THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 29, pp. 20315–20325, July 21, 2006
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ERK1/2 Activation Mediates Aβ Oligomer-induced Neurotoxicity via Caspase-3 Activation and Tau Cleavage in Rat Organotypic Hippocampal Slice Cultures*

Received for publication, February 2, 2006, and in revised form, April 21, 2006 Published, JBC Papers in Press, May 18, 2006, DOI 10.1074/jbc.M601016200

Young Hae Chong[‡1], Yoo Jeong Shin[‡], Eun Ok Lee[‡], Rakez Kayed[§], Charles G. Glabe[§], and Andrea J. Tenner[§]

From the [‡]Department of Microbiology, College of Medicine, Division of Molecular Biology and Neuroscience, Ewha Medical Research Institute, Ewha Womans University, 911-1, Mok-6-dong, Yangcheonku, Seoul, 158-710, Korea and the [§]Department of Molecular Biology and Biochemistry, Center for Immunology, University of California, Irvine, California 92697

In this study, we investigated the molecular basis for the altered signal transduction associated with soluble amyloid β-protein (Aβ) oligomer-mediated neurotoxicity in the hippocampus, which is primarily linked to cognitive dysfunction in Alzheimer disease (AD). As measured by media lactate dehydrogenase levels, and staining with propidium iodide, acute exposure to low micromolar concentrations of the Aβ1–42 oligomer significantly induced cell death. This was accompanied by activation of the ERK1/2 signal transduction pathway in rat organotypic hippocampal slices. Notably, this resulted in caspase-3 activation by a process that led to proteolytic cleavage of Tau, which was recently confirmed to occur in AD brains. Tau cleavage likely occurred in the absence of overt synaptic loss, as suggested by the preserved levels of synaptophysin, a presynaptic marker. Moreover, among the pharmacological agents tested to inhibit several kinase cascades, only the ERK inhibitor significantly attenuated Aβ1–42 oligomer-induced toxicity concomitant with the reduction of activation of ERK1/2 and caspase-3 to a lesser extent. Importantly, the caspase-3 inhibitor also decreased Aβ oligomer-induced cell death, with no appreciable effect on the ERK signaling pathway, although such treatment was effective in reducing caspase-3 activation and Tau cleavage. Therefore, these results suggest that local targeting of the ERK1/2 signaling pathway to reduce Tau cleavage, as occurs with the inhibition of caspase-3 activation, may modulate the neurotoxic effects of soluble Aβ oligomer in the hippocampus and provide the rationale for symptomatic treatment of AD.

Alzheimer disease (AD)[2] neuropathology is characterized by key features that include fibrillar amyloid β-protein (Aβ) deposition into dense senile plaques, the formation of neurofibrillary tangles composed of hyperphosphorylated Tau, and the loss of neurons and synapses in the affected brain region leading to the progressive loss of cognitive function (1, 2). Recent evidence suggests that soluble, prefibrillar, and oligomeric forms of Aβ are acutely toxic (3) and can interfere with synaptic plasticity in the brain, suggesting that this form of the peptide may be responsible for episodic memory deficits, an early symptom of AD, which is linked to hippocampal pathology (4). In fact, soluble Aβ oligomers, also referred as Aβ-derived diffusible ligands, strikingly elevated in the AD brain (5–8) are also described in human amyloid precursor protein transgenic mice AD models (9) and can inhibit the long term potentiation (LTP) of synaptic efficiency (10, 11). The pathogenic relevance of this form of Aβ has been substantiated by a newly identified Arctic familial AD mutation, which has an increased propensity to oligomerize (12, 13), and by *in vitro* data demonstrating potent neurotoxicity upon exposure to soluble oligomer or protofibrils (3, 14, 15). Moreover, the ultrastructural localization of Aβ oligomer within neuritic processes and at synaptic terminals in AD brains (16, 17) further supports the hypothesis that soluble Aβ oligomers may play a crucial role as the earliest effector that causes synaptic dysfunction and early memory loss associated with dementia in AD (4, 18). Hence, considerable interest is now focused on understanding the neurotoxic mechanisms elicited by soluble Aβ oligomer in order to generate effective therapeutic strategies targeting this molecule for the treatment of AD.

Among the mitogen-activated kinase (MAPK) family, a role for the extracellular signal-regulated kinase (ERK)1/2 signaling pathway, in neuronal death, has been proposed recently in AD (19–21). Indeed, fibrillar Aβ1–42 can induce ERK activation, and sustained activation of the ERK1/2 signaling pathway, mediated by fibrillar Aβ, can lead to abnormal phosphorylation of Tau, the generation of dystrophic neurites, and progressive neuronal degeneration (22, 23). However, the signaling events associated with neurotoxicity triggered by soluble Aβ oligomer in the AD brain are largely unknown.

On the other hand, a potential role for the proteolytic cleavage of Tau, the microtubule-stabilizing protein, in fibrillar Aβ-induced neuronal degeneration, has also been implicated. A previous study showed that truncated Tau was present in AD brains but not in age-matched controls (24). In addition, the cleavage of Tau, by caspases, has been demonstrated both in

* This work was supported by Grant A040042 from the Biomedical Brain Research Center of the Korea Health 21 R&D Project funded by the Ministry of Health and Welfare, Republic of Korea (to Y. H. C.) and by National Institutes of Health Grant NS 35144 (to A. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 822-02-2650-5739; Fax: 822-02-2653-8891; E-mail: younghae@ewha.ac.kr.

[2] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-protein; MAPK, mitogen-activated protein kinase; MEK1, MAPK kinase kinase; ERK, extracellular signal-responsive kinase; JNK, c-Jun NH_2-terminal kinase; LDH, lactate dehydrogenase; PI, propidium iodide; LTP, long term potentiation; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; AFC, 7-amino-4-trifluoromethyl coumarin.

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neurons treated with aggregated Aβ and in AD brains (25–28). Moreover, these studies provided evidence supporting a key role of truncated Tau either by caspases or calpain in fibrillar Aβ-induced neurodegeneration and enhancement of pathological Tau filament assembly in neurons. Interestingly, Aβ-mediated Tau cleavage appears to occur as an early event that may precede hyperphosphorylation, in the evolution of the AD tangle pathology (27). These findings imply that aberrant Tau truncation, in addition to Tau hyperphosphorylation, may represent novel cellular mechanisms functionally linking amyloid deposition and neurofibrillary tangles in AD. However, despite such extensive studies of the fibrillar form of Aβ, as a key neuropathological factor in AD, there is little known about the precise role of the soluble Aβ oligomer in the modulation of neuronal loss and the molecular basis for the altered signal transduction associated with neurotoxicity in response to this molecule in the hippocampus, a region that is particularly vulnerable to the ravages of AD and that underlies the type of memory deficit detected in early AD (29). Furthermore, few studies have tried to understand how Aβ oligomer affects Tau cleavage and how the Aβ oligomer may modulate this process at the molecular level.

Therefore, this study investigated the intracellular signaling mechanisms triggered by soluble Aβ oligomer, and determined how they correlate with the Aβ oligomer-mediated neurotoxic effect on the hippocampus. By using a rat organotypic hippocampal slice culture, which maintains the cytoarchitecture of the intact brain, direct toxicity of Aβ1–42 oligomer was determined by measuring the propidium iodide (PI) uptake and lactate dehydrogenase (LDH) release. We also assessed whether caspase-3 activation mediates Aβ1–42 oligomer-induced neuronal cell death in the hippocampus. Finally, the Aβ oligomer-triggered signaling cascade, and specifically ERK1/2 activation, was assessed to determine its relation to Aβ oligomer-induced apoptotic cell death. To this end, we analyzed the effect of the Aβ oligomer on the proteolysis of Tau, recently identified as a downstream substrate of caspase-3 (26, 27), and we evaluated the modulation of Tau proteolysis by the ERK inhibitor in comparison with the caspase-3 inhibitor. Our data constitute the first evidence that Aβ oligomer-induced toxicity in the hippocampus occurs through the activation of the ERK1/2 signaling cascade. In addition, this study posits a possible link between Aβ oligomer-mediated toxicity and Tau cleavage, which occurs through caspase-3 activation upon stimulation of the ERK1/2 signaling cascade, in AD pathology.

EXPERIMENTAL PROCEDURES

Materials—Specific inhibitors of several protein kinases, including U0126 (an inhibitor MAPK kinase kinase (MEK1/2)), SB202580 (an inhibitor of p38 MAPK), SP600125 (an inhibitor of Jun NH_2-terminal kinase (JNK)), SB216763 (an inhibitor of GSK-3β), and LY294002 (an inhibitor of phosphatidylinositol 3-kinase) were all obtained from Calbiochem. The general caspase inhibitor, Z-VAD-fmk, the caspase-3 specific inhibitor, Ac-DEVD-CHO, and the calpain inhibitor, calpeptin, were also obtained from Calbiochem. PI, nucleic acid stain, was obtained from Molecular Probes (Eugene, OR). Anti-Aβ 6E10 was purchased from Signet Labs, Inc. (Dedham, MA). Anti-β-actin antibody and other chemicals were obtained from Sigma.

Preparation of Homogeneous Populations of Amyloid Peptide Monomer and Oligomer—Aβ1–42 peptide was synthesized as described previously (3). Soluble Aβ1–42 monomers and oligomers were prepared as described previously (30). Briefly, 1.0 mg of Aβ1–42 peptide was dissolved in 400 μl of hexafluoroisopropanol for 10–20 min at room temperature, and 100 μl of the resulting seedless solution was added to 900 μl of double-distilled water in a siliconized Eppendorf tube. After a 10–20-min incubation at room temperature, the samples were centrifuged for 15 min at 14,000 × *g*, and the supernatant fraction (pH 2.8–3.5) was transferred to a new tube and subjected to a gentle stream of N_2 for 10 min to evaporate the hexafluoroisopropanol. Vehicle was prepared the same way except with no peptide. The monomer solutions were used immediately after evaporation of hexafluoroisopropanol. For soluble oligomers, the samples were then stirred at 500 rpm using a Teflon-coated micro stir bar for 48 h at 22 °C. Aβ1–42 oligomer was confirmed by dot blot assay using oligomer-specific A11 antibody (3) and was further verified by Western blotting using 6E10 antibody as described below.

Hippocampal Slice Cultures—All experimental procedures were carried out under protocols approved by the University of California, Irvine, Institutional Animal Care and Use Committee as described previously (31). Briefly, hippocampal slice cultures were prepared from 10-day-old Sprague-Dawley rat pups (Charles River Breeding Laboratories, Inc., Wilmington, MA). Slices were cut at 400 μm on a McIlwain tissue chopper, transferred to Millicell (Millipore Corp., Bedford, MA) membrane inserts (0.4 μm), and placed in 6-well culture plates. The upper surfaces of the slices were exposed to a humidified 37 °C atmosphere containing 5% CO_2. Slice culture media consisted of basal Eagle's medium with Earle's balanced salt solution, 20% heat-inactivated horse serum, and the following supplements: 20 mM NaCl, 5 mM $NaHCO_3$, 1.7 mM $MgSO_4$, 0.2 mM $CaCl_2$, 26.7 mM HEPES, 26.6 mM L-glutamine, 48 mM L-(+)-glucose, 100 units/ml penicillin, and 100 mg/liter streptomycin, pH 7.25. The medium was changed every other day. Slices were examined periodically for viability, and any dark or abnormal slices were discarded.

Experimental Treatment—On days 10–11 post-dissection, treatment of the slices was started. All reagents were added to serum-free medium (no horse serum), which was equilibrated at 37 °C, 5% CO_2 before their addition to the slices. Monomeric or oligomeric Aβ1–42 was added to slice cultures as described previously (31) with some modification. Briefly, slices were pretreated with various pharmacological agents, as described in the text. All concentrations were selected on the basis of the maximal effects of these drugs on their specified targets. Peptide was then added to cultures in serum-free medium at various concentrations up to 2 μM as noted in the text. Vehicles were treated the same way except with no peptide. The effect of vehicle alone on cell viability was not detectable. At the indicated times after treatment initiation, slices were rinsed twice in 1× phosphate-buffered saline, then harvested by removing the Millicell membrane insert after freezing samples on dry ice, and processed for immunoblotting as described below.

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Assessment of Neuronal Cell Death—To visualize neuronal cell death, hippocampal slices were stained by adding PI into the culture medium at a concentration of 5 μg/ml (5 μg/ml of media) throughout the Aβ or vehicle treatment, and the degree of hippocampal neuronal death was evaluated by microscopic observation of PI uptake as described previously (32, 33). PI is a polar compound that is not toxic to neurons and is impermeable to an intact cell membrane, but it penetrates damaged cell membranes of dying cells and binds to nuclear DNA to generate a bright red fluorescence. PI fluorescence images were captured at different time points after treatment as indicated. The PI images were recorded with an Axiovert 200 inverted microscope (Carl Zeiss Light Microscopy, Göttingen, Germany) with an AxioCam (Zeiss) digital camera controlled by AxioVision program (Zeiss). Images were analyzed with the KS 300 analysis program (Zeiss) and neuronal cell death, in the different cultures, was expressed as an arbitrary unit of PI uptake.

Lactate Dehydrogenase Activity—LDH enzymatic activity in the culture medium was used to evaluate the extent of cellular damage produced in cultured slices subjected to the different treatments as described previously (34). Briefly, culture medium was collected after incubation of slices, as indicated in the figures, and from the vehicle-treated control cultures. 100-μl aliquots of culture media were taken for determination of LDH activities using Tox-7 (Sigma) according to the manufacturer's directions. The activity was expressed as the relative percentage of neuronal death using respective values for vehicle- or Aβ1–42-treated slice cultures as 100%.

Dot Blot Assay—The monomeric and oligomeric Aβ peptides were prepared as described above and applied to nitrocellulose membrane. Dot blot assay was performed as described previously (3) with minor modifications. Briefly, the membrane was blocked with 3% nonfat milk in TBS-T (137 mM NaCl, 20 mM Tris base, and 0.1% Tween 20; pH 7.4) at room temperature for 1 h. The membrane was washed with TBS-T and probed with anti-oligomer A11 antibody (1:5,000) or 6E10 (1: 5,000) in 1% nonfat milk for 1 h at room temperature. After washing, it was probed with anti-rabbit horseradish peroxidase- or anti-mouse horseradish peroxidase-conjugated antibody solution (1:3,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The blots were developed by using ECL chemiluminescence system (Amersham Biosciences).

PAGE and Western Blotting—Immunoblotting was carried out as recently described in detail (31, 34). Briefly, slices were homogenized in ice-cold extraction buffer (10 mM triethanolamine, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.15 M NaCl, 0.3% Nonidet P-40) supplemented with the protease inhibitors pepstatin (2 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and the phosphatase inhibitor sodium orthovanadate (1 mM). The protein concentration was determined by the method of BCA (Pierce). Equal amounts (20 μg) of sample proteins were separated according to their molecular weight on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (Millipore, Bedford, MA). After transfer, membranes were blocked with 3% milk in TBS-T for 0.5 h. Membranes were probed with the primary antibody diluted with 1% milk and incubated overnight at 4 °C. The following antibodies were used: anti-Tau ((clone Tau-5); 1:1000; Pharmingen), anti-phosphorylated ERK1/2 (1:1000; Promega, Madison, WI), anti-ERK1/2 (1:1000; Promega, Madison, WI), anti-synaptophysin (1:3000; DAKO, Carpinteria, CA), and anti-β-actin (1:5000; Sigma). The signal was obtained using the ECL system after incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Densitometric values were normalized using β-actin as internal controls as indicated. Aβ oligomer present in the slice culture media after 24 h of incubation was detected under nondenaturing condition using 6E10 antibody, which detects all forms of Aβ.

Caspase-3 Activity Assay—Caspase-3 activity was measured using the fluorometric caspase-3 activity assay kit (Calbiochem) according to the manufacturer's instructions. The fluorescence was measured after cleavage of the caspase-3 substrate (DEVD) labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin (AFC), to AFC by caspase-3. Briefly, hippocampal slices, treated with 2 μM Aβ1–42 oligomer as described above, were harvested in extraction buffer and incubated on ice for 20 min. After centrifugation at 16,000 × *g* for 10 min, the supernatant was incubated with the caspase-3 substrate (DEVD-AFC) for 1 h at 37 °C. The fluorescence was assessed using a fluorescent plate reader with a 400 nm excitation and a 505 nm emission according to the manufacturer's protocol. Caspase-3 activity is expressed as a relative percentage of vehicle- or Aβ1–42-treated samples as 100%.

Tau in Vitro Cleavage Assay—For experiments using purified caspase-3 (Sigma), total lysates were prepared from hippocampal slices and then centrifuged for 10 min at 16,000 × *g*. The supernatants (20 μl) were then incubated with recombinant caspase-3 (Sigma) for 1 h at 37 °C, as described previously (35). The reactions were terminated by adding 1 volume of 2× SDS sample buffer and boiled for 5 min. Samples were subjected to Western blot analysis using Tau-5 antibody that recognizes total Tau.

Protease Inhibitor Treatment—To inhibit caspase activation, the caspase inhibitors, Z-VAD-fmk or Ac-DEVD-CHO were added to the medium of hippocampal slice cultures 1 h prior to and for the duration of the Aβ treatment. The supernatants, prepared as described above, were subjected to Western blot analysis using Tau antibodies.

Statistical Analysis—Data were expressed as the means ± S.E. of the values from the number of experiments indicated in the corresponding figures. Differences between groups were examined for statistical significance using one-way analysis of variance with an unpaired Student's *t* test. A *p* value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Treatment of Cultured Hippocampal Slices with Aβ1–42 Oligomer Increases PI Uptake and LDH Release—Homogeneous populations of Aβ monomer and prefibrillar Aβ oligomers were prepared as described above. As shown in Fig. 1*A*, the oligomeric Aβ preparation was confirmed by dot blot assay using a conformationally directed antibody against amyloid oligomers (larger than tetramer), A11, which is highly specific for

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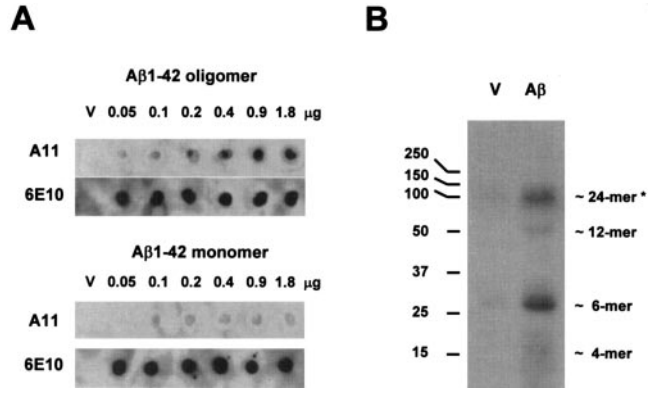

FIGURE 1. **Characterization of oligomer-specific immunoreactivity.** *A,* in this dot blot assay, increasing concentrations of peptides were applied to a nitrocellulose membrane and probed either with A11 oligomer-specific antibody or 6E10. A11 recognized only the Aβ1–42 oligomer, whereas 6E10 recognizes both Aβ oligomer and Aβ monomer. *B,* representative Western blot showing Aβ1–42 oligomer species in the supernatant solution of the slice culture after 24 h of incubation with vehicle only (*V*) or Aβ1–42 oligomer (2 μM) for 24 h. 30 μl of each supernatant was analyzed by immunoblotting using 6E10 under nondenaturing conditions. The band around ~100 kDa (~24-mer) of Aβ oligomer was clearly detected in the culture medium from Aβ1–42 oligomer-treated slices.

toxic oligomer subspecies as characterized (3, 36). The monomeric preparation contained a small amount of detectable Aβ oligomers. This Aβ oligomer preparation was shown to have an approximate molecular mass of ~90 kDa by size exclusion chromatography, contained very little material of lower molecular mass, and consisted of spherical vesicles with diameters of ~3–5 nm (30). Furthermore, Western blot analysis revealed that this Aβ oligomer was detected as a stable, SDS-resistant band, corresponding to an ~24-mer, in the culture medium from Aβ oligomer-treated slices after 24 h of incubation (Fig. 1*B*).

In order to investigate the direct toxic effect of the Aβ1–42 oligomer on neuronal viability, in the organotypic hippocampal slices, we first examined the degree of cell death by measuring the PI uptake and LDH release. PI uptake has been confirmed as an accurate quantitative measure of neuronal death in hippocampal slice cultures with other measures of neuronal death, including release of LDH (32, 33). The organotypic hippocampal slice culture is a good model system to study the mechanisms of neurodegeneration. This is because several features of hippocampal circuitry, including the maturation of synapses, receptors, and intrinsic fiber pathways, are preserved *in vivo*, and the preparation is well suited for prolonged pharmacological treatment and recovery, which would be difficult to perform in an intact animal system (37, 38). As shown in Fig. 2, the amount of LDH in the media containing hippocampal slices treated with the Aβ1–42 oligomer was significantly increased in a dose- and time-dependent manner above control levels, indicating that cell death had occurred. Exposure to 1 μM Aβ1–42 oligomer for 24 h increased the percentage of LDH release to approximately 3.5-fold over the vehicle-treated controls (Fig. 2*A*), and there was approximately a 6-fold increase in the degree of cell death when the concentration of Aβ1–42 oligomer was increased to 2 μM (Fig. 2*B*). Consistent with the LDH results, Aβ1–42 oligomer caused a time-dependent increase in PI uptake in the hippocampus, which was first

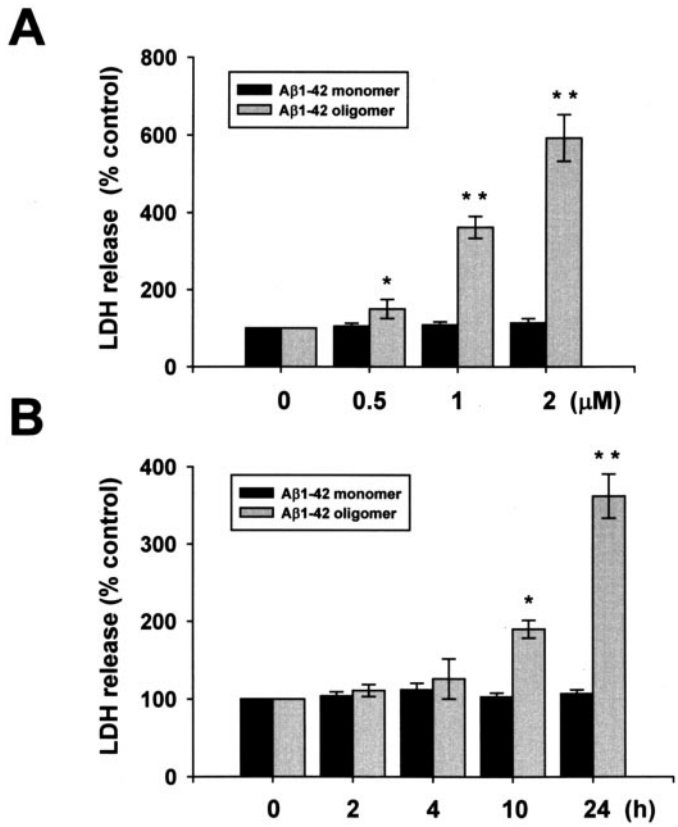

FIGURE 2. **LDH release in response to the Aβ1–42 oligomer in cultured media of hippocampal slices.** *A,* dose response of LDH release by the Aβ1–42 monomer or the Aβ1–42 oligomer was examined by incubation with increasing concentrations of peptides as indicated for 24 h. *B,* in time course experiments over a 24-h incubation LDH activity in cultured media from hippocampal slices was assayed after exposing rat hippocampal slice cultures to 1 μM of Aβ1–42 monomer or Aβ1–42 oligomer for the indicated times. Values are expressed as a percentage of the vehicle-treated controls, considering the values obtained in the hippocampal slices as 100%. The *numbers* represent the means ± S.E. ($n = 2$–9). *, $p < 0.05$; **, $p < 0.001$, different from the vehicle-treated controls.

detectable within 10 h and continued to increase at 24 h (Fig. 3, *B* and *C*). Exposure to 0.5 μM Aβ1–42 oligomer for 24 h induced PI uptake, which was evident in the hippocampal CA1, a region important for memory formation but not seen in vehicle-treated slices. The degree of PI uptake clearly increased, and the area of staining extended to other hippocampal regions when the concentration of Aβ1–42 oligomer was increased to 2 μM (Fig. 3, *A* and *C*). In parallel, slices were also exposed to the Aβ1–42 monomer under the same experimental conditions, but there was no observed increase in cell death with this peptide as measured by either PI uptake or LDH release (Fig. 2 and Fig. 3). These results suggest that the Aβ1–42 oligomer had a greater neurotoxicity than the Aβ1–42 monomer in organotypic hippocampal slices. These findings are consistent with other recent studies (14, 39).

Aβ1–42 Oligomer-induced Cell Death Was Accompanied by Caspase-3 Activation—To further clarify the mechanism by which Aβ1–42 oligomer causes cell death in the hippocampal slices, we evaluated the activation of caspase-3, a key executor in apoptosis, by using the caspase activation assay. The results indicate that Aβ1–42 oligomer induced caspase-3 activity, whereas Aβ1–42 monomer had no appreciable effect (Fig. 4*A*).

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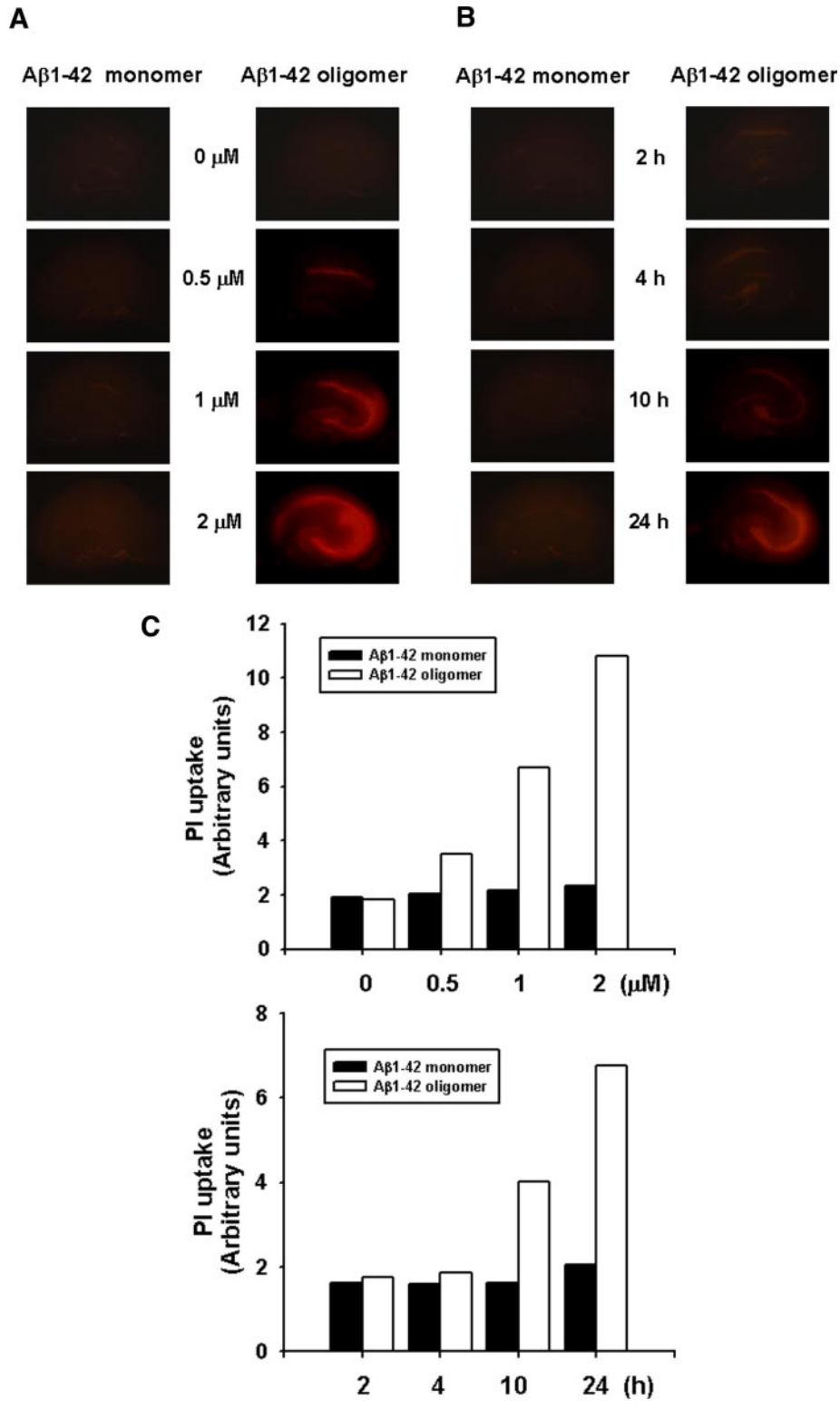

FIGURE 3. **PI uptake upon exposure to Aβ1–42 oligomer in hippocampal slices.** Dose- (*A*) and time-dependent (*B*) responses of PI uptake in hippocampal slices were recorded under conditions identical to those described for Fig. 2. PI fluorescence was observed as described under "Experimental Procedures." Representative data from two to five experiments with similar results are shown. *C*, the PI uptake of dose- (*A*) and time-dependent (*B*) responses was quantitatively analyzed as detailed under "Experimental Procedures" and is shown as graphs. PI uptake values are expressed as arbitrary units.

This activation pattern of caspase-3 is closely correlated with the pattern of cell death obtained by both the LDH assay and the PI assay presented above (Fig. 2 and Fig. 3). In addition, the potential role of caspase-3 in the Aβ1–42 oligomer-induced toxicity was also confirmed by the caspase inhibitors Z-VAD-fmk and Ac-DEVD-CHO. Pretreatment of Z-VAD-fmk, which is relatively specific for caspase-1, -3, and -4, or Ac-DEVD-CHO, a specific caspase-3 inhibitor, almost completely prevented the Aβ1–42 oligomer-induced caspase-3 activation compared with Aβ1–42 oligomer-treated cultures (Fig. 4*B*). Furthermore, these caspase-3 inhibitors, partially but significantly, reduced Aβ1–42 oligomer-induced cell death as evaluated by LDH release (Fig. 4*C*). These data indicate that Aβ1–42 oligomer-induced cell death was accompanied by activation of caspase-3 and that the cell death seen in the hippocampal slices, treated with relatively low concentrations of Aβ1–42 oligomer, appeared, at least in part, to be due to apoptosis. In addition, partial protection from Aβ1–42 oligomer-induced toxicity, observed with the caspase inhibitors, suggests that multiple mechanisms, including necrosis as well as apoptosis, may be involved in the hippocampal toxicity elicited by the Aβ1–42 oligomer.

U0126 Partially but Significantly Reduced the Hippocampal Toxicity in Response to the Aβ1–42 Oligomer—To unravel the signaling pathway, upstream of caspase-3 activation, leading to cell death observed upon exposure of the Aβ1–42 oligomer to the hippocampal slices, we investigated the results of blocking various signaling pathways with pharmacological agents. By using inhibitors of certain kinase cascades to test their involvement in hippocampal signaling, we first determined their effects on cell death in response to oligomeric Aβ1–42 by using the LDH release assay. Among the MAPK inhibitors tested, U0126, known to selectively block the activity of MAPK kinase kinase (MEK1/2), activator of ERKs (ERK1/2), partially but significantly decreased Aβ1–42 oligomer-mediated toxicity (Fig. 5). However, SB202580, a specific inhibitor of p38 MAPK, and SP600125, an inhibitor of Jun NH_2-terminal

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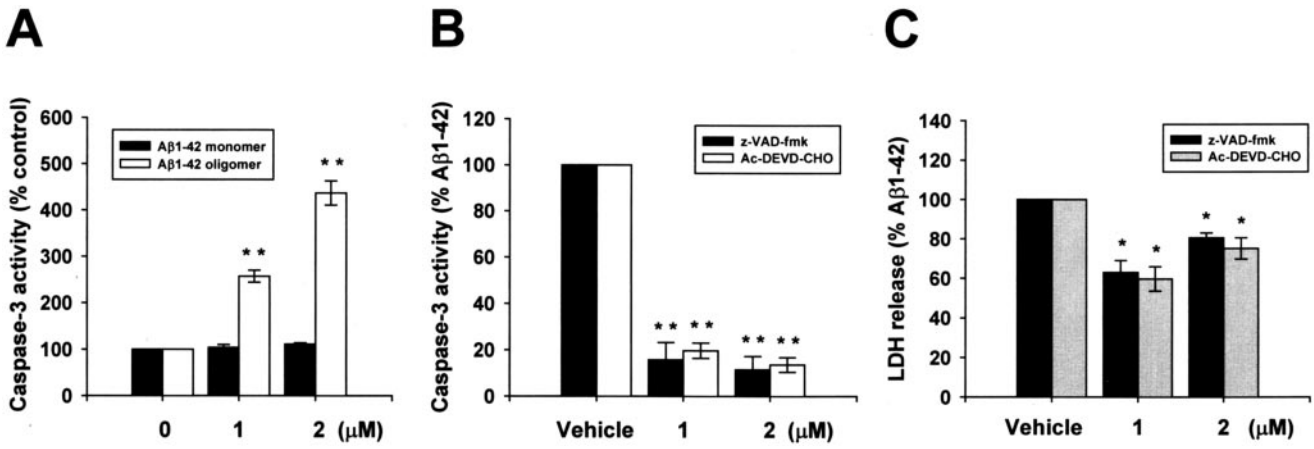

FIGURE 4. **Activation of caspase-3 in response to Aβ1–42 oligomer in hippocampal slices.** *A, in vitro* protease activity assays were performed to measure activation of caspase-3 using cell extracts obtained from hippocampal slices, which were treated with Aβ1–42 monomer or Aβ1–42 oligomer as indicated for 24 h. *B,* effects of caspase-3 inhibitors on the caspase-3 activity in response to Aβ1–42 oligomer. Slices were treated with caspase-3 inhibitors, Z-VAD-fmk, or Ac-DEVD-CHO at the concentrations indicated, 1 h before the incubation with 1 μM of Aβ1–42 oligomer for 24 h. *C,* effects of caspase-3 inhibitors on LDH release in response to Aβ1–42 oligomer in cultured media obtained under the same experimental conditions as described in *B*. Values are expressed as a percentage of the vehicle-treated controls (*A*) or of the Aβ1–42 oligomer-treated slices (*B* and *C*). Each number represents the mean ± S.E. from two to three different experiments. *, $p < 0.05$; **, $p < 0.01$, different from the vehicle-treated control or the Aβ1–42 oligomer-treated samples.

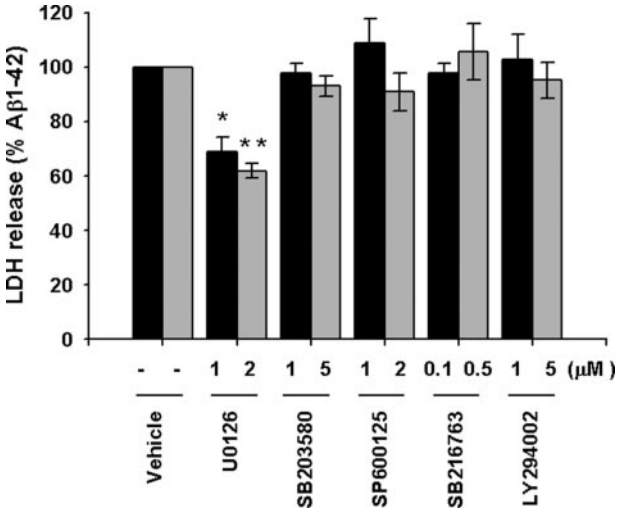

FIGURE 5. **Effects of various protein kinase inhibitors on Aβ1–42 oligomer-induced toxicity in hippocampal slice cultures.** Hippocampal slices were incubated with specific protein kinase inhibitors, including U0126, SB203580, SP600125, SP216763, or LY294002, for 1 h at the concentrations indicated and then treated with 1 μM Aβ1–42 oligomer for 24 h. LDH release assay was conducted to determine the effects of these pharmacological agents on Aβ1–42 oligomer-induced toxicity as described in Fig. 2. Values are expressed as a percentage of the Aβ1–42 oligomer-treated slices. Data are the means ± S.E. from two to four different experiments. *, $p < 0.05$; **, $p < 0.001$, different from the vehicle-treated controls.

kinase (JNK), failed to reduce the cell death because of Aβ1–42 oligomer treatment. Likewise, SB216763, an inhibitor of GSK-3β, and LY294002, an inhibitor of phosphatidylinositol 3-kinase, an activator of Akt, showed only minimal effects. Based on the evidence presented in previous papers (40–42) where these inhibitors were used, the concentrations employed in the current experiment should have been enough to inhibit their respective kinases without significantly affecting cell viability.

Aβ1–42 Oligomer Consistently Induced ERK1/2 Activation in Hippocampal Slices Undergoing Apoptotic Cell Death—To further delineate the potential involvement of ERK signaling in Aβ1–42 oligomer-induced toxicity in hippocampal slices, we analyzed whether ERK signal transduction is activated. Consistent with the pharmacological study presented above, hippocampal slice cultures treated acutely with Aβ1–42 oligomer resulted in the sustained activation of ERK1/2 in a dose- and time-dependent manner as compared with the vehicle-treated control (Fig. 6, *A* and *B*). As expected, U0126 consistently suppressed Aβ1–42 oligomer-mediated activation of ERK1/2 (Fig. 7*A*). U0126 also significantly down-regulated caspase-3 activity induced by Aβ1–42 oligomer; this was concomitant with decreased ERK1/2 activation (Fig. 7*C*). By contrast, Ac-DEVD-CHO, which efficiently blocked Aβ1–42 oligomer-induced activation of caspase-3 as presented above (Fig. 4*B*), did not exhibit the down-regulation of Aβ1–42 oligomer-induced activation of ERK1/2 (Fig. 7*B*). These results are consistent with the idea that Aβ1–42 oligomer-induced apoptotic cell death, in the hippocampus, is in part mediated through the activation of the ERK signaling pathway, which in turn activates caspase-3.

Aβ1–42 Oligomer-induced Tau Cleavage Is Consistent with Caspase-3 Activation—Recent studies provide direct evidence supporting a key role for cleaved Tau in the mechanisms leading to fibrillar Aβ-induced neurodegeneration and tangle formation in AD (25–27). Therefore, we reasoned that Tau would be a downstream target of caspase-3, which is activated by ERK1/2 signal transduction in the hippocampus in response to oligomeric Aβ1–42. To test this premise, hippocampal slices were treated with the Aβ1–42 oligomer, and the level of Tau was monitored. Analysis with Tau-5 antibody that recognizes total Tau revealed significant breakdown of this protein in a dose- and time-dependent manner (Fig. 8, *A* and *B*). The intact Tau appears in all lanes as 69- to 55-kDa bands. However, levels of Tau breakdown products were increased significantly and progressively elevated during the time course of Aβ1–42 oligomer treatment. This pattern of Tau cleavage corresponded to that of caspase-3 activity, which is induced by the ERK1/2 signaling pathway as described (Fig. 4 and Fig. 6). By contrast,

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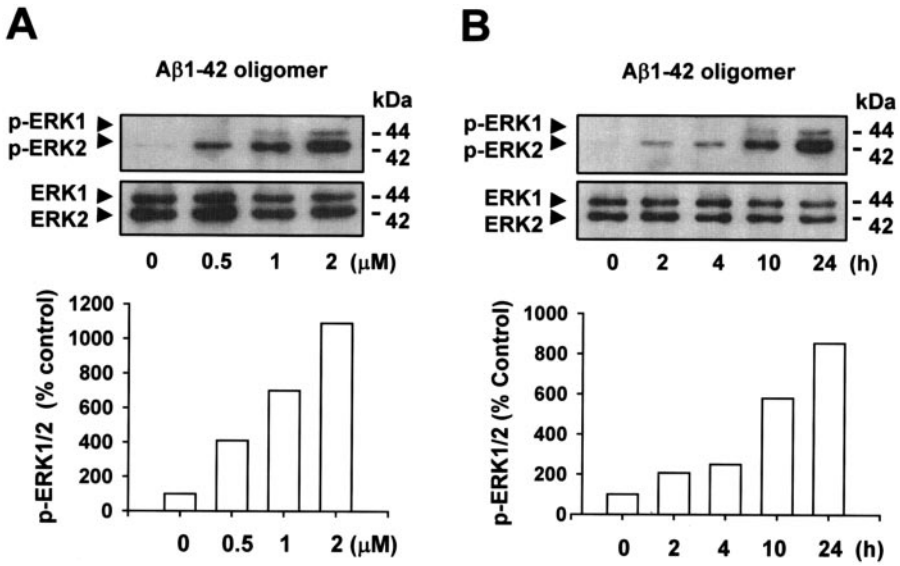

FIGURE 6. **ERK1/2 activation in response to Aβ1–42 oligomer in hippocampal slice cultures.** *A* and *B*, representative Western blots showing Aβ1–42 oligomer-induced ERK activation in a dose- and time-dependent manner under conditions identical to those described for Fig. 2. Equal amounts of total cell lysates were immunoblotted for phosphorylation of ERK1/2 using antibodies specific for the phosphorylated forms of ERK1/2. Approximately equal loading of each lane was confirmed using phosphorylation-independent antibodies of ERK1/2. The *lower panels* correspond to the quantification of ERK/1/2 activation normalized to the total amount of ERK1/2 of *A* and *B*, respectively.

synaptophysin levels, which correlate closely with synapse number and hence are commonly used to assay for loss of synapses (35, 43, 44), were not significantly altered, and cleavage of synaptophysin was not clearly seen under the same experimental conditions. To compare Aβ1–42 oligomer-mediated Tau cleavage pattern in hippocampal slices to that generated by caspase-3, we subjected the total lysate of hippocampal slices to *in vitro* caspase-3 digestion. Indeed, *in vitro* caspase-3 digestion prominently generated the Tau cleavage pattern observed in the hippocampal slices exposed to Aβ1–42 oligomer, whereas caspase-3 failed to cleave synaptophysin (Fig. 8*C*), which was truncated by calpain-1 generating a breakdown product with a mass of ~30 kDa.[3] Finally, in order to further confirm whether caspase-3 activation triggered by Aβ1–42 oligomer treatment could induce the proteolysis of Tau in the hippocampal slices, we treated them with cell-permeable inhibitors of caspase-3 or calpain-1 prior to Aβ1–42 oligomer incubation. The caspase-3 inhibitor Ac-DEVD-CHO significantly prevented Tau cleavage induced by the Aβ1–42 oligomer, whereas the calpain-1 inhibitor, calpeptin, did not block Tau cleavage (Fig. 8*D*). These results imply that the Aβ1–42 oligomer can induce proteolytic cleavage of Tau, a downstream target for the caspase-3 that is activated, at least in part, by the ERK signaling pathway in hippocampal slices undergoing Aβ1–42 oligomer-induced apoptotic cell death. Our results also suggest that synaptophysin is not a primary target for caspase-3.

DISCUSSION

The results presented in this paper verify a critical role for the soluble Aβ oligomer in inducing neurotoxicity in rat organotypic hippocampal slice cultures. This Aβ-induced neurotoxicity may be mediated, at least in part, through activation of the ERK1/2 signal transduction pathway, which results in caspase-3 activation leading to proteolytic cleavage of Tau. Several lines of observation support this conclusion. First, Aβ1–42 oligomer conferred a potent neurotoxicity in the hippocampus, which was accompanied by increased, sustained activation of the ERK1/2 pathway leading to caspase-3 activation in a dose- and time-dependent manner. Second, among the pharmacological agents used to inhibit several kinase cascades, only MEK-ERK1/2 inhibitor significantly attenuated Aβ1–42 oligomer-induced toxicity concomitant with the reduction of activation of ERK1/2 and caspase-3 to a lesser extent. Third, cleavage of Tau was detected in the hippocampal slice cultures exposed to the Aβ1–42 oligomer that causes caspase-3 activation. Finally, the caspase-3 inhibitors, which were effective in reducing both caspase-3 activation and Tau cleavage, significantly attenuated Aβ1–42 oligomer-induced cell death without appreciable effect on the ERK1/2 signaling pathway. Therefore, these data imply that one mechanism, by which the soluble Aβ oligomer promotes neurotoxicity, at least in the hippocampus, is via a sustained increase in activation of ERK1/2 and caspase-3 and by cleavage of Tau. Remarkably, even at the relatively low concentrations of soluble Aβ oligomer, selective region-specific toxicity was evident in the hippocampal CA1, a division important for cognition and memory, which is in good agreement with the selective neurodegeneration distinctly manifest in AD (45).

Recent studies clearly demonstrated that specific oligomeric forms of Aβ, including the ~12-mer as well as ~24-mer, are highly associated with cognitive decline in HuAPPsw (Tg2576) and 3xTg-AD mice models (36, 46, 62). Trimers and hexamers were excluded as components of toxic Aβ oligomer because they were present before memory impairment (36). In our study, we demonstrated that ~24-mer along with lesser quantity of ~12-mer of Aβ oligomers were detected as stable, SDS-resistant species in the culture medium from Aβ1–42 oligomer-treated slices even after 24 h of incubation. These findings together provide collective evidence that these high molecular weight Aβ oligomers may represent the primary pathogenic structure responsible for mediating Aβ toxicity in AD. This idea is further strengthened by the recent reports showing that intracerebroventricular injection of oligomers inhibits LTP (10, 11) and specifically disrupts cognitive function (47) and that the concomitant injection of the anti-Aβ antibodies 6E10 or A11 with Aβ oligomers neutralizes the oligomer-induced LTP dysfunction and Aβ pathology (48, 62).

[3] Y. H. Chong, Y. J. Shin, E. O. Lee, R. Kayed, C. G. Glabe, and A. J. Tenner, unpublished observations.

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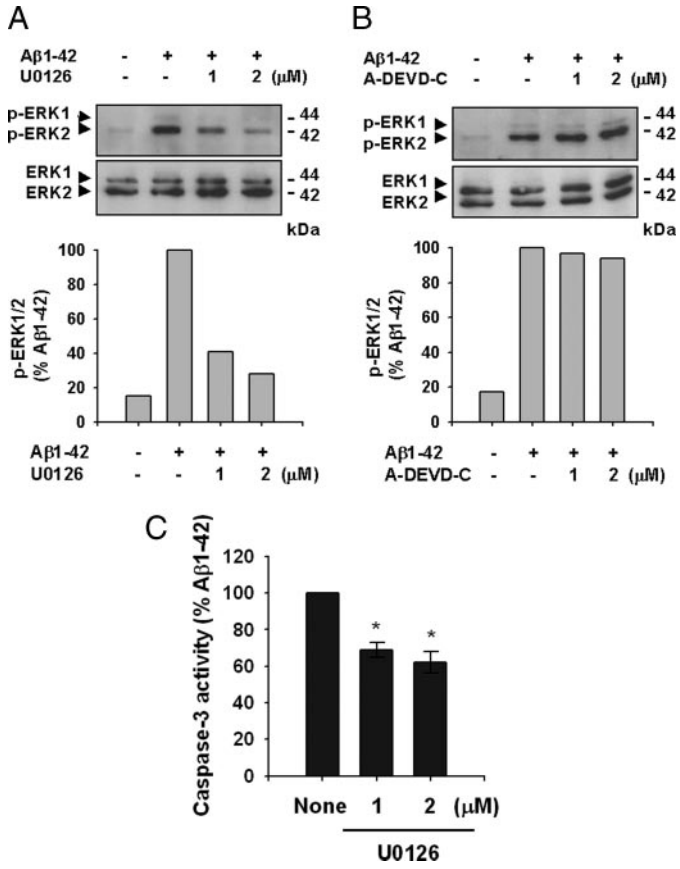

FIGURE 7. **U0126 suppresses activation of ERK1/2 and caspase-3 activation in response to Aβ1–42 oligomer in hippocampal slices.** *A* and *B*, representative Western blots showing effects of U0126 (*A*) or Ac-DEVD-CHO (*B*) on oligomeric Aβ1–42-induced ERK1/2 activation in hippocampal slices. Slices were incubated with U0126 or Ac-DEVD-CHO (A-DEVD-C) for 1 h at the concentrations indicated and then treated with 1 μM Aβ1–42 oligomer for 24 h. Immunoblots were performed as described in Fig. 6. The *lower panels* correspond to the quantification of ERK1/2 activation normalized to the total amount of ERK1/2 of *A* and *B*, respectively. *C*, the effect of U0126 on Aβ1–42 oligomer-induced activation of caspase-3. Slices were treated with U0126 at the concentrations indicated for 1 h before the incubation with Aβ1–42 oligomer for 24 h. Caspase-3 activity assays were performed as described in Fig. 4. Values are expressed as a percentage of the Aβ1–42 oligomer-treated slices. Each number represents the mean ± S.E. from two to three different experiments. *, $p < 0.05$, different from the Aβ1–42 oligomer-treated samples.

The presence of ~24-mer along with a lower mass of oligomer species in the culture media as shown in this study may indicate the possibility of dissociation of toxic oligomers in some situations. Alternatively, some of the Aβ oligomers in our preparation are not yet SDS-resistant. Thus, further biochemical experiments will be necessary to better address these conformational changes during incubation time. Importantly, soluble Aβ oligomers have been isolated from post-mortem brains of AD patients (5–8) and are particularly found within the neuritic processes and at synaptic terminals in AD brains (16, 17), strongly supporting *in vivo* the relevance of this study.

Our data demonstrating that Aβ1–42 oligomers elicited greater neurotoxicity than the Aβ1–42 monomer, in the organotypic hippocampal slices, are consistent with the recent studies demonstrating *in vitro* that the oligomeric Aβ conformation is ~10-fold more neurotoxic than the plaque-forming fibrillar Aβ assembly and ~40-fold more toxic than the monomeric Aβ (14, 39). Given that Aβ1–42 oligomer-induced cell death was accompanied by the activation of caspase-3, the cell death seen in the hippocampal slices treated with Aβ1–42 oligomer appeared, at least in part, to be due to apoptosis. This is consistent with earlier studies showing evidence of apoptosis in AD brains (49) and apoptotic cascades triggered by Aβ in a neuronal cell culture model (50). However, almost complete inhibition of caspase-3 activity by inclusion of Z-VAD-fmk or Ac-DEVD-CHO, the caspase-3 inhibitors, led to partial protection from oligomeric Aβ1–42-induced toxicity. This strongly suggests that there are multiple mechanisms underlying Aβ1–42 oligomer toxicity in the hippocampus. Several studies have reported that both apoptosis and necrosis may occur in parallel during neurodegeneration (51). In addition, biphasic cell death, beginning with acute necrosis followed by a delayed apoptosis, the hybrid forms of neuronal death, and/or switch from apoptosis to necrosis strongly support this view (52, 53). Further experiments are necessary to clearly establish the connection between apoptosis and necrosis induced by the Aβ oligomer in the hippocampus.

This study constitutes the first report demonstrating an intracellular pathway by which the Aβ1–42 oligomer triggers ERK1/2 activation, caspase activation, Tau cleavage, and apoptotic cell death in the hippocampus, suggesting a key role for this molecule in AD pathology. The fact that the MEK-ERK1/2 inhibitor U0126 precluded ERK1/2 activation, significantly ameliorating Aβ1–42 oligomer-induced cell death, indicates that this event is initially triggered by MEK-ERK1/2. Importantly, our finding that suppression of caspase-3 activation by U0126 was only partial indicates the involvement of multiple signaling events in Aβ1–42 oligomer-induced apoptosis. Few studies have explored the signaling mechanism underlying Aβ oligomer-mediated neurotoxicity, although there is one study that provides evidence for the involvement of the protein kinase C signaling pathway (54). On the other hand, a recent study implies that Aβ oligomer-evoked inhibition of LTP is mediated via activation of the kinases JNK, Cdk5, and p38 MAPK in hippocampal slices (11). However, our study demonstrating that Aβ oligomer-mediated toxicity in the hippocampus was only reduced by U0126, not by other protein kinase inhibitors, suggests a lack of direct involvement of JNK, p38 MAPK, or GSK-3β. This diversity of signaling pathways triggered by the soluble Aβ oligomer might be due to the physical state of the peptides and the time of exposure to the stimulus, as suggested by others (55), or possibly from the culture conditions and treatment schemes. Indeed, our results may be only relevant to the high molecular weight Aβ oligomers such as ~24-mer present in our oligomer preparation because Aβ-derived diffusible ligands or low molecular weight oligomers did not appear to cause neurotoxicity through the same mechanism (11, 54). Nevertheless, our findings are consistent with a recent study that demonstrated activation of the ERK signaling pathway following acute Aβ1–42 oligomer treatment of cultured hippocampal slices, although the resulting toxicity was not examined (55). Moreover, the potential role of sustained activation of the ERK signaling pathway in Aβ oligomer-mediated toxicity in the hippocampus, as seen in this study, is strongly supported by high levels of activated ERK1/2 detected around amyloid deposits in

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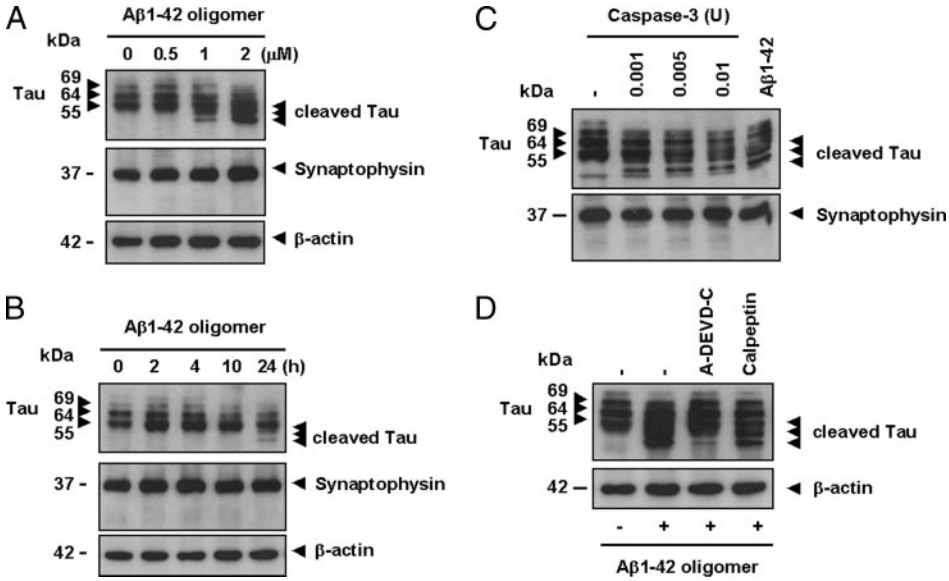

FIGURE 8. **Tau cleavage is consistent with caspase-3 activation in response to Aβ1–42 oligomer in hippocampal slices undergoing Aβ-induced toxicity.** *A* and *B,* representative immunoblots of Tau and synaptophysin content in whole cell extracts prepared from hippocampal slices as described in Fig. 2. *C,* representative immunoblot of *in vitro* Tau proteolysis by caspase-3. Tau or synaptophysin present in total lysate of hippocampal slices treated with vehicle only for 24 h are digested with increasing concentrations of caspase-3 for 1 h as indicated. For comparison, whole cell extract prepared from slices cultured with 1 μM Aβ1–42 oligomer for 24 h as described under "Experimental Procedures" and Fig. 2 was run next to caspase-3-treated samples. *D,* effect of specific caspase-3 inhibitor, Ac-DEVD-CHO (*A-DEVD-C*), on Tau proteolysis triggered by the Aβ1–42 oligomer in hippocampal slices. Western blot analysis of Tau content in whole cell extracts prepared from hippocampal slices treated with the caspase-3 inhibitor, Ac-DEVD-CHO (1 μM), or the calpain-1 inhibitor, calpeptin (1 μM), for 1 h prior to the addition of Aβ1–42 oligomer (1 μM) is shown. Total lysates were prepared from slices after 24 h of treatment as described under "Experimental Procedures." Representative gels from two to three experiments with similar results are shown. Uniformity of gel loading was confirmed with β-actin as the standard.

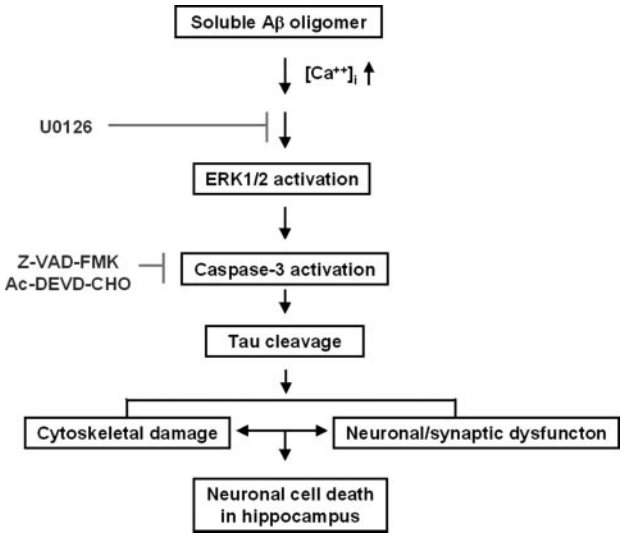

FIGURE 9. **A schematic of the proposed role of Aβ1–42 oligomer in hippocampal toxicity by the ERK1/2 signaling pathway leading to caspase-3-activation and Tau cleavage.** This scheme presents a molecular mechanism by which low micromolar concentrations of Aβ1–42 oligomer may cause a significant increase in the activation of the ERK signaling pathway, which in turn activates caspase-3 in the hippocampus. In the experimental paradigm employed here, the role of Ca^{2+} as an upstream effector responsible for activation of ERK in the machinery of apoptotic cell death remains unsettled despite our previous knowledge that dysregulation of Ca^{2+} ions, through cytotoxic pores created by Aβ oligomer, might play a crucial role in Aβ oligomer-mediated toxicity (3, 30). Tau truncation by active caspase-3 might be associated with Aβ1–42 oligomer-induced cell death in the hippocampus by leading to cytoskeletal damage and neuronal/synaptic dysfunction, which is associated with memory deficits as seen in early AD patients.

AD brains (20), as well as the sustained activation of ERK in hippocampal neurons in response to fibrillar Aβ-induced neurotoxicity (23, 25, 56). In addition, our observation that the changes in ERK1 phosphorylation, indicative of kinase activation, were not as robust as ERK2 closely corresponds to earlier reports that ERK2 is implicated as a critical factor for hippocampal synaptic plasticity or learning and memory (57, 58).

The present study provides evidence that the Aβ1–42 oligomer can induce cell toxicity via proteolytic cleavage of Tau, a downstream target for the caspase-3 that is activated by the ERK signaling pathway. Indeed, two proteases proposed to play a role in the pathogenesis of AD are caspase-3 and calpain-1 (26, 27, 59), which are families of cysteine proteases that have important roles in the initiation, regulation, and execution of cell death (60, 61). Our finding that caspase-3, rather than calpain-1, plays a primary role in Tau cleavage, which is linked to the toxic action of Aβ1–42 oligomer in the hippocampus, is consistent with previous reports that Tau, the dendritic and axonal constituent, is considered the preferred substrate for caspases leading to the generation of dystrophic neurites in response to fibrillar Aβ treatments (25–28, 59). In rat primary cortical neurons, Tau cleavage was observed by 10 μM fibrillar Aβ (26), which is a 10-fold higher dose than Aβ oligomer used in this study. In addition, caspase-3-cleaved Tau was recently confirmed to occur in AD brains as an early event, and thus is likely to be in part associated with the development of tangle pathology within AD brains (26, 27), and could be a marker for hippocampal neuronal cell death.

Several studies have demonstrated that fibrillar Aβ peptides promote pathological Tau filament assembly in neurons by triggering caspase cleavage of Tau, and generating a proteolytic product that assembles more rapidly and more extensively into Tau filaments *in vitro* than wild-type Tau (25–28). This supports the hypothesis that amyloid deposition, neurofibrillary tangles, and caspase activation could share a common pathway. Furthermore, in AD brains, activated ERK1/2 and phosphorylated Tau colocalize with amyloid-rich areas (20). A recent study has demonstrated that a single intrahippocampal injection of a specific oligomeric antibody efficiently cleared Aβ pathology and, more importantly, Tau pathology in an *in vivo* model of AD (62). This finding along with our results strongly implicates a link between Aβ oligomerization and Tau pathology. Accordingly, the interference of Aβ oligomerization might be a valid therapeutic target for AD treatment (48, 62, 63). In addition, our finding that hippocampal slice cultures treated acutely with

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Aβ oligomer did not exhibit synaptophysin cleavage was clearly confirmed by the lack of synaptophysin proteolysis by caspase-3 *in vitro*. These observations suggest that Tau cleavage might occur independently from synapse loss in the hippocampus; this might precede proteolytic cleavage of synaptophysin as described recently (35, 64).

In conclusion, we propose that an apoptotic route, in hippocampal slices after Aβ1–42 oligomer treatment, might involve Tau cleavage, possibly leading to cell structure deregulation, upon caspase-3 activation through the ERK signaling pathway as summarized in Fig. 9. The data generated from this study will help elucidate how soluble Aβ oligomer affects the signal pathway in the hippocampus, possibly shedding light on how elevated Aβ oligomer causes hippocampal dysfunction leading to the episodic memory deficits associated with early AD. Thus, control of ERK signaling or use of specific caspase proteolytic inhibitors might be useful in preventing, at least partially, Aβ oligomer-induced neurotoxicity in the hippocampus.

Acknowledgments—We thank Karntipa Pisalyaput and Jalene Imaoka for their technical assistance.

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Young Hae Chong, Yoo Jeong Shin, Eun Ok Lee, Rakez Kayed, Charles G. Glabe and Andrea J. Tenner

J. Biol. Chem. 2006, 281:20315-20325.
doi: 10.1074/jbc.M601016200 originally published online May 18, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M601016200

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