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A novel compound RS-0466 reverses β-amyloid-induced cytotoxicity through the Akt signaling pathway in vitro

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Abstract

β-Amyloid peptide is the principal protein in the senile plaques of Alzheimer's disease and is considered to be responsible for the pathology of Alzheimer's disease. Several studies have shown that β-amyloid is cytotoxic, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an indicator of viability in cells. Utilizing the MTT assay, we screened an in-house library to find compounds that suppress β-amyloid-induced inhibition of MTT reduction. From among the screening hits, we focused on 6-ethyl-*N*,*N*′-bis(3-hydroxyphenyl)[1,3,5]triazine-2,4-diamine (named RS-0466), which had been newly synthesized in our laboratory. This compound was found to be capable of significantly inhibiting β-amyloid-induced cytotoxicity in HeLa cells and of reversing the decrease of phosphorylated Akt induced by β-amyloid. Furthermore, RS-0466 reversed the β-amyloid-induced impairment of long-term potentiation in rat hippocampal slices. These results raise the possibility that RS-0466 or its derivatives have potential as a therapeutic agent for Alzheimer's disease patients, and its effect is at least in part mediated by activation of Akt.

Keywords: β-Amyloid; Alzheimer's disease; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Akt; Long-term potentiation; Hippocampus; RS-0466

1. Introduction

Alzheimer's disease is the most frequent neurodegenerative disorder and is characterized by two different types of fibrillar deposits, consisting of senile plaques and neurofibrillary tangles. A severe loss of basal forebrain cholinergic neurons and cortical cholinergic innervations are observed in its late stages (Anderton et al., 1998; Selkoe, 2001). Extracellular senile plaques are composed of β-amyloid protein, which is a 40–42 amino acid peptide fragment of the β-amyloid precursor protein. The potential role of β-amyloid as a neurotoxic agent has been demonstrated in vitro (Pike et al., 1993; Yankner et al., 1990) and in vivo (Emre et al., 1992; Moechars et al., 1999), which supports the hypothesis that β-amyloid triggers a neurotoxic cascade, thereby causing neurodegeneration in Alzheimer's disease patients. Although several promising approaches have been suggested such as immnunotherapy (Thatte, 2001) and β-sheet breaker compounds (Findeis and Molineaux, 1999), available Alzheimer's disease-specific therapy is currently limited to that based on the enhancement of cholinergic function, the clinical effect of which is not sufficient (Grundman and Thal, 2000). Therefore, substantial therapeutic intervention is long overdue.

In many kinds of cell culture models, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is widely used as an indicator of β-amyloid-induced cytotoxicity (Liu and Schubert, 1997). The advantage of an MTT assay is that inhibition of MTT reduction is observed following application of nanomolar concentrations of β-

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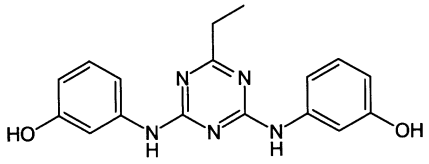

Fig. 1. Chemical structure of RS-0466. $C_{17}H_{17}N_5O_2$, mol. wt.: 323.35.

amyloid. Considering the physiological concentration of β-amyloid in the brain of Alzheimer's disease patients (Andreasen et al., 2001; Scheuner et al., 1996), MTT reduction might be a useful biological phenomenon for investigating the role of nanomolar concentrations of β-amyloid and, thus, the assay seems to be suitable for screening therapeutic agents for Alzheimer's disease.

In this study, we screened an in-house library to find compounds that prevent β-amyloid-induced cytotoxicity, utilizing the MTT assay. From among the screening hits, we focused on 6-ethyl-*N*,*N*′-bis(3-hydroxyphenyl)[1,3,5]-triazine-2,4-diamine (named RS-0466, Fig. 1), which has been newly synthesized in our laboratory. Furthermore, we investigated the pharmacological profile of RS-0466 in a long-term potentiation protocol. Long-term potentiation in the hippocampus is one form of synaptic plasticity and is thought to be a cellular mechanism underlying learning and memory (Malinow et al., 2000), the functions of which are severely damaged in Alzheimer's disease.

2. Materials and methods

2.1. Agents

β-Amyloid-(1–40) and β-amyloid-(1–42) (HCl salt) were purchased from AnaSpec (San Jose, CA, USA). Both β-amyloid-(1–40) and β-amyloid-(1–42), each at a concentration of 500 μM, were dissolved in 2 mM HCl and stored in small aliquots at −20 °C. RS-0466 was synthesized in our laboratory and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 30 mg/ml or in ethanol at a concentration of 5 mg/ml. Thioflavin T and MTT were from Wako (Osaka, Japan). For Western blotting, protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany), rabbit polyclonal anti-Akt and anti-phosphorylated Akt (Ser 473) antibodies were from Cell Signaling (Beverly, MA, USA), and an anti-rabbit immunoglobulin G antibody was from Amersham (Buckinghamshire, UK).

2.2. Cell culture

HeLa cells were suspended in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum. They were plated at a density of 1000 cells/well on 96-well tissue culture plates (catalogue number 3595, Corning, NY, USA), and cultured in a humidified atmosphere (5% CO_2–95% room air) at 37 °C. One day after plating, the culture medium was changed to EMEM supplemented with 5% fetal bovine serum. To generate fibrils, β-amyloid-(1–40) and β-amyloid-(1–42) were each diluted to 100 μM in water, NaCl and HEPES (final concentrations [in mM]: NaCl 150, HCl 0.4 and HEPES 20), and then incubated at room temperature for 1–2 days. After a change of medium, RS-0466 DMSO and/or the preincubated β-amyloid were added to the medium. The final concentration of DMSO was ≦0.01%. The HeLa cells were further cultured for 1 day and assayed for MTT reduction.

Primary hippocampal neurons were prepared from the hippocampi of 18-day-old embryos of Wistar rats (Japan SLC, Shizuoka, Japan) as described previously (Kaneko et al., 1995). The hippocampi were dissociated by gentle pipetting. Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM pyruvate and 20 mM HEPES. They were plated at a density of 18,000 cells/well on poly-L-lysine-coated 96-well tissue culture plates (catalogue number 354516, Becton Dickinson and Company, NJ, USA) and cultured in a humidified atmosphere (5% CO_2–95% room air) at 37 °C. One hour after plating, the culture medium was changed to a SUMILON Nerve Cell Culture System (SUMILON Medium, Sumitomo Bakelite). On in vitro day 4, the preincubated β-amyloid was added, and 1 h after the addition of β-amloid, RS-0466 or vehicle (DMSO) was added to the medium. The final concentration of DMSO was ≦0.01%. The hippocampal neurons were further cultured for 4 days and assayed for MTT reduction.

2.3. MTT assay

An MTT reduction assay was performed as described previously (Kaneko et al., 1995). Briefly, MTT was dissolved in phosphate-buffered saline at 5 mg/ml, and 5-μl aliquots were added to 96-well plates containing 50 μl medium per well. After incubation for 3 h at 37 °C, the absorbance at 570 nm (reference at 650 nm) of solubilized MTT-formazan product was measured with a microplate reader.

2.4. Western blotting

Cells were rinsed with Tris-buffered saline (TBS) and solubilized in sample buffer (TBS containing 2% sodium dodecyl sulfate (SDS), 80 mM Tris–HCl, 15% glycerol, 0.01% bromophenol blue and protease inhibitor cocktail, pH 7.4). The amount of protein in lysates was assayed and then the lysates were immediately boiled for 5 min. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gelelectrophoresis under reducing conditions, followed by transfer onto a polyvinylidene difluoride membrane at 1 mA/cm^2 for 3 h at room temperature.

The membranes were incubated in TBS containing 0.1% Tween 20 (TBS-T) and 10% skimmed milk at room temperature for 1 h and then with anti-Akt antibody (1:500 dilution in TBS-T and 5% skimmed milk) or anti-phosphorylated Akt antibody (1:1000 dilution) at 4 °C overnight. The membranes were washed in TBS-T for 10 min three times and further incubated with peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:1000 dilution in TBS-T and 5% skim milk) at room temperature for 1 h. Immunoreactive proteins were visualized using an enhanced chemiluminescence kit (Amersham). Levels of phosphorylated Akt obtained in three independent experiments were quantified with NIH Image software (developed by the National Institutes of Health, MD, USA). The immunoreactivity of phosphorylated Akt was normalized to total Akt. Data are expressed as a percentage of control, which represents the level of phosphorylated Akt in MTT-stimulated cells.

2.5. Slice preparation and compound treatment

Recording of field excitatory postsynaptic potentials (fEPSPs) in rat hippocampal slices was carried out as described in our previous report (Nakagami et al., 2002). Briefly, 3–4-week-old male Wistar rats (SLC) were anesthetized with ether and rapidly decapitated. Transverse hippocampal slices, 400 μm thick, were prepared using a microslicer (Dosaka E.M., Kyoto, Japan). Slices were maintained in oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF) at 30 °C for at least 1 h prior to pretreatment with β-amyloid and/or compound. ACSF had the following composition (in mM): NaCl 127, KCl 1.6, KH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10. After the recovery period, the slices were transferred to a small chamber filled with the same ACSF containing β-amyloid-(1–42) and/or RS-0466. β-Amyloid-(1–42) preincubated for 1–2 days, and RS-0466 dissolved in ethanol were used in the 5 h-pretreatment. The compounds were vortexed and diluted to desired final concentrations in ACSF immediately before pretreatment in each experiment. Control slices were also transferred to the same chamber filled with only ACSF.

2.6. Electrophysiological recordings

After pretreatment, the slices were transferred to a recording chamber in which they were continuously perfused with warmed (30 °C) and oxygenated ACSF at a rate of 1.5–2.0 ml/min. To thoroughly remove the β-amyloid- and/or compound-containing ACSF, the slices were perfused for at least 30 min before recording. The Schaffer collaterals were stimulated with a bipolar electrode, and the evoked fEPSPs were extracellularly recorded from the stratum radiatum of the CA1 region with a glass capillary microelectrode filled with 0.9% NaCl. A rectangular pulse of 50 μs duration (20–40 μA) was delivered every 30 s with an intensity that evoked a fEPSP of 50–60% of the maximum. The degree of paired-pulse facilitation was initially determined at an interpulse interval of 50 ms, and data are expressed as the percentage increase in the slope of the second fEPSP compared to the first. All data were collected using a MacLab/2e system (ADInstruments, Australia) and analyzed online using the program Scope, Ver. 3.5.

2.7. Statistical analysis

All data in this study are expressed as means ± S.E.M. Significant differences of data were calculated by Tukey's test after analysis of variance. Probability values of $P < 0.05$ were considered to represent significant differences.

3. Results

3.1. Effect of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction in HeLa cells

We investigated the effect of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction in HeLa cells. Application of 100 ng/ml (approximately 22.1 nM) β-amyloid-(1–42) suppressed MTT reduction by 32.4%, which was almost the same in as our previous report (Fig. 2; Kaneko et al., 1995). The effect of RS-0466 alone was investigated in the range of 0.37–10 μg/ml. Although 10 μg/ml RS-0466 inhibited MTT reduction, cytotoxicity was not observed up to 3.3 μg/ml. Finally, we examined the effect of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction and found that RS-0466 has significantly ameliorated β-amyloid-(1–42)-induced cytotoxicity. Similar results were obtained with β-amyloid-(1–40) instead of β-amyloid-(1–42) (data not shown).

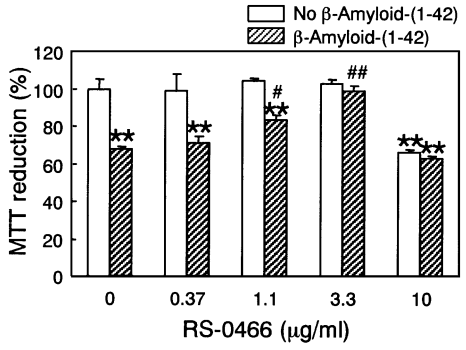

Fig. 2. Effects of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction in HeLa cells. Cultures were treated with RS-0466 for 1 day in the absence (open columns) or presence (hatched columns) of 100 ng/ml (approximately 22.1 nM) β-amyloid-(1–42). $^{**}P < 0.01$ vs. no-β-amyloid-(1–42) group without RS-0466, $\#P < 0.05$, $\#\#P < 0.01$ vs. β-amyloid-(1–42) group without RS-0466, Tukey's test, $n = 8–10$ from three independent experiments.

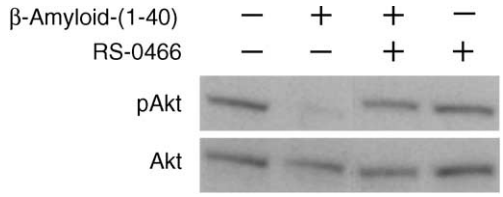

Fig. 3. Effect of RS-0466 on β-amyloid-(1–40)-induced inhibition of Akt phosphorylation in HeLa cells. Cultures were treated with 3.3 μg/ml RS-0466 for 1 day in the absence or presence of 100 ng/ml (approximately 22.1 nM) β-amyloid-(1–40), and then incubated with MTT for 30 min. Total Akt was estimated by using an anti-Akt antibody, which recognizes both phosphorylated and non-phosphorylated forms of Akt.

3.2. Effect of RS-0466 on β-amyloid-(1–40)-induced inhibition of Akt phosphorylation

The above result raised the possibility that RS-0466 works not in the extracellular space but in the intracellular space and/or on the cellular membrane. Among many cellular signaling molecules, we focused on Akt, which is a serine/threonine kinase with a broad spectrum. More recently, we have reported that β-amyloid-(1–40) significantly inhibits MTT-induced Akt phosphorylation (Kubo et al., 2002). In accordance with the report, HeLa cells contained a significant level of phosphorylated Akt in the absence of β-amyloid-(1–40) after incubation with MTT for 30 min (Fig. 3); however, treatment with 1 μg/ml β-amyloid-(1–40) resulted in a decrease in the level of phosphorylated Akt (17.6 ± 1.6%). This decrease in phosphorylated Akt was significantly reversed by addition of 3.3 μg/ml RS-0466 (100.2 ± 23.2%).

3.3. Effect of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction in hippocampal neurons

We have previously confirmed that a low concentration of β-amyloid (100–500 nM) significantly inhibited MTT reduction in primary hippocampal neurons (Kaneko et al., 1995). In this experiment, hippocampal neurons were treated with 0.5 μg/ml (approximately 110.7 nM) β-amyloid-(1–42) to detect β-amyloid-(1–42)-induced cytotoxicity. As shown in Fig. 4, this treatment produced 52.3% inhibition of MTT reduction. The effect of RS-

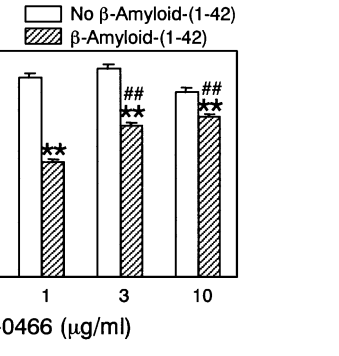

Fig. 4. Effects of RS-0466 on β-amyloid-(1–42)-induced inhibition of MTT reduction in hippocampal neurons. Cultures were treated with RS-0466 for 4 days in the absence (open columns) or presence (hatched columns) of 0.5 μg/ml (approximately 110.7 nM) β-amyloid-(1–42). $^{**}P<0.01$ vs. no-β-amyloid-(1–42) group without RS-0466, $\#\#P<0.01$ vs. β-amyloid-(1–42) group without RS-0466, Tukey's test, $n=5–15$ from three independent experiments.

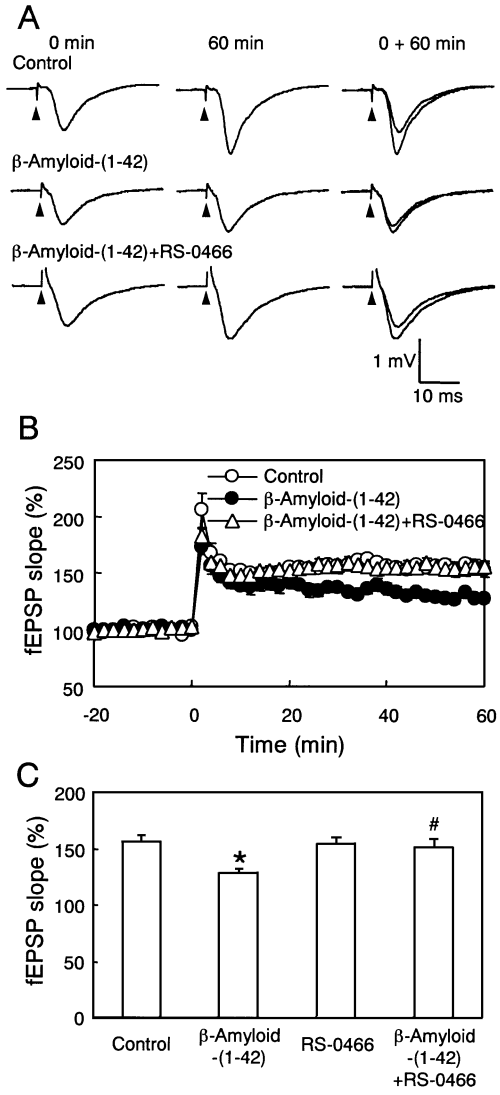

Fig. 5. Effect of RS-0466 on β-amyloid-(1–42)-induced impairment of long-term potentiation. (A) Typical fEPSPs recorded in the stratum radiatum of the CA1 area by stimulating the Schaffer collaterals. The fEPSPs immediately before and 60 min after high-frequency stimulation are superimposed on the right. Test stimulation was delivered at the times indicated by the arrowheads. (B) The time course of long-term potentiation in control slices (open circles; $n=5$), slices pretreated with 500 nM β-amyloid-(1–42) for 5 h (closed circles; $n=9$), and slices pretreated with 500 nM β-amyloid-(1–42) and 1 μg/ml RS-0466 for 5 h (open triangles; $n=5$). High-frequency stimulation (100 pulses at 100 Hz) was applied at time 0. Long-term potentiation was plotted as a percentage of the baseline fEPSP slope. (C) Summary of the effect of pretreatment with β-amyloid-(1–42) and/or RS-0466 on long-term potentiation. The average percentage of the fEPSP slope 0–60 min after high-frequency stimulation was calculated for each slice. $^{*}P<0.05$ vs. control slices, $\#P<0.05$ vs. β-amyloid-(1–42)-pretreated slices, Tukey's test, $n=4–9$.

0466 alone was investigated in the range of 1–10 μg/ml, and RS-0466 significantly protected hippocampal neurons against β-amyloid-(1–42)-induced cytotoxicity at concentrations of 3 and 10 μg/ml, indicating that the protective effect of RS-0466 is not a phenomenon peculiar to cell lines.

3.4. Effect of RS-0466 on β-amyoid-(1–42)-induced impairment of long-term potentiation

Because RS-0466 was demonstrated to protect HeLa cells and hippocampal neurons against β-amyloid-(1–42)-induced cytotoxicity, we further investigated the profile of RS-0466 using hippocampal slices, which better reflect physiological states than dispersed cells. In this study, we used an experimental protocol of β-amyloid-(1–42) pretreatment to detect β-amyloid-(1–42)-induced impairment of long-term potentiation. In the control slices, application of high-frequency stimulation produced a robust potentiation of fEPSPs, which lasted for 60 min and reached up to 160% (Fig. 5A and B, open circles). Subsequently, hippocampal slices were pretreated with 500 nM β-amyloid-(1–42) for 5 h. This concentration and application period did not induce cell death in primary hippocampal neurons (our unpublished data). The same high-frequency stimulation was delivered at time 0, but fEPSPs gradually declined to the 130% level in β-amyloid-(1–42)-pretreated slices (Fig. 5A and B, closed circles). The baseline response without high-frequency stimulation did not change over a 60-min period in the β-amyloid-(1–42)-pretreated slices (data not shown), and there was no apparent difference between the control slices and β-amyloid-(1–42)-pretreated slices in paired-pulse facilitation can interpulse interval of 50 ms; 126.4 ± 4.4%, $n = 5$ vs. 127.4 ± 6.9%, $n = 5$. In this experimental model, the effect of RS-0466 on the β-amyloid-(1–42)-induced impairment of long-term potentiation was examined. Pretreatment with 1 μg/ml RS-0466 alone did not affect long-term potentiation, and RS-0466 blocked the impairment of long-term potentiation induced by pretreatment with β-amyloid-(1–42) (Fig. 5A and B, open triangles). Comparison of slices showed that the magnitude of potentiation 0–60 min after high-frequency stimulation was significantly smaller in β-amyloid-(1–42)-pretreated slices than in control slices (Fig. 5C). Furthermore, RS-0466 significantly reversed the impairment of long-term potentiation induced by pretreatment with β-amyloid-(1–42).

4. Discussion

In this study, we have demonstrated for the first time using the MTT assays, that RS-0466, a newly synthesized compound, ameliorates β-amyloid-induced cytotoxicity in HeLa cells. We also investigated whether RS-0406 has potential as a β-sheet breaker by using a thioflavin T assay, but the compound did not affect β-amyloid fibrillogenesis (data not shown). It seems likely that RS-0466 works directly in HeLa cells, because RS-0466 did not show the profile of a fibril breaker and reversed the decrease in phosphorylated Akt induced by β-amyloid. Furthermore, RS-0466 blocked β-amyloid-induced impairment of long-term potentiation in vitro. These results raise the possibility that RS-0466 or one of its derivatives has potential as a therapeutic agent for Alzheimer's disease patients, and its effect is at least in part mediated by activation of Akt.

RS-0466 reversed the decrease in phosphorylated Akt by β-amyloid. Phosphoinositides are phosphorylated by phosphoinositide 3-kinase (PI3K), and activation of Akt is triggered via these products of PI3K. Several reports suggest that the PI3K–Akt pathway is particularly important for promoting cell survival (Brunet et al., 2001). Therefore, it is likely that the effect of RS-0466 on phosphorylated Akt contributes to the inhibition of β-amyloid-induced cytotoxicity. Recently, Zhang et al. (2001a,b) reported that α-lipoic acid and estradiol protect neurons against β-amyloid-induced cytotoxicity by activation of Akt. Tang et al. (1996) reported that treatment with estradiol reduced the risk and delayed the onset of Alzheimer's disease. To the best of our knowledge, chemical compounds, which maintain the level of phosphorylated Akt, and thereby protect cells from the cytotoxicity of β-amyloid, have not been reported yet. Furthermore, RS-0466 reversed the decrease in phosphorylated Akt induced by amylin, which is secreted from pancreatic islet β-cells and converted to amyloid deposits in type 2 diabetes (our unpublished data). Therefore, RS-0466 could be a major breakthrough in the development of therapeutic agents for amyloidosis such as Alzheimer's disease and type 2 diabetes. To find and develop a more suitable therapeutic agent, it should be elucidated how RS-0466 affects the level of phosphorylated Akt. This mechanism is possibly through signals and/or receptors involved in β-amyloid-induced cytotoxicity, by modification of Ca^{2+} homeostasis or by suppression of reactive oxygen species, etc.

Although our results imply the involvement of the Akt pathway in β-amyloid-induced cytotoxicity, it is also important to consider that the decrease in phosphorylated Akt alone is not necessarily sufficient for cytotoxicity. For example, it has been demonstrated that inhibition of the cyclin-dependent kinase (Giovanni et al., 1999) or the c-Jun N-terminal kinase (JNK) pathway (Troy et al., 2001) prevents neuronal death induced by β-amyloid. Therefore, the Akt pathway is presumably involved in the cytotoxicity; however, several other contributing components of β-amyloid-induced cell death should be also considered.

RS-0466 reversed the β-amyloid-induced impairment of long-term potentiation in rat hippocampal slices. This finding is important from the point of view that the positive effect of RS-0466 is observed not only at the dispersed cell level but also at the slice level. More recently, it has been reported that PI3K activity is required for long-term potentiation expression (Sanna et al., 2002). We are also currently

investigating whether phosphorylated Akt is decreased by treatment with β-amyloid decreases similar to the result in HeLa cells, but such an effect has not been observed so far. The change in phosphorylated Akt in slices under our experimental conditions seems much smaller than that observed in dispersed cells. This might be mainly due to the difference in experimental conditions such as preparations, concentrations of β-amyloid, time of treatment, etc.

It is commonly accepted that the inhibition of MTT reduction by β-amyloid is a useful indicator of β-amyloid-induced cytotoxicity; however, the cellular compartment responsible for MTT reduction remains to be determined. Recent findings demonstrated that enhancement of the exocytosis of MTT formazan (Liu and Schubert, 1997) or induction of lysosomal acidification (Kane et al., 1999) by β-amyloid might be involved in β-amyloid-induced inhibition of MTT reduction. We also reported that β-amyloid alters the distribution of early endosomal trafficking (Kubo et al., 2002). As with the MTT assay, it remains to be solved how pretreatment with β-amyloid impairs long-term potentiation. Lambert et al. (1998) reported that pretreatment with β-amyloid-derived diffusible ligands inhibited hippocampal long-term potentiation in vitro and Fyn, a non-receptor tyrosine kinase, is considered to be involved in the toxicity. We also demonstrated that glutamate exacerbates β-amyloid-induced impairment of long-term potentiation, suggesting that common and/or synergistic cascades between glutamate and β-amyloid exist (Nakagami and Oda, 2002a). Although the mechanisms of the above two assays still remain to be solved, these experimental protocols are important because a fast screening tool for β-amyloid inhibitors is indispensable for the development of new therapeutic drugs for Alzheimer's disease (Levine and Scholten, 1999). Further investigation concerning the mechanisms might help to provide insight into new therapeutic targets for the development of anti-Alzheimer's disease drugs.

In summary, we have succeeded in obtaining a novel compound, RS-0466, which has a pharmacological profile including inhibition of β-amyloid-induced cytotoxicity and impairment of long-term potentiation. Further detailed study, especially in vivo study, is certainly needed; however, at present, our results raise the possibility that RS-0466 could be a therapeutic agent for Alzheimer's disease by modulating the Akt signaling pathway.

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