Full Paper

Role of Caspase-12 in Amyloid β -Peptide-Induced Toxicity in Organotypic Hippocampal Slices Cultured for Long Periods

Kumiko Ishige¹, Noritaka Takagi¹, Toru Imai¹, Wolf Dieter Rausch², Yasuhiro Kosuge¹, Tetsuroh Kihara¹, Kuniko Kusama-Eguchi³, Hiroko Ikeda⁴, Alexander Rudolf Cools⁵, John Lionel Waddington⁶, Noriaki Koshikawa⁴, and Yoshihisa Ito^{1,*}

¹Research Unit of Pharmacology, and ³Research Unit of Molecular Cell Biology, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi 274-8555, Japan

²Institute for Medical Chemistry, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210, Vienna, Austria

⁴Department of Pharmacology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan

⁵Department of Cognitive Neuroscience, Radboud University of Nijmegen, 6500 HB Nijmegen, The Netherlands

⁶Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, St. Stephen's Green, Dublin 2, Ireland

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Abstract. Amyloid β ($A\beta$) toxicity has been implicated in cell death in the hippocampus, but its specific mechanisms are poorly understood. In this study, $A\beta$ -induced cell death was investigated in organotypic hippocampal slice cultures (OHCs) that were cultured for various periods in vitro. There were no obvious histological differences among slices cultured for 3 to 7 weeks in vitro. Although there was little neurotoxicity after treatment with $A\beta_{25-35}$ in OHCs cultured for relatively shorter periods (3–5 weeks), age-dependent cell death was evident in OHCs cultured for relatively longer periods (6–7 weeks) after exposure to $A\beta_{25-35}$. In OHCs cultured for 7 weeks, *S*-allyl-L-cysteine (SAC), a component of aged garlic extract, protected the cells in areas CA1 and CA3 and the dentate gyrus from $A\beta_{25-35}$ -induced toxicity. The immunoreactivity of cleaved caspase-12 was increased whereas that of glucose-regulated protein 78 was not altered after exposure to $A\beta_{25-35}$. The increases in the cleaved caspase-12 were also reversed by simultaneously applied SAC. These results suggest that OHCs cultured for relatively longer periods are more susceptible to $A\beta$ -induced toxicity and that the $A\beta$ -induced cell death involves caspase-12-dependent pathways. It is also suggested that SAC is able to protect against the $A\beta$ -induced neuronal cell death through the inhibition of the caspase-12-dependent pathway.

Keywords: amyloid β -peptide, *S*-allyl-L-cysteine, L-glutamate, organotypic hippocampal slice culture, caspase-12

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that causes progressive loss of cognitive abilities. A central feature of the pathology of AD is profound loss of neurons in the CNS. The amyloid precursor protein can be proteolytically cleaved into multiple fragments, many of which have distinct biological actions. Amyloid β -peptide ($A\beta$) has been shown

to have a cytotoxic effect on cultured neurons (1–4), and one hypothesis for the pathogenesis of AD is that this is caused by “ $A\beta$ -induced free radical-mediated neurotoxicity” (5–7).

The endoplasmic reticulum (ER) is an organelle that regulates the folding and assembly of secretory and membrane proteins, lipid metabolism, the cellular response to stress, and the homeostasis of intracellular calcium (8). Certain pathological stress conditions can disrupt homeostasis in the ER, including loss of the ER intraluminal oxidative environment and depletion of intracellular calcium stores, leading to accumulation of misfolded proteins in the ER, a condition referred to as

*Corresponding author. yoshiito@pha.nihon-u.ac.jp

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ER stress (9). ER stress has been shown to play a pivotal role in A β -induced neurotoxicity (10) and AD pathology (11, 12). In our previous experiments, we also showed that exposure of cultured hippocampal neurons to A β _{1–40} or A β _{25–35} resulted in an increase of cell death in a time- and concentration-dependent manner and in destruction of neuronal integrity with a significant increase in the level of glucose-regulated protein 78 (GRP78), known to be a molecular chaperone that regulates protein folding and translocation into the ER (3, 4), suggesting that the cellular insults elicited by these agents involves ER stress in cultured hippocampal neurons. However, the exact molecular mechanisms that underlie A β -induced neuronal death are still not completely understood.

Caspase-12 is localized specifically on the cytoplasmic side of the ER and is thought to play a pivotal role in ER stress-induced cell death in the rodent brain (10, 13). Recently it has been shown that exposing cultured hippocampal neurons to A β induces activation of caspase-12 (10).

The organotypic hippocampal culture (OHC) is a system that is useful for studying the mechanisms of neurodegeneration (14). This is because several features of hippocampal circuitry are preserved, and the preparation is well suited for studies on prolonged pharmacological treatment and recovery, which would be difficult to perform in an intact animal experiment. We have reported that OHC cultured for 3 weeks showed little neurotoxicity after treatment with A β _{1–40} or A β _{25–35} (25 or 50 μ M) alone for 48 h, whereas exposure of OHC to A β in combination with ibotenic acid, a potent *N*-methyl-D-aspartate (NMDA) agonist, induced severe cell death when compared with the effect of ibotenic acid alone (15). This phenomenon was not observed in OHC cultured for 2 weeks or shorter periods.

Aged garlic extract (AGE) that has been aged for at least 10 months has been shown to have biological activity in several areas, including improvements in impaired learning and memory (16, 17), neurotrophic effects (18), and antioxidant activity (19). *S*-Allyl-L-cysteine (SAC), an active organosulfur compound present in AGE, has been shown to have neurotrophic effects (20) in cultured neurons and neuroprotective activities (21) in the rodent brain. In our studies, we have found that SAC protects against A β -induced cell death, but does not protect against 4-hydroxynonenal-induced cell death in either NGF-differentiated PC12 cells (22) or cultured hippocampal neurons (3). We have also reported that SAC protects hippocampal neurons in the CA3 area and the dentate gyrus (DG) from the cell death induced by A β in combination with ibotenic acid with no change in the CA1 area in OHC (15).

In order to examine whether A β alone causes cell death in the OHC, A β -induced cell death was investigated in OHCs that were cultured for various periods in vitro. We also demonstrated that OHCs cultured for relatively longer periods are more susceptible to A β -induced toxicity and that SAC is able to protect against the A β -induced neuronal cell death that is mediated predominantly through ER stress and the caspase-12-dependent pathway in OHCs.

Materials and Methods

Materials

The chemicals and reagents used in this study were purchased from the following suppliers: Hanks' balanced salts solution (HBSS) and ibotenic acid were obtained from Sigma (St. Louis, MO, USA); A β _{25–35} (Peptide Institute, Osaka); propidium iodide (PI) (Molecular Probes, OR, USA); minimal essential medium (MEM) (Invitrogen, Carlsbad, CA, USA); toluidine blue LDH-Cytotoxic Test kit (Wako Pure Chemicals, Osaka); ethyl ether (Showa Ether Co., Osaka); anti-caspase-12 polyclonal antibody (Exalpha Biologicals, Maynard, MA, USA); anti-GRP 78 polyclonal antibody (StressGen, Victoria, Canada); anti-rabbit-IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). *S*-Allyl-L-cysteine was synthesized by Wakunaga Pharmaceutical Co. (Osaka) and was kindly provided free of charge.

Preparation of organotypic hippocampal slice cultures (OHCs)

The OHC was prepared as described previously (15). Under sterile conditions, the hippocampi were dissected out from anesthetized 6–7-day-old Wistar/ST rats. Transverse slices (400- μ m-thick) were prepared using a McIlwain tissue chopper (The Mickle Laboratory Engineering, Gomshall, UK) and were placed on a membrane insert (Millicell-CM; Millipore, Billerica, MA, USA). The culture medium that was used was MEM containing 30 mM HEPES, 30 mM D-glucose, 5 mM NaHCO₃, 2.5 mM MgCl₂, 0.5 mM L-ascorbate, 2 mM CaCl₂, 25% horse serum, and 25% HBSS. Cultures were maintained at 32°C in a humidified incubator with 5% CO₂, and the medium was changed every 2 or 3 days. Slices cultured for 3, 4, 5, 6, and 7 weeks in vitro were used for assessment of A β toxicity.

Toluidine blue staining

Cell staining was performed as described by Noraberg et al. (23). The OHC was treated with toluidine blue

(1 mg/ml) for 15 min, and then the slice was washed with ethanol followed by H₂O. The slice was treated with toluidine blue (1 mg/ml) for 15 min again and then re-washed with ethanol. The stained slice was observed by under phase-contrast microscopy and photographed.

Drug treatments and assessment of neuronal death

A β_{25-35} is a short synthetic peptide that is suitable for use in A β toxicity studies, and it possesses properties similar to A β_{1-40} and A β_{1-42} (24, 25). Treatment of the OHC with A β_{25-35} was done as described by Bruce et al. (26). A 100- μ L aliquot of A β_{25-35} or ibotenic acid was applied directly to the top of the slices. Selected slices were treated with SAC at the same time as A β . After 48 h of this treatment, the medium was changed to Locke's solution (154.0 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 3.6 mM NaHCO₃, and 5.0 mM HEPES, pH 7.4) and PI solution was added to the medium (final concentration = 50 μ g/ml). Thirty minutes after the addition, the degree of hippocampal neuronal death was evaluated by microscopic evaluation of PI uptake. Sections were photographed, and an analysis of specific areas was done, initially by first selecting the areas of the hippocampal regions (CA1, CA3, and DG) on the image. The intensity of the fluorescence was quantitatively analyzed using Scion Image. The neuronal death in the selecting areas in the different cultures was expressed as relative percentage of the neuronal death in control OHCs (15).

LDH release from OHC

After slices were treated with A β for 48 h, the LDH released from OHCs was quantified using a LDH-Cytotoxic Test kit according to the manufacturers' instructions. In this assay, nicotinamide adenine dinucleotide (NAD) is reduced to nicotinamide adenine dinucleotide, reduced form (NADH) through the conversion of lactate to pyruvate by LDH, and then NADH reduces tetrazolium dyes to formazan dyes in the presence of diaphorase. Briefly, a 50- μ L aliquot of culture medium was mixed with 50 μ L of the LDH substrate mixture in a 96-well plate. After incubation for 0.5 h at room temperature, the reaction was stopped by adding 100 μ L of 0.5 M HCl, and the absorbance was measured with a microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA, USA) at a test wavelength of 570 nm. The background absorbance obtained from the culture medium was subtracted from the absorbance of each sample.

Western blotting

Forty-eight hours after the treatment with A β in the absence or presence of SAC (100 μ M), these slices were

rinsed twice with ice-cold PBS and then lysed in sample buffer [125 mM Tris-HCl (pH 6.0), 3% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.2% bromophenol blue]. Aliquots from each sample (15 μ g protein/lane) were subjected to 10 or 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The blots were blocked in blocking buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 5% skim milk] for 1 h at room temperature and then treated with anti-caspase-12 antibody (diluted 1:1000) overnight at 4°C. The blots were washed repeatedly in Tris-buffered saline (20 mM Tris-HCl, pH 7.6 and 137 mM NaCl) containing 0.05% Tween 20, and then HRP-conjugated secondary antibody (diluted 1:20000) was added for 1 h. Immuno-reactive bands were detected by ECL. Optical density on the blots was measured with Scion Image (Scion Corporation, Frederick, MD, USA).

Statistics

Student's *t*-test was used when two groups were compared. When more than three groups were being compared, an analysis of variance (ANOVA) with the Bonferroni test for *post hoc* comparison was used.

Results

A β - and ibotenic acid-induced toxicities in OHCs cultured for various time periods

When hippocampal slices were cultured for various time periods (3, 4, 5, 6, and 7 weeks; Fig. 1A), they showed no morphological changes. There was also no difference in basal cell viability among the OHCs (Fig. 1B). We did not use slices cultured for 8 weeks or longer because they became thinner in comparison with younger ones (data not shown). In order to investigate the direct toxicity of A β_{25-35} , we examined the degree of cell death by measuring the PI uptake in the OHCs cultured for various time periods. We assessed whether there was any effect of 50 μ M A β_{25-35} on cell survival. In the OHCs cultured for 3 or 4 weeks in vitro, weak PI fluorescence intensity was detected after 48 h of treatment in all areas of the hippocampus (Fig. 1C). When the slices were cultured for 6–7 weeks in vitro, the degree of PI fluorescence (Fig. 1C) was clearly time-dependent, and the most obvious PI fluorescence was seen in area CA1. LDH release from slices cultured for various time periods after exposure to A β was also concentration- and time-dependent in OHCs cultured for 6 and 7 weeks but not in younger ones (Fig. 2).

A previous study from our laboratory has shown that when OHCs cultured for 3 weeks was treated with ibotenic acid, an NMDA agonist, at various concentra-

(A) Toluidine blue staining

(B) PI uptake

(C) Effect of A β

Fig. 1. Characterization of OHCs cultured for various time periods (3–7 weeks) and effect of A β_{25-35} on neuronal survival in these OHCs. A: Toluidine blue staining. The OHC was treated with toluidine blue (1 mg/ml) for 15 min, and then the slice was washed with ethanol followed by H₂O. The slice was again treated with toluidine blue (1 mg/ml) for 15 min and then re-washed with ethanol. The stained slice was observed by phase-contrast microscopy and photographed. B: Time-course changes in PI staining of OHCs cultured for various time periods. The OHC was prepared as described in Materials and Methods, and the PI fluorescence was also observed as described. The fluorescence intensity was analyzed quantitatively using Scion Image. Ten 50 $\times$ 50 pixel areas were chosen in each area of the hippocampus at random and an average of the red color intensity of 10 areas was calculated. Background intensity was subtracted from the average value. Each value represents the mean $\pm$ S.E.M. for 4 experiments. C: Characterization of neuronal death in the OHC induced by A β_{25-35} . A 100- μ l aliquot containing A β_{25-35} (25 μ M) was applied directly to the top of the slices. Representative data from 4 different experiments are shown. Graph: The fluorescence intensity was analyzed quantitatively as shown above. Each value represents the mean $\pm$ S.E.M. for 4 experiments. ** P <0.01, as compared with the respective value at 3 weeks.

tions for 48 h, the degree of PI fluorescence was clearly concentration-dependent within the range 5–125 μ M (15). Therefore, the degree of neuronal death induced by ibotenic acid was also compared among OHCs cultured

for 3–7 weeks. After treatment with 65 μ M ibotenic acid for 48 h, a significant increase in PI fluorescence was observed in the OHCs cultured for 3–7 weeks. Although no difference in ibotenic acid-induced

Fig. 2. Time-course changes in $A\beta$ -induced release of LDH from OHCs cultured for various time periods. The OHC was prepared as described in Materials and Methods; 100- μ l aliquots containing $A\beta_{25-35}$ (25 and 50 μ M) were applied directly to the top of slices and incubated for 48 h. After exposure, the amount of LDH released into the medium was assayed as described in Materials and Methods. Each value represents the mean $\pm$ S.E.M. for 4 experiments. * P <0.05, ** P <0.01, as compared with the respective value at 3 weeks

neuronal death was observed in area CA1 among these OHCs, a significant increase in PI fluorescence was detected after 4 weeks and longer in area CA3 and after 5 weeks and longer in the DG in the OHCs (Fig. 3).

Characterization of direct toxicity of $A\beta$ in OHC cultured for 7 weeks and protective effects of SAC

When slices that had been cultured for 7 weeks were exposed to $A\beta_{25-35}$ at concentrations of 25 and 50 μ M for 48 h, the degree of PI fluorescence and the LDH release from the OHCs were concentration-dependent (Figs. 2 and 4). When the OHCs were exposed to $A\beta_{35-25}$, the reverse peptide of $A\beta_{25-35}$, at 50 μ M for 48 h, there was no effect on PI fluorescence in any area of the hippocampus (Fig. 4). This suggests that the toxicity of $A\beta_{25-35}$ was specific for the sequence of the $A\beta$ peptide.

We also assessed the effect of SAC, an active component of AGE, on the neuronal death induced by $A\beta_{25-35}$ and ibotenic acid in OHCs that had been cultured for 7 weeks. SAC (100 μ M) dramatically attenuated the level of PI fluorescence induced by 50 μ M $A\beta_{25-35}$ (Fig. 5). In contrast, SAC had no effect on the level of PI fluorescence induced by 65 μ M ibotenic acid (Fig. 5).

$A\beta$ induced an increase in the level of cleaved caspase-12 without affecting the level of GRP78

In order to address the potential role of ER stress in $A\beta$ -induced neuronal death observed in post-mature slices, the level of GRP78, an ER-associated signal protein, after exposure to 50 μ M $A\beta_{25-35}$ was investigated in OHCs cultured for various time periods (Fig. 6). There

Fig. 3. Time-course changes in ibotenic acid-induced neurotoxicity of OHCs cultured for various time periods. PI fluorescence was observed as described in Materials and Methods. Representative data from 4 different experiments are shown. Graph: The fluorescence intensity was analyzed quantitatively using Scion Image. Ten 50 $\times$ 50 pixel areas were chosen in each area of the hippocampus at random and an average of the red color intensity of 10 areas was calculated. Background intensity was subtracted from the average value. Each value represents the mean $\pm$ S.E.M. for 4 experiments. ** P <0.01, *** P <0.001, as compared with the respective value at 3 weeks.

was no significant difference in the immunoreactivity among $A\beta$ -treated slices cultured for 3, 5, and 7 weeks. SAC (100 μ M) also had no effect on the immunoreactivity of GRP 78 in these OHCs.

To evaluate the role of caspase-12, an ER-specific caspase in rodents, under $A\beta_{25-35}$ -treated conditions in these OHCs, we investigated the expression of pro- and cleaved caspase-12 proteins in OHCs cultured for 3, 5, and 7 weeks after exposure to 50 μ M $A\beta_{25-35}$. As shown in Fig. 7, a significant increase in the immunoreactivity of cleaved caspase-12 (42 kDa) was observed in slices cultured for 7 weeks as compared with that in the slices cultured for 3 weeks, whereas no such increase was observed in OHC cultured for 5 weeks in vitro. In

Fig. 4. Characterization of direct toxicity of A β in OHC cultured for 7 weeks. The OHC was prepared as described in Materials and Methods; 100- μ l aliquots containing A β_{25-35} (25 and 50 μ M) and A β_{35-25} , the reverse peptide, were applied directly to the top of the slices. PI fluorescence was observed as described in Materials and Methods. Representative data from 3 different experiments are shown. Graph: The fluorescence intensity was analyzed quantitatively using Scion Image. Ten 50 $\times$ 50 pixel areas were chosen in each area of the hippocampus at random and an average of the red color intensity of 10 areas was calculated. Background intensity was subtracted from the average value. Each value represents the mean $\pm$ S.E.M. for 3 experiments. ** P < 0.01, as compared with the control.

contrast, there was no difference in pro-caspase-12 immunoreactivity (60 kDa) among these groups. Furthermore, A β_{25-35} -induced increase in the immunoreactivity in OHC cultured for 7 weeks in vitro was reversibly suppressed by SAC (100 μ M).

Discussion

Previous investigations from our laboratory have shown that there is little neurotoxicity in OHCs cultured

Fig. 5. Protective effect of SAC against A β_{25-35} - and ibotenic acid (IBO)-induced neurotoxicity in OHC cultured for 7 weeks. The OHC was prepared as described in Materials and Methods; 100- μ l aliquots containing A β (25 μ M) or ibotenic acid (65 μ M) in the absence and presence of SAC (100 μ M) were applied directly to the top of the slices. PI fluorescence was observed as described in Materials and Methods. Representative data of A β_{25-35} - and ibotenic acid-induced neurotoxicity from 3 different experiments are shown. Graph: Protective effect of SAC against A β_{25-35} -induced neurotoxicity. The fluorescence intensity was analyzed quantitatively using Scion Image. Ten 50 $\times$ 50 pixel areas were chosen in each area of the hippocampus at random and an average of the red color intensity of 10 areas was calculated. Background intensity was subtracted from the average value. Each value represents the mean $\pm$ S.E.M. for 3 experiments. ** P < 0.01, as compared with the control; ## P < 0.01, as compared with A β_{25-35} alone.

for 3 weeks after treatment with A β_{25-35} and A β_{1-40} (25 or 50 μ M) alone for 48 h (15), although primary cultured neurons in the hippocampus have been shown to be

Fig. 6. Lack of effects of A β and SAC on GRP78 protein expression in OHC cultured for 7 weeks. The slices were cultured for the indicated time periods, and then the OHCs were treated with A β_{25-35} (50 μ M) in the absence and presence of SAC for 48 h. After the treatment, the OHCs were treated with lysate buffer, and Western blotting was performed as described in Materials and Methods. Anti-tubulin antibody was used as an internal control. Photographs show representative Western blots of GRP78 and tubulin. Representative data from 5 different experiments are shown. The graph shows the relative densities of bands on the blots estimated quantitatively using Sion Image. Values represent the mean $\pm$ S.E.M. for 5 independent experiments.

vulnerable to A β (3, 4). In the present study using slices cultured for various time periods (3–7 weeks), we demonstrated that A β_{25-35} -induced neuronal death is sequence-specific and dependent on the culture duration of these OHCs. When the slices were cultured for 6–7 weeks in vitro, the degree of PI fluorescence and the amount of LDH released from the slices were significantly increased after exposure to A β_{25-35} for 48 h, whereas these phenomena were not observed in OHCs cultured for 5 weeks and shorter. Exposure of OHC to ibotenic acid (65 μ M), a potent NMDA agonist, for 48 h induced neuronal cell death mainly in area CA1 in slices cultured for 3–7 weeks, and an age-dependent increase of PI fluorescence intensity was seen in slices cultured for 4 weeks or longer in area CA3 and in slices cultured for 5 weeks or longer in the DG. These results suggest that in post-mature cultures, the age-dependency of vulnerability to A β differs from that of vulnerability to ibotenic acid that activates NMDA receptors. It seems that the A β -induced increase in the fluorescence intensity observed in OHCs is originated from neuronal cells since most of the PI stained cells were located in

neuronal subarea of the hippocampus (CA1, CA2, and DG). However, we have not investigated the percentage of fluorescence intensity derived from glial cells in this study.

Prolonged ER stress leads to cell death and is linked to the pathogenesis of some neurodegenerative disorders that feature misfolded proteins, including AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (27). Recent studies have shown that exposure of primary cultured hippocampal neurons to A β induces the expression of GRP78, a molecular chaperone that regulates protein folding and translocation into the ER (28), and activation of caspase-12 (3), which is localized specifically on the cytoplasmic side of the ER and is thought to play a pivotal role in ER stress-induced cell death in the rodent brain (13, 29, 30). The present data demonstrated clearly that A β -induced neuronal death involved an increase in cleaved form of caspase-12 (42 kDa) in OHCs cultured for 7 weeks, whereas no increase in these immunoreactivities was observed in slices cultured for 3 and 5 weeks. In contrast, the change of immunoreactivity of GRP78 was not observed in OHCs cultured for 3, 5, and 7 weeks after exposure to A β . The present results also showed that in the absence of A β , neither cell viability nor the immunoreactivities of GRP78 and caspase-12 were increased in the slices cultured for longer time periods. Taken together, it seems that the mechanism of the A β -induced neuronal death in OHCs cultured for longer periods is different from that of the neuronal death observed in primary hippocampal neurons.

An abrupt increase in intracellular Ca²⁺ and cellular disturbance of Ca²⁺ homeostasis have been shown to play critical role in A β -induced neuronal death (31, 32). It has been shown that proteolysis of caspase-12 involves calpain activated by Ca²⁺ release in the vicinity of the ER (33). A recent study in our laboratory revealed that A β_{25-35} increased calpain activity and the cleaved form of caspase-12 in mature OHCs (cultured for 3 weeks) in the presence of a lower concentration of tunicamycin, an ER stressor, whereas levels of ER chaperones, GRP78 and GRP94, were not affected by A β_{25-35} (T. Imai et al., submitted). Taken together, it is suggested that A β induces an increase in intracellular Ca²⁺ level, thus leading to the activation of calpain and caspase-12-dependent pro-apoptotic pathways. However, further studies will be necessary to elucidate changes of intracellular Ca²⁺ level in the vicinity of the ER in OHCs cultured for relatively longer periods after exposure to A β . Moreover, downstream pathways of the caspase-12-dependent pro-apoptotic pathways including a change of caspase-3 activity should be elucidated.

Recently, a human colon cDNA library has been

Fig. 7. Increase in expression of the cleaved form of caspase-12 protein induced by A β and SAC-induced inhibition of the increase in OHC cultured for 7 weeks. The slices were cultured for the indicated time periods, and then the OHCs were treated with A β_{25-35} (50 μ M) in the absence and presence of SAC for 48 h. After the treatment, the OHCs were treated with lysis buffer, and Western blotting was performed as described in Materials and Methods. Anti-tubulin antibody was used as an internal control. Photographs show representative Western blots of caspase-12 (pro- and cleaved forms) and tubulin. Representative data from 5 different experiments are shown. The minor band just under the pro-caspase-12 is a non-specific band. The graph shows the relative densities of bands on the blots estimated quantitatively by Sion Image. Values represent the mean $\pm$ S.E.M. for 5 independent experiments. * P <0.05, as compared with the control; # P <0.05, as compared with A β_{25-35} alone.

screened using the sequence of mouse caspase-12 as a probe, and caspase-4 was found to be a candidate for the ER-dependent caspase (34). Caspase-4 was shown to be localized on the ER and mitochondria in SK-N-SH and HeLa cells, and it was cleaved by ER stress inducers such as tunicamycin or thapsigargin, but not non-ER stress inducers such as staurosporine or etoposide (34). A recent study from our laboratory showed that pro-caspase-4 was detected in SK-N-SH cells, and the cleaved form was induced by the treatment with an ER stressor, tunicamycin (Oda et al., submitted). Studies on cellular insults responsible for ER stress-induced cell death in human origin cells is also in progress in our laboratory.

SAC, an organosulfur compound possessing a thioallyl group, present in AGE, has been shown to protect neurons against various toxic or ischemic conditions (7, 21, 35). Previous investigations in our laboratory

have also shown that SAC protects NGF-differentiated PC12 cells (22) and cultured hippocampal neurons (3) against A β - and tunicamycin-induced toxicities. However, it has no effect on 4-hydroxynonenal-induced and survival factor deprivation-induced cell death, which have been shown to be independent on ER-stress and caspase-12 in these cells. Moreover, we have shown recently that SAC does not protect cerebellar granule neurons from A β toxicity because the cell death is not dependent on a caspase-12–pathway but instead depends on a CHOP-mediated one (4). These results suggest that SAC has a unique effect on the vicinity of the ER and that it could protect against the cell death triggered by caspase-12. In this study using OHCs, we also demonstrated that SAC protected hippocampal cells against A β -induced neurotoxicity in all hippocampal areas in older cultures and that it concomitantly attenuated an increase in the cleaved form of caspase-12

after exposure to A β in these OHCs. In contrast, ibotenic acid did not affect the levels of cleaved caspase-12 and GRP78 (data not shown), and SAC had no effect on ibotenic acid-induced neuronal death in OHCs cultured for 7 weeks. Taken together, it is evident that SAC is able to protect against the A β -induced neuronal cell death that is mediated predominantly through the caspase-12-dependent pathway, regardless of the culture system employed. These results also suggest that ER-stress itself did not play a pivotal role in A β -induced neurotoxicity in OHCs cultured for relatively longer periods.

In conclusion, the present results suggest that OHCs cultured for relatively longer periods are more vulnerable to A β -induced toxicity and that the caspase-12-mediated pathway is involved in A β -induced cell death in OHC. Both the present results and previous findings also indicate that SAC could provide a significant neuroprotective effect against A β -induced toxicity, not only in dissociated hippocampal neurons and PC 12 cells but also in neurons in the OHCs.

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