

Protection of Amyloid β Protein (25-35)-induced Neuronal Cell Damage by Methanol Extract of New Stem of *Phyllostachys nigra* Munro var. *henonis* Stapf in Cultured Rat Cortical Neuron

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ABSTRACT : *Caulis Bambusae in Taenia* is widely used in Korea and China due to its various pharmacological activity. The present study aims to investigate the effect of the methanol extract of *Caulis Bambusae in Taenia* (CB) from *Phyllostachys nigra* Munro var. *henonis* Stapf (Gramineae) on amyloid β protein (25-35) (A β (25-35)), a synthetic 25-35 amyloid peptide, -induced neurotoxicity using cultured rat cortical neurons. CB, over a concentration range of 10-50 $\mu\text{g}/\mu\text{l}$, inhibited the A β (25-35) (10 μM)-induced neuronal cell death, as assessed by a 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and the number of apoptotic nuclei, evidenced by Hoechst 33342 staining. CB (50 $\mu\text{g}/\mu\text{l}$) inhibited glutamate release into medium induced by 10 μM A β (25-35), which was measured by HPLC. Pretreatment of CB (50 $\mu\text{g}/\mu\text{l}$) inhibited 10 μM A β (25-35)-induced elevation of cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$), which was measured by a fluorescent dye, fluo-4 AM, and generation of reactive oxygen species. These results suggest that CB prevents A β (25-35)-induced neuronal cell damage *in vitro*.

Key words : *Caulis Bambusae in Taenia*, Neuroprotection, amyloid β protein (25-35); Neurotoxicity; Cortical neurons

INTRODUCTION

Alzheimer's disease (AD) is characterized by neuronal loss and extracellular senile plaque, whose major constituent is amyloid β protein (A β), a 39-43 amino acid peptide derived from amyloid precursor protein (Ivins *et al.*, 1999). Both *in vitro* (Iversen *et al.*, 1995) and *in vivo* (Chen *et al.*, 1994) studies have reported the toxic effects of A β or A β peptide fragments suggesting an important role of A β in the pathogenesis of AD. In cultures, A β can directly induce neuronal cell death (Ueda *et al.*, 1994) and can render neurons vulnerable to excitotoxicity (Koh *et al.*, 1990) and oxidative insults (Goodman & Matton, 1994). The mechanisms underlying A β -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 ($[\text{Ca}^{2+}]_i$), and oxidative stresses (Forloni, 1993; Gray & Patel, 1995; Ueda *et al.*, 1997; Ekinci *et al.*, 2000). NMDA receptor acts either as selective substrate of

A β binding or as mediator of A β -triggered glutamate excitotoxicity (Harkany *et al.*, 1999). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to Ca^{2+} . Extensive elevation of the $[\text{Ca}^{2+}]_i$ may lead directly to cellular dysfunction, overexcitation or death (Horn *et al.*, 1999). Therefore, Ca^{2+} influx through NMDA receptor activation by A β exposure may be a critical role in A β -induced neurotoxicity, as proved by a report that the neurotoxic effect of A β was reduced by NMDA receptor antagonist, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine (MK-801) (Tibor *et al.*, 1999). Several lines of evidence support the involvement of oxidative stress as an active factor in A β -mediated neuropathology, by triggering or facilitating neurodegeneration through a wide range of molecular events that disturb neuronal homeostasis (Ekinci *et al.*, 2000). However, the clinical benefit of NMDA receptor antagonists and direct blockers of neuronal Ca^{2+} channels is debatable, since they lack convincing effectiveness or have serious side-effects (Ferber & Krieglstein, 1996; Li *et al.*, 2002). There

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are much efforts to develop beneficial agents from medicinal plants to achieve neuroprotection.

Caulis Bambusae in Taenia (CB) is middle layer of new stem of *Phyllostachys nigra* Munro var. *henonis* Stapf (Gramineae) and called Jukyueu in Korean, and bamboo shavings in English. Although CB effectively treats a number of somatic complaints, such as nausea and vomiting, it also has a pronounced psychotropic effect (Flaws, 2002). Therefore, it has been prescribed for cough due to heat and phlegm, vomiting, palpitation, insomnia, mental-emotional complaints associated with fear and fright and stroke with impairment of consciousness, included in traditional oriental medicinal prescription in Korea and China (Pharmacopoeia in china, 1997). It is also known to be prescribed in traditional chinese medicine for the treatment of pain of various kinds (Zhang *et al.*, 2002; Jia *et al.*, 2003). However, there is not enough experimental evidence to prove the medicinal effect of CB. Therefore, to extend the knowledge on the pharmacological actions of CB in the CNS, the present study examined whether methanol extract of CB has the neuroprotective action against A β (25-35)-induced cell death in primarily cultured rat cortical neurons. It was also examined the effect of CB on the A β (25-35)-induced cytosolic calcium concentration ([Ca²⁺]_c) elevation, glutamate release and reactive oxygen species (ROS) generation.

MATERIALS AND METHODS

Materials

CB was purchased from an oriental drug store in Taegu, Korea, and identified by professor K.-S. Song, Kyungpook National University. A β (25-35) was purchased from Bachem (Bubendorf, Switzerland). 2-Mercaptoethanol, trypsin (from bovine pancreas), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), o-phthalaldehyde (OPA), Dulbecco's modified Eagle's medium (DMEM), Joklik-modified Eagle's medium, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-4 AM, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) and Hoechst 33342 dye were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from JRH Biosciences (Lenexa, Kansas, USA). All other chemicals used were of the highest grade available.

Preparation of methanol extract of CB

CB (300 g) was extracted three times in a reflux condenser for 24 h each with 2 l of methanol. The solution was combined, filtered through Whatman NO. 1 filter paper, and concentrated using a rotary vacuum evaporator. The yield was about 10% (w/w).

Primary culture of cortical neurons

Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic day 16 \pm 1. Fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed into Joklik-modified Eagle's medium containing trypsin (0.25 mg/ml). After slight trituration through a 5-ml pipette five to six times, the cells were incubated for 10 min at 37°C. Dissociated cells were collected by centrifugation (1,500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (44 mM), glucose (22.73 mM), penicillin (40 U/ml), gentamicin (50 μ g/ml), KCl (5 mM) and 10% fetal bovine serum at a density of about 2×10^6 cells/ml. Cells were plated onto poly-L-lysine coated 12 well-plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, and coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of cytosolic Ca²⁺ concentration ([Ca²⁺]_c), ROS and apoptosis. After 2 days incubation, the medium was replaced with a new growth medium in which the concentrations of fetal bovine serum and KCl were changed to 5% and 15 mM, respectively. Cultures were kept at 37°C in a 5% CO₂ atmosphere, changing the medium twice a week.

Neurotoxicity experiments

A β (25-35) stock solution of 2 mM was prepared in sterile distilled water, stored at -20°C, and incubated for more than 2 days at 37°C to aggregate before use. CB was dissolved in methanol with the concentration of 50 mg/ml and further diluted with experimental buffers. The final concentration of methanol was 0.1%, which did not affect cell viability. Neurotoxicity experiments were performed on neurons grown for 5~7 days *in vitro*. The culture medium was removed and replaced with serum-free growth medium. Cells were then incubated for 20 min in the medium, and incubated for a further 24 h (unless otherwise indicated) in the presence of 10 μ M A β (25-35) at 37°C to produce neurotoxicity. CB was applied to cells 15 min before the treatment with A β (25-35), and was also present in the medium during the incubation period with A β (25-35). For some experiments, a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM CaCl₂ at pH 7.4 was used.

Analysis of cell viability

MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase (Lee *et al.*, 2005). Therefore, the amount of

formazan produced is proportional to the number of viable cells. After completion of incubation with 10 μM A β (25-35) for 24 h, the culture medium was replaced by a solution of MTT (0.5 mg/ml) in serum-free growth medium. After a 4 h incubation at 37°C, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using microplate reader (Bio-Tek ELx808, Vermont, USA). Serum-free growth medium was used as blank solution.

Measurement of apoptotic cell death

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. After the exposure to 10 μM A β (25-35) in serum-free growth medium for 24 h, cells on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, then stained with Hoechst 33342 dye at the concentration of 1 mg/ml in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). The dye was excited at 340 nm, and emission was filtered with a 510 nm barrier filter. To quantify the apoptotic process, neurons with fragmented or condensed DNA and normal DNA were counted. Data was shown as apoptotic cells as a percentage of total cells.

Measurement of $[\text{Ca}^{2+}]_c$

Cells grown on coverslips were loaded with 3 μM fluo-4 AM (dissolved in dimethylsulfoxide (DMSO)) in serum-free growth medium for 45 min at 37°C in the CO₂ incubator, and washed with the incubation buffer. The coverslips containing fluo-4 AM labeled cells were mounted on a perfusion chamber containing incubation buffer, subjected to a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany), and then scanned every 3 second with a 488 nm excitation argon laser and a 515 nm longpass emission filter. After the baseline of $[\text{Ca}^{2+}]_c$ was observed for 50 sec, 10 μM A β (25-35) was added to the perfusion chamber for the measurement of $[\text{Ca}^{2+}]_c$ change. In order to test the effect of CB on the A β (25-35)-induced $[\text{Ca}^{2+}]_c$ change, cells were pretreated with CB (50 mg/ml) 15 min before the treatment with 10 μM A β (25-35) after being loaded with fluo-4 AM and washed. CB was also present in the perfusion chamber during the $[\text{Ca}^{2+}]_c$ mea-

surement period. All images, about 200 images, from the scanning were processed to analyze changes of $[\text{Ca}^{2+}]_c$ in a single cell level. The results were expressed as the relative fluorescence intensity (RFI) (Lee *et al.*, 1998).

Measurement of glutamate concentration

After being washed and equilibrated for 20 min with the incubation buffer, cells were incubated with the buffer containing 10 μM A β (25-35) for 6 h at 37°C. At the end of the incubation, glutamate secreted into the medium from the treated cells was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (BAS MF series, Indiana, USA) (Ellison *et al.*, 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 μm ; 4.6 \times 100 mm) after pre-derivatization with OPA/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 $\mu\text{A/V}$, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min.

Measurement of ROS generation

The microfluorescence assay of 2',7'-dichlorofluorescein (DCF), the fluorescent product of H₂DCF-DA, was used to monitor the generation of ROS. Cells grown on coverslips were washed with phenol red-free DMEM three times and incubated with the buffer containing 10 μM A β (25-35) at 37°C for 24 h. The uptake of H₂DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with 10 μM A β (25-35). After being washed, coverslips containing cortical cells loaded with H₂DCF-DA were mounted on the confocal microscope stage, and the cells were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) using 488 nm excitation and 510 nm emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. Challenge of H₂DCF-DA and measurement of fluorescence intensity was performed in the dark.

Statistical analysis

Data were expressed as mean \pm SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Turkey's tests. P values of <0.05 were considered to be significant.

RESULTS

CB protects neurons against cell death induced by A β (25-35).

To assess A β (25-35)-induced neuronal cell death, the MTT assay was performed. MTT assay is extensively used as a sensitive, quantitative and reliable colorimetric assay for cell viability. In previous experiments (Ban & Seong, 2005), we have demonstrated that A β (25-35) over the concentration range of 5–20 μ M produced a concentration-dependent reduction of cell viability in cultured cortical neuron. For the present experiments, the concentration of 10 μ M was used for the determination of A β (25-35)-induced neuronal cell damage. Fig. 1 shows the inhibitory effect of CB on a 10 μ M A β (25-35)-induced decrease of MTT reduction. MTT reduction rate decreased to $71.2 \pm 4.1\%$ when using 10 μ M A β (25-35). CB concentration-dependently reduced the A β (25-35)-induced decrease of MTT reduction over a concentration range of 10–50 μ g/ml showing $109.4 \pm 6.0\%$ with 50 μ g/ml, a complete protection against the neuronal damage.

An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of A β (25-35). Cell nuclei stained by Hoeschst 33342 enables the occurrence of DNA condensate to be detected, a characteristic feature of apoptosis. In cells treated with 1 μ M A β (25-35), chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable cells. As shown in Fig. 2, the proportion of apoptotic cells was calculated. The treatment

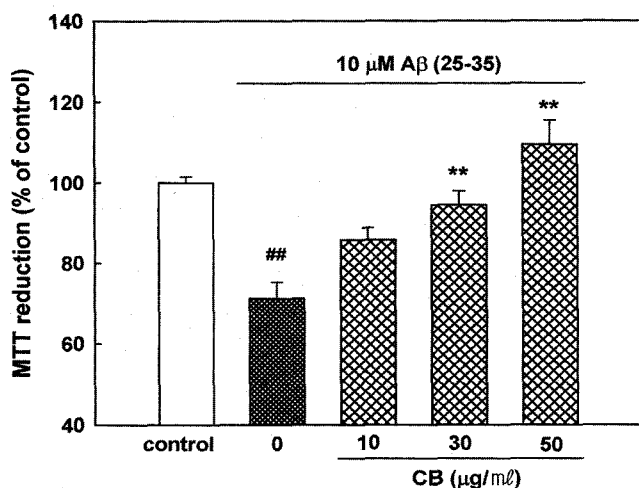


Fig. 1. Inhibitory effect of CB on A β (25-35)-induced cell death in cultured cortical neurons. Neuronal death was measured by the MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean \pm SEM values of the data obtained from three independent experiments performed in triplicate. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μ M A β (25-35).

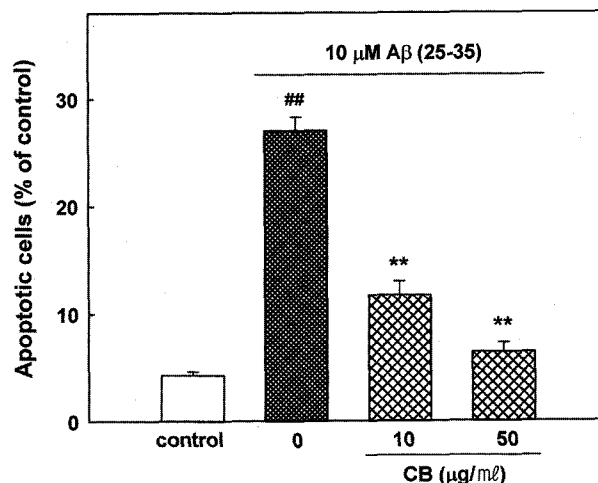


Fig. 2. Inhibitory effect of CB on A β (25-35)-induced apoptosis of cultured cortical neurons as measured by Hoechst 33342 staining. Apoptotic cells produced by 10 μ M A β (25-35) were counted from five to six fields per well. Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean \pm SEM values of the data obtained from four independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μ M β (25-35).

of cells with 10 μ M A β (25-35) produced apoptosis of $27.0 \pm 1.3\%$ of the total population of cultured cortical neurons, as compared with $4.3 \pm 0.4\%$ of apoptotic neurons in control cultures. CB (50 μ g/ml) significantly decreased the A β (25-35)-induced apoptotic cell death, showing $6.4 \pm 0.9\%$. CB alone did not affect cell viability (data not shown).

CB inhibits A β (25-35)-induced elevation of [Ca²⁺]_i.

The increase of [Ca²⁺]_i has been postulated to be associated with A β -induced cell death in many studies. In cultured cortical cells, treatment with 10 μ M A β (25-35) produced relatively slow and gradual increase of [Ca²⁺]_i. A maximal fluorescence intensity of about 180, compared to a base of 100, with the [Ca²⁺]_i elevation was measured about 5 min after the A β (25-35) application. After peaking, the fluorescence level was decreased gradually. In contrast, pretreatment with CB (50 μ g/ml) significantly inhibited the elevation of [Ca²⁺]_i induced by 10 μ M A β (25-35) throughout the measurement period. CB did not affect basal [Ca²⁺]_i (data not shown).

CB inhibits A β (25-35)-induced elevation of glutamate release

Glutamate released into the extracellular medium was quantified 6 h after the incubation of cells with 10 μ M A β (25-35). As shown in Fig. 4, 10 μ M A β (25-35) markedly elevated the basal glutamate level from $1.32 \pm 0.30 \mu$ M of

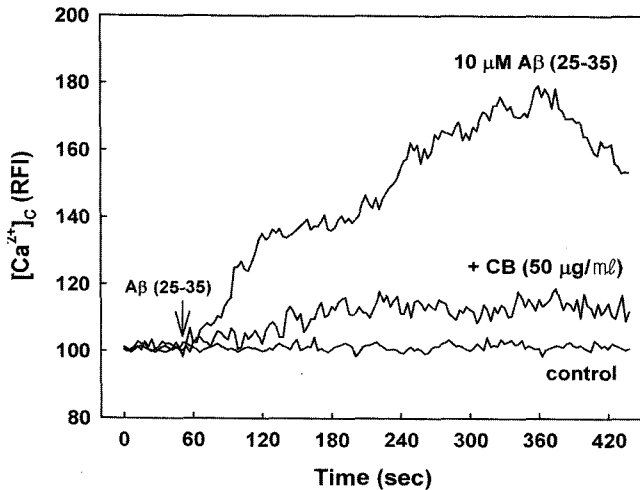


Fig. 3. Change of $[Ca^{2+}]_c$ in response to A β (25-35) in the presence or absence of CB in cultured cortical neurons. $[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative.

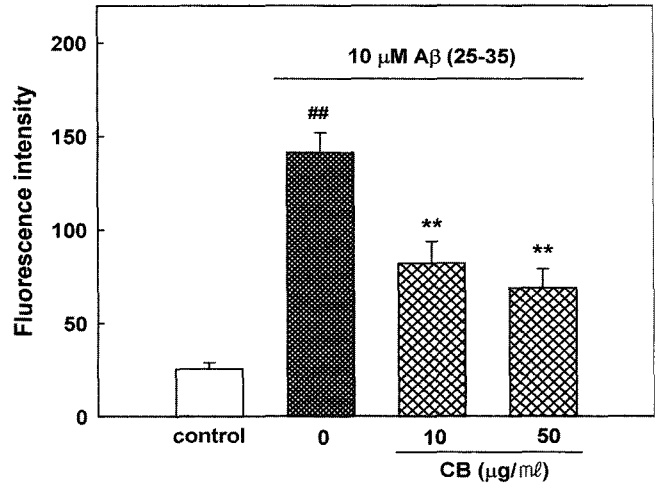


Fig. 5. Inhibitory effect of CB on A β (25-35)-induced ROS generation in cultured cortical neurons. Results are expressed as mean \pm SEM of relative fluorescence intensity obtained from three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 μ M A β (25-35).

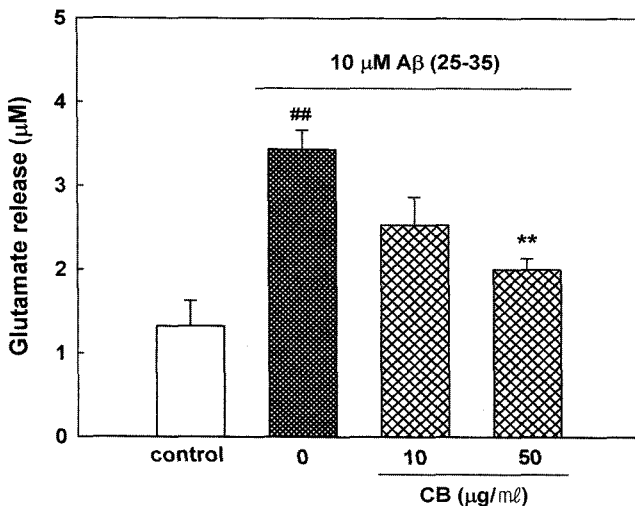


Fig. 4. Inhibitory effect of CB on A β (25-35)-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with ECD. Results are expressed as mean \pm SEM values of the data obtained in three independent experiments performed in two to three wells. ## $p < 0.01$ compared to control. ** $p < 0.01$ compared to 10 M A (25-35).

control cells to $3.43 \pm 0.23 \mu$ M. CB (50 μ g/ml) strongly blocked the A β (25-35)-induced elevation of glutamate release showing $2.01 \pm 0.13 \mu$ M.

CB inhibits A β (25-35)-induced ROS generation

A β (25-35) increased the glutamate release and the concentration of $[Ca^{2+}]_c$. Furthermore, the pathological condition

induced by A β (25-35) is associated with accelerated formation of ROS. In A β (25-35) (10 μ M)-treated cells for 24 h, the fluorescence intensity increased about 5.5 folds to 141.3 ± 10.7 compared to control cells of 25.6 ± 3.3 . The A β (25-35)-induced increase of ROS generation was significantly inhibited by CB (50 μ g/ml) showing 68.9 ± 10.2 of fluorescence intensity. CB alone did not show direct reaction with H₂DCF-DA to generate fluorescence (data not shown).

DISCUSSION

A β is a major contributor to the pathogenesis of AD. A β -induced neurotoxicity has been attributed in various studies to Ca^{2+} influx, and generation of ROS (Behl *et al.*, 1994; Arias *et al.*, 1995; Miranda *et al.*, 2000). In our previous studies, it was confirmed that A β (25-35) caused neuronal cell death, which was blocked by the treatment with MK-801, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor (Ban & Seong, 2005). This result implies the involvement of NMDA-glutamate receptor activation, an increase of Ca^{2+} influx and generation of ROS in A β (25-35)-induced neurotoxicity in cultured cortical neurons, as evidenced in other studies (O'Mahony *et al.*, 1998). Regardless of the relative contribution of these events to A β (25-35)-induced neurotoxicity, the primary event following A β (25-35) treatment in 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). Furthermore, A β (25-35)-induced elevation of $[Ca^{2+}]_c$ and neurotoxicity were inhibited by MK-801, suggesting Ca^{2+} influx through NMDA receptor-coupled VDCC plays a critical role in the neurotoxicity (Tibor *et al.*, 1999). It has been reported that vitamin-E, an antioxidant, blocked the A β -induced generation of ROS, but not Ca^{2+} influx, and reduction of extracellular Ca^{2+} inhibited the A β -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). Confirming these reports, it was demonstrated that the significant increase of ROS generation took more than 1 h, while the elevation of $[Ca^{2+}]_c$ occurred within seconds after the treatment with 10 μ M A β (25-35). In addition, L-NAME, a NOS inhibitor, failed to inhibit the A β (25-35)-induced increase in $[Ca^{2+}]_c$ in the short period of measurement in contrast to the complete inhibition of verapamil on the A β (25-35)-induced ROS generation in the previous data (Ban & Seong, 2005). However, in many experiments, free radicals are responsible for the increase of $[Ca^{2+}]_i$. The ROS-induced membrane damage causes further Ca^{2+} influx and resultant accentuated Ca^{2+} influx in turn will induce the generation of further ROS (Cotman *et al.*, 1992). Many researchers have demonstrated that A β triggered apoptotic degeneration in *in vitro* neuronal experiment (Harkany *et al.*, 1999; Yan *et al.*, 1999). Cultured cortical neurons exposed to 10 μ M A β (25-35) for more than 24 h showed increased chromatin condensation, a typical characteristic of apoptotic cell death in the present work. The A β (25-35)-induced apoptotic neuronal death was also blocked by MK-801, verapamil and L-NAME (Ban & Seong, 2005).

The present study provides evidence that A β (25-35)-induced injury to rat cortical neurons can be prevented by CB. CB was able to reduce the A β (25-35)-induced neuronal apoptotic death, $[Ca^{2+}]_c$ increase, glutamate release, and ROS generation. Many reports demonstrated that A β neurotoxicity is mainly due to massive Ca^{2+} influx through VDCC probably coupled to NMDA receptor (Ueda *et al.*, 1997; Harkany *et al.*, 1999). In the present study, A β (25-35) elicited gradual and significant $[Ca^{2+}]_c$ increase, which was blocked by CB. CB also significantly inhibited the A β (25-35)-induced glutamate elevation. This result indicates that the sustained inhibition on $[Ca^{2+}]_c$ elevation by CB resulted in the decrease of the A β (25-35)-induced glutamate release. The elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading is still a matter for further research. ROS generation undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca^{2+} influx in the cytosol (Pereira & Oliveira, 2000). Many reports demonstrated the involvement of ROS formation in

A β -induced neurotoxicity (Miranda *et al.*, 2000; Cardoso *et al.*, 2002). CB decreased the A β (25-35)-induced increase of ROS generation. It was not elucidated whether CB suppressed ROS generation through the inhibition of $[Ca^{2+}]_c$ increase, or vice versa, in the present study. We demonstrated that L-NAME, an inhibitor of ROS generation, failed to show an inhibition on the A β (25-35)-induced $[Ca^{2+}]_c$ increase occurred in seconds to minutes, while verapamil, a Ca^{2+} channel antagonist, completely blocked ROS generation in a previous experiment using cultured cortical neurons (Ban & Seong, 2005). Therefore, it is suggested that CB inhibited the A β (25-35)-induced ROS generation via the blockade of $[Ca^{2+}]_c$ increase. It is thus concluded that CB may prevent the A β (25-35)-induced apoptosis of neuronal cell by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release and generation of ROS.

CB clears heat, cools the blood, transforms phlegm, and stops vomiting. Besides these precious somatic activities, CB has been suggested to have pronounced psychotropic effect and prescribed for insomnia and stroke (Flaws, 2002). These results imply the possibility of CB having neuroprotective effect. A β is believed to play a central role in the pathophysiology of AD (Hsiao *et al.*, 1995; Holcomb *et al.*, 1998). Although it is still controversial whether increased A β formation is sufficient to cause nerve cell degeneration in AD, neurotoxic effects of A β have been demonstrated in both *in vitro* and *in vivo* (Chen *et al.*, 1994; Iversen *et al.*, 1995). CB completely blocked A β (25-35)-induced neuronal cell death in the present study. This CB-induced neuroprotection might be related to the prevention of AD progression. CB has been known to contain many pharmacologically active components such as pentosan, lignan, cellulose and triterpene. We isolated various active components including friedelin, a triterpene, from CB. It, however, must be made further studies to clarify the active components to which CB-induced neuroprotection is attributable.

In conclusion, we demonstrated a novel pharmacological action of CB and its mechanism in the present study. The protection against A β (25-35)-induced neurotoxicity by CB may help to explain at least its inhibitory actions on the progression of AD, and furthermore provide the pharmacological basis of its clinical usage in treatment of neurodegeneration in AD.

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