HS0608. Many reports demonstrated the involvement of ROS formation in Aβ-induced neurotoxicity (Miranda *et al*., 2000; Morais Cardoso *et al*., 2002). It has been reported that vitamin-E, an antioxidant, blocked the Aβ-induced generation of ROS, but not Ca^{2+} influx, and reduction of extracellular Ca^{2+} inhibited the Aβ-induced increase in intracellular Ca^{2+} as well as generation of ROS, indicating that ROS generation is a consequence of Ca^{2+} accumulation (Ekinci *et al*., 2000). HS0608 also decreased the Aβ (25-35)-induced increase of ROS generation. These results indicate that HS0608 might prevent Aβ (25-35)–induced Ca^{2+} entry through VDCC- and/or NMDA-receptor–coupled channels to inhibit ROS generation and then neuronal death, although the mechanism by which HS0608 blocks the channels is not clear. *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix, the constituents of HS0608, have been reported to possess antioxidant principles, curcumin, hispolon, and baicalein, baicalin and wogonin (Chang *et al*., 2009; Guo *et al*., 2008; Su *et al*., 2008; Zhang *et al*., 2006), repectively, suggesting that inhibition of Aβ (25-35)–induced neuronal death by HS0608 might be due to their ROS scavenging activity. Further study to elucidate the precise mechanism should be performed.

Many researchers have demonstrated that Aβ triggers apoptotic degeneration in *in vitro* neuronal experiments (Ekinci *et al*., 2000; Yan *et al*., 1999). In the present work, cultured cortical neurons exposed to Aβ (25-35) for 36 h showed increased chromatin condensation, a typical feature of apoptotic cell death, which was reduced by HS0608. Aβ-induced apoptosis was also associated with COX-2 upregulation, and COX has been suggested to be an important source of ROS in the pathologic brain (Chan, 2001; Jang and Surh, 2005). *Curcuma longae* radix, *Phellinus linteus*, and *Scutellariae* radix have been reported to inhibit expression of inflammatory mediators including NFκB, COX-2 and iNOS (Chang *et al*., 2009; Guo *et al*., 2008; Kim *et al*., 2007; Yune *et al*., 2009). Therefore, the protective effect of HS0608 on the Aβ-induced neurotoxicity might result from the inhibition of the inflammatory process. The molecular mechanism for the prevention of neuronal apoptosis by HS0608 should be further clarified.

I.c.v. injection of Aβ (25-35) into experimental rodents induces memory impairment in different behavioral paradigms, including spontaneous alternation, the water maze, and passive avoidance (Maurice *et al*., 1996; Um *et al*., 2006). Aβ (25-35) preferentially impairs spatial and non-spatial short-term memory, and these effects remain evident up to 6 months after even a single i.c.v. injection of the peptide (Stepanichev *et al*., 2003). Memory impairment in the passive avoidance test was also confirmed in mice 7 days after the i.c.v. injection of Aβ (25-35) in the present work. Chronic treatment with HS0608 effectively protected the mice against Aβ (25-35)-induced memory deficit. This result was consistent with its protective effect on Aβ (25-35)-induced neurotoxicity *in vitro*. Aβ accumulation associated with cognitive impairment in AD is accompanied by an increase in cholinesterase activity (Atack *et al*., 1983). Therefore, it is suggested that HS0608 may increase cholinergic activity.

On the other hand, other studies have indicated that oxidative stress is responsible for the onset of the cognitive dysfunction as well as the progression of AD (Butterfield *et al*., 2001; Kontush, 2001). Elevated levels of Aβ induce oxidative stress, increasing the appearance of ROS such as superoxide and NO and subsequently producing $ONOO^-$ by a rapid interaction, could mediate the damage seen in AD (Kontush, 2001; Smith *et al*., 1997). A scavenger of $ONOO^-$ protects against Aβ (25-35)-induced memory impairment (Alkam *et al*., 2007), and antioxidants such as α-tocopherol protect against cytotoxicity *in vitro* as well as against learning and memory deficits induced by Aβ (Yamada *et al*., 1999). In the present study, 10 μM Aβ (25-35) significantly increased the ROS level in cultured neurons, and this was inhibited by HS0608. In addition, the constituents of HS0608 contain antioxidant components (Chang *et al*., 2009; Guo *et al*., 2008; Su *et al*., 2008; Zhang *et al*., 2006). Therefore, it is possible that the favorable effect of HS0608 on Aβ (25-35)-induced cognitive deficits can be attributed to the inhibition of ROS generation. Meanwhile, the attenuation of memory impairment by HS0608 is likely to be a corollary of its inhibition

of Aβ (25-35)-induced $[Ca^{2+}]_i$, because memantine was demonstrated to have therapeutic benefits on AD due to its affinity for the NMDA receptor Ca^{2+} channel (Wenk, 2006)). Moreover, the possible contribution of the blocking effect of donepezil, an acetylcholinesterase inhibitor, on the voltage-gated Ca^{2+} channels to the neuroprotective effect in AD was reported (Solntseva *et al*., 2007). In support of this hypothesis, verapamil, an L-type Ca^{2+} channel blocker, inhibited Aβ (25-35)-induced memory impairment in a previous study (Cho *et al*., 2009).

The three constituent plants of HS0608 and their active principles have been reported for neuroprotective activities. *Curcuma longae* radix and curcumins have been shown to protect against Aβ-induced cognitive deficits, cerebral ischemia and heavy metals-induced neurotoxicity and have anti-depressant activity (Dairam *et al*., 2007; Frautschy *et al*., 2001; Shukla *et al*., 2008; Wang *et al*., 2005; Yu *et al*., 2002). Recently, *Phellinus linteus* has been demonstrated to reduce infarction of ischemic rats (Suzuki *et al*., 2009). Neuroprotective effects of *Scutellariae* radix and its active components, wogonin, baicalein and baicalin, have been widely studied (Heo *et al*., 2009; Mu *et al*., 2009; Wang *et al*., 2004; Zhang *et al*., 2006). Therefore, the preparation of HS0608 might reveal synergistic effect of these three plants in protection of Aβ-induced memory impairment.

In conclusion, the protection against Aβ (25-35)-induced neuronal cell damage in culture and Aβ (25-35)-induced memory deficit *in vivo* may explain the inhibitory action of HS0608 on the progression of AD. Further studies should determine the specific components in three plants of HS0608 that are responsible for preventing the memory impairment.

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