Upstream Signaling Pathways Leading to the Activation of Double-stranded RNA-dependent Serine/Threonine Protein Kinase in β-Amyloid Peptide Neurotoxicity*

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One of the hallmarks of Alzheimer’s disease is extracellular accumulation of senile plaques composed primarily of aggregated β-amyloid (Aβ) peptide. Treatment of cultured neurons with Aβ peptide induces neuronal death in which apoptosis is suggested to be one of the mechanisms. We have demonstrated previously that Aβ peptide induces activation of double-stranded RNA-dependent serine/threonine protein kinase (PKR) and phosphorylation of eukaryotic initiation factor 2α (eIF2α) in neurons in vitro. Degenerating neurons in brain tissues from Alzheimer’s disease patients also displayed high immunoreactivity for phosphorylated PKR and eIF2α. Our previous data have also indicated that PKR plays a significant role in mediating Aβ peptide-induced neuronal death, because neurons from PKR knockout mice and neuroblastoma SH-SY5Y cells stably transfected with dominant negative mutant of PKR are less susceptible to Aβ peptide toxicity. Therefore, it is important to understand how PKR is activated by Aβ peptide. We report here that inhibition of caspase-3 activity reduces phosphorylation of PKR and to a certain extent, cleavage of PKR and eIF2α in neurons exposed to Aβ peptide. Calcium release from the endoplasmic reticulum and activation of caspase-8 are the upstream signals modulating the caspase-3-mediated activation of PKR by Aβ peptide. Although in other systems HSP90 serves as a repressor for PKR, it is unlikely the candidate for caspase-3 to affect PKR activation in neurons after Aβ peptide exposure. Elucidation of the upstream pathways for PKR activation can help us to understand how this kinase participates in Aβ peptide neurotoxicity and to develop effective neuroprotective strategy.

One of the pathological hallmarks of Alzheimer’s disease (AD) is extracellular accumulation of senile plaques composed primarily of aggregated β-amyloid (Aβ) peptide. Aβ peptide is a heterogeneous 39–43-amino acid peptide generated by sequential cleavage of amyloid precursor protein by β-secretase and γ-secretase. It is generally considered that Aβ peptide plays a pivotal role in the pathogenesis of AD (for review see Ref. 2). Treatment of cultured neurons with Aβ peptide has been shown to induce apoptosis (3) and necrosis (4). Neurons undergoing Aβ peptide-induced apoptosis are morphologically characterized by membrane blebbing, cell shrinkage, DNA fragmentation, and chromatin condensation and biochemically by an ordered activation of a conserved family of cysteine proteases called caspases (5–7). In the brains of AD patients, it has been demonstrated that numerous tangle-bearing neurons and non-tangle-bearing neurons display apoptotic features with DNA fragmentation (8, 9).

Caspases have been considered to play important roles in coordinating apoptosis (10, 11). Aβ peptide is able to induce selective activation of caspases including caspase-2, -3, -6, -8, -9, and -12 (12–16). Among all the caspases, caspase-3 has been shown to play important roles in the execution phase of apoptosis (17, 18). Caspase-3 has been reported to be activated in brain tissues from AD patients (19) and in Aβ peptide-treated neurons (13, 20). Although the significance of other caspases such as caspase-2 cannot be neglected (15), inhibition of caspase-3 activation has been shown to protect neurons from Aβ peptide-induced apoptosis, implying that caspase-3 might represent one of the key players in mediating Aβ peptide-induced apoptosis (20).

Recently, we found that activation of double-stranded RNA (dsRNA)-dependent protein kinase (PKR) and phosphorylation of eukaryotic initiation factor 2α (eIF2α) are observed in the degenerating neurons of the brains from AD patients (21) and in cultured neuronal cells challenged with Aβ peptide (22). We have also reported that PKR plays a crucial role in mediating Aβ peptide-induced neuronal death, because primary cortical neurons from PKR knockout mice and neuroblastoma SH-SY5Y cells stably transfected with dominant negative mutant PKR are less susceptible to Aβ peptide toxicity (22). Human PKR consists of 551 amino acids that form two functional domains: an N-terminal dsRNA binding regulatory domain and a C-terminal kinase catalytic domain (23). Following virus infection, dsRNAs produced by viruses activate PKR after binding to its regulatory domain. As a result, PKR becomes auto-

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1 The abbreviations used are: AD, Alzheimer’s disease; Aβ, β-amyloid; dsRNA, double-stranded RNA; PKR, dsRNA-dependent protein kinase; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; XeC, Xestospongin C; HSP, heat shock protein; ANOVA, analysis of variance.

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phosphorylated to further promote its activity. Increasing lines of compelling evidence have shown that PKR can be activated without the presence of dsRNA. Instead, non-dsRNA molecules including polyinosinates (23), PACT (PKR-activating protein) (24), and cellular stresses such as serum-deprivation and calcium depletion from the endoplasmic reticulum (ER) (25) can activate PKR. Activated PKR can phosphorylate eIF2α at serine 51. The phosphorylation of eIF2α inhibits the guanine nucleotide exchange factor eIF2B by preventing the exchange of GDP for GTP on eIF2 so that global protein translation of mRNAs using 5’-cap initiation codon is inhibited leading to apoptosis (26). In addition to the translational regulation, PKR also mediates apoptosis by controlling the activation of several transcriptional factors (25, 27–29), regulation of selective pro-apoptotic molecules such as Bax or Fas (22, 25, 30), and selective activation of caspases (25, 31, 32).

Although many studies have been focusing on downstream pathways of PKR (25, 31, 32), how PKR is activated by Aβ peptide in neurons is still unclear. In the present study, we aim to examine the upstream signaling pathways of PKR triggered by Aβ peptide in neurons. Our previous report (22) has shown that activation of caspase-3 occurs within a short period of time after exposure of Aβ peptide. Therefore, we further investigated whether this early activation of caspase-3 is required for the activation of Aβ peptide-induced neuronal apoptosis. We found that inhibition of caspase-3 activity attenuated apoptosis by controlling the activation of several transcriptional factors (25, 27–29), regulation of selective pro-apoptotic molecules such as Bax or Fas (22, 25, 30), and selective activation of caspases (25, 31, 32).

Upstream Signals of PKR Activation Induced by Aβ Peptide

EXPERIMENTAL PROCEDURES

Primary Cell Cultures of Cortical Neurons—Cell cultures were carried out as described previously (22, 33). Briefly, cerebral cortices of 17-day-old embryos of Sprague-Dawley rats (The Laboratory Animal Service was then centrifuged at 14,000 R/min, followed by Aβ peptide-induced neuronal apoptosis (25). The phosphorylation of eIF2α (100 mM), EDTA (1 mM), EGTA (1 mM), NaF (1 mM), Na4P2O7 (20 mM), putrescine (100 μM), streptomycin (50 μg/ml)-coated 6-well plates at 2.2 × 106 cells/well. Neurons were cultured in Eagle’s minimal essential medium (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen), glucose (18 mM), l-glutamine (2 mM), insulin (5 μg/ml), progesterone (0.02 μM), purinucle (100 μM), selenium (30 μM), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37 °C in a humidified 5% CO2 atmosphere. Deoxyfluorouridine/uridine (2 μM) was added to the cultures to prevent the growth of non-neuronal cells. Neurons were cultured for 7 days prior to treatments.

Treatments—Cortical neurons at 7 days in vitro were pretreated with a cell-permeable caspase-3 inhibitor (DEVDF-CHO; Calbiochem) at 100 μM for 1 h, a cell-permeable caspase-8 inhibitor (IETD-CHO; Calbiochem) at 40 μM for 1 h, or an isoform 1,4,5-trisphosphate receptor antagonist Xestospongin C (XeC; Calbiochem) at 1 μM for 2 h in serum-free medium, followed by Aβ peptide 25–35 (Aβ25−35 peptide; Sigma) at 25 μM or Aβ peptide 1–42 (Aβ1−42 peptide; Bioprotein Company, San Diego, CA) at 25 μM in serum-free medium. The peptides were incubated in autoclaved Milli-Q water at 37 °C for 3 days prior to use.

Western Blot Analysis—After treatments, neurons were scratched and lysed in ice-cold E1A buffer containing HEPES (pH 7.6, 50 mM), NaCl (250 mM), Nonidet P-40 (0.1%), EDTA (5 mM), protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Sigma). The lysate was then centrifuged at 14,000 × g for 30 min at 4 °C. Quantification of protein content in the supernatant was measured by using a protein assay kit (Bio-Rad). 150 μg of cellular proteins were pre-cleared with Protein G-Sepharose beads (Protein G-Sepharose 4 Fast Flow; Amersham Biosciences). The unbound proteins were then incubated with a monoclonal PKR antibody (100 μg protein/1.5 μg antibody; BD Biosciences) overnight at 4 °C. Protein G-Sepharose was then added and mixed for 2 h at 4 °C. After washing the beads four times with E1A buffer, the immunoprecipitated samples were subjected to Western blot analysis according to the method mentioned before.

Caspase Activity Assay—After treatments, neurons were scratched and lysed in E1A buffer without protease and phosphatase inhibitors for caspase activity assays (BIOSOURCE). The lysate was then centrifuged at 14,000 × g for 30 min at 4 °C. 50 μg of cellular proteins from the supernatant were used for different caspase activity assays. For colorimetric activity assays, substrates for caspase-3 (Ac-Asp-Glu-Val-Asp-p-nitroanilide, Ac-DEVD-pNA; Calbiochem) or caspase-8 (Ac-Asp-Glu-Thr-Asp-p-nitroanilide, Ac-IETD-pNA; Calbiochem) were incubated with the protein extracts for 2 h at 37 °C to yield a yellow-brown product p-nitroanilide, which was measured by a spectrophotometric reader at 405 nm. For fluorogenic activity assays, fluorogenic signals from the cleaved product of the caspase-7 substrate (MCA-Val-Asp-Glu-Val-Asp-Glu-Asp-Glu-Lys–(DNP)-NH2; MCA-VDQDVDDR–(DNP)-NH2; Calbiochem) were measured with excitation and emission wavelengths at 320 and 405 nm, respectively, after 2 h of incubation at 37 °C.

Measurement of Intracellular Free Calcium Levels—Intracellular free calcium levels ([Ca2+]i) were determined by fluorescence imaging with acetoxymethyl-fura 2 (fura 2-AM; Calbiochem) using the methods described previously (35). Briefly, after pretreatment with XeC, cultured neurons were incubated with 5 μM fura 2-AM for 30 min at 37 °C for dye loading, followed by washing with Hanks’ balanced saline supplemented with HEPES (10 mM) twice and glucose (10 mM) and a 5-min calcium-incubation prior to calcium imaging. [Ca2+]i, in 20–25 neuronal cell bodies per microscopic field was monitored under an inverted microscope prior to and after exposure of neurons to Aβ25−35 peptide at 25 μM. The mean of [Ca2+]i, from at least four separate cultures was determined from the ratio of the fluorescence emissions using two different excitation wavelengths (340 and 380 nm) according to the formula [Ca2+]i = Kd[(F−F0)/(F0/F−F0)].

Statistical Analysis—Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance, Tukey’s test was used as an a posteriori test according to a statistical program SigmaStat® (Jandel Scientific, Chicago, IL). The level of significance was p < 0.05. For the comparison of significance between two groups, Student’s t test was conducted at the level of significance of p < 0.05 or p < 0.001 with SigmaStat® (Jandel Scientific). Results are expressed as the means ± S.E. from at least three independent experiments.
RESULTS

Aβ Peptide Induced Early Activation of Caspase-3—We have shown previously (22) that PKR and eIF2α play significant roles in Aβ peptide-induced neurodegeneration in vitro and in degenerating neurons of the brains from AD patients (21). However, how Aβ peptide activates PKR in neurons is still unclear. In the present study, we aim to examine the upstream signaling pathways leading to the activation of PKR in Aβ peptide neurotoxicity. Because we have shown that PKR is activated less than 2 h upon the treatment with Aβ peptide in primary cortical neurons (22), we search for any possible candidates that may start to participate in the Aβ peptide-triggered apoptotic process in a short moment of time. A time course study of caspase-3 specific activity indicated that caspase-3 was significantly (p < 0.05) activated in neurons 1 h after the treatment with Aβ25–35 peptide at 25 μM (0.29 ± 0.01 pmol/min/μg; see Fig. 1a) when compared with the corresponding control (0.23 ± 0.01 pmol/min/μg; see Fig. 1a). The Aβ25–35 peptide-induced caspase-3 activity progressively augmented in a time-dependent manner from 1 to 16 h (Fig. 1a).

To study the role of caspase-3 in the activation of PKR in Aβ peptide neurotoxicity, one of the possible ways is to inhibit caspase-3 by a specific caspase-3 inhibitor (DEVD-CHO). We at first verified the effectiveness of this caspase-3 inhibitor in our experimental model. Caspase-3 activity induced by Aβ25–35 peptide in cultured neurons at 2 and 7 h was significantly blocked by 100 μM DEVD-CHO, which was added 1 h prior to the exposure of Aβ25–35 peptide (Fig. 1b). DEVD-CHO at 100 μM did not show any toxicity to the neurons (data not shown). In addition to the blocking effect on caspase-3, DEVD-CHO might also inhibit caspase-7. Despite the cross-inhibitory effects, our results demonstrated that caspase-7 was not significantly (p > 0.05) activated 2 and 7 h after the treatment with Aβ25–35 peptide at 25 μM (Fig. 1c). Therefore, the inhibitory effect of DEVD-CHO on caspase-7 could be neglected in our present study.

Inhibition of Caspase-3 Activity Attenuated Aβ Peptide-induced Phosphorylation of PKR and eIF2α—Having shown that caspase-3 was early activated by Aβ peptide, and the caspase-3 activity can be significantly inhibited by DEVD-CHO, we then study whether modulation of caspase-3 activity can mediate the activation of PKR-eIF2 pathway. Western blot analysis demonstrated that 25 μM Aβ25–35 peptide induced an increased phosphorylation of PKR at threonine 446 and 451 in neurons when compared with the corresponding controls (Fig. 2a). Pretreatment of neurons with 100 μM DEVD-CHO for 1 h markedly reduced the Aβ25–35 peptide-induced PKR phosphorylation (Fig. 2a). 100 μM DEVD-CHO per se did not induce PKR phosphorylation (Fig. 2a). There was no other band detected near the region of 38 to 48 kDa (the kinase fragment), indicating that no cleaved PKR was phosphorylated at threonine 446 and 451 (data not shown). Fig. 2b shows that 25 μM Aβ25–35 peptide induced phosphorylation of eIF2α at serine 51 in neurons, and pretreatment of neurons with 100 μM DEVD-CHO for 1 h markedly reduced the eIF2α phosphorylation. In controls, there was also a mild expression of the phosphorylated PKR (Fig. 2a) and phosphorylated eIF2α (Fig. 2b), which was possibly because of the effects of serum deprivation during experimental treatments.

Inhibition of Caspase-3 Activity Blocked Aβ Peptide-induced Cleavage of eIF2α and PKR—Previous reports have shown that PKR and eIF2α can be cleaved by caspase-3. To further dem-

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Fig. 1. DEVD-CHO completely blocked Aβ25–35 peptide-induced activation of caspase-3 in primary cortical neurons. 50 μg of cellular proteins from cultured cortical neurons were utilized for the measurements of the activity of caspases. a, a time course study shows the changes in caspase-3 activity in the neurons treated with or without 25 μM Aβ25–35 peptide for 1, 3, 7, and 17 h. Results expressed as the specific activity at a unit of picomoles of colorimetric pNA generated per min per μg of protein are shown as the mean ± S.E. from three independent experiments. Significant difference between the corresponding control and Aβ25–35 peptide-treated neurons is indicated by * p < 0.05 and ***, p < 0.001 (Student’s t test). b, with or without 1-h pre-incubation of a cell-permeable inhibitor of caspase-3 DEVD-CHO at 100 μM, activity of caspase-3 in neurons in the presence or absence of 25 μM Aβ25–35 peptide for 2 and 7 h was measured. Results expressed as -fold of control are shown as the mean ± S.E. from three independent experiments. Significant difference is indicated by *, p < 0.05 versus control (one-way ANOVA with Tukey’s test). c, caspase-7 activity assays were performed from the neurons with or without exposure to 25 μM Aβ25–35 peptide for 2 and 7 h. Results expressed as -fold of control are shown as the mean ± S.E. from three independent experiments. No significant difference between the corresponding control and Aβ25–35 peptide-treated neurons is indicated by N.S., p < 0.05 (Student’s t test).
onstrate that caspase-3 is actively involved in neurons exposed to Aβ peptide, we study whether caspase-3-mediated cleavage of PKR and eIF2α occurs in our model. There is an additional form of eIF2α, migrating with a slightly faster electrophoretic mobility than the full-length eIF2α in Western blot analysis (Fig. 3, a and b). This additional form of eIF2α is its cleaved form. The difference in the molecular mass between the full-length and cleaved form of eIF2α proteins shown in Fig. 3, a and b is about 2–3 kDa, which is in agreement with the previous findings (36–38). In controls, in addition to the appearance of the full-length form of eIF2α, the cleaved bands were also observed (Fig. 3, a and b), which is possibly related to the effects of serum deprivation during experimental treatments. This is because some proteases including caspasps and calpain could be activated in cultured neurons under the serum-free condition (39–41). Because the complete inhibition of caspase-3 activity cannot fully prevent the cleavage of eIF2α in the controls, there might be some other proteases responsible for this cleavage under the serum-free condition. Neurons exposed to Aβ25–35 peptide appeared to have the cleaved form of eIF2α only, indicating that the full-length form of the protein was totally cleaved (Fig. 3, a and b). Aβ25–35 peptide, a reverse peptide of Aβ25–35 as a negative control, did not induce cleavage of eIF2α when compared with the corresponding control (Fig. 3b). Aβ25–35 peptide-induced cleavage of eIF2α was blocked by pretreating neurons with 100 μM DEVD-CHO for 1 h (Fig. 3a).

Fig. 3. Inhibition of Aβ25–35 peptide-induced caspase-3 activity blocked the cleavage of eIF2α and PKR in primary cortical neurons. Representative gels of Western blot analysis from three independent experiments show the immunoreactivity toward phosphorylated (threonine 446 and 451) PKR (a) and phosphorylated (serine 51) eIF2α (b). β-Actin was used as the control for the Western blot experiments.

The cleavage of PKR induced by Aβ25–35 peptide did not appear as complete cleavage as seen in eIF2α, because a large portion of the full-length form of PKR was detected (Fig. 3, c and d). Aβ25–35 peptide, a reverse peptide of Aβ25–35 as a negative control in this experiment, did not induce proteolysis of PKR when compared with the corresponding control (Fig. 3d). Inhibition of caspase-3 activity by using 50 or 100 μM DEVD-CHO blocked Aβ25–35 peptide-induced cleavage of PKR in neurons, indicating that caspase-3 is responsible for this cleavage (Fig. 3c).

Modulation of Aβ Peptide-induced Calcium Release from the ER to Cytosol Attenuated Caspase-3-mediated Activation of PKR—We have reported previously (22) that a membrane-permeable calcium chelator BAPTA-AM can reduce Aβ peptide-induced PKR activation in neurons, suggesting that changes in [Ca²⁺]ᵢ play a role in the activation of PKR. However, how [Ca²⁺]ᵢ mediates the activation of PKR in Aβ peptide neurotoxicity is still unclear. Our data showed that Aβ25–35 peptide induced a rapid increase in [Ca²⁺]ᵢ in neurons (Fig. 4a). Neurons exposed to Aβ25–35 peptide maintained a high [Ca²⁺]ᵢ, when compared with the control (Fig. 4a). Pre-incubation of neurons with 1 μM XeC, an antagonist of inositol 1,4,5-trisphosphate receptor that can modulate calcium release from the ER.
indicates that pretreatment with XeC 1 h prior to the exposure to Aβ peptide (12). Fas and Fas ligand have also been shown to be involved in Aβ peptide-induced neurotoxicity (43). Because caspase-3 can be activated or processed by caspase-8 (44, 45), we tried to analyze whether caspase-8 activation was also responsible for the caspase-3-mediated activation of PKR.

Our results showed that caspase-8 was significantly (p < 0.05) activated 1 h after the exposure of Aβ peptide at 25 μM in neurons (Fig. 5a). A caspase-8 inhibitor IETD-CHO at 40 μM significantly (p < 0.05) inhibited 25 μM Aβ peptide-induced caspase-3 activation at 2 h (Fig. 5b). The concentrations of IETD-CHO higher than 40 μM were toxic to the cultured neurons (data not shown). To study whether modulation of caspase-8 activity can affect the caspase-3-mediated PKR-eIF2α pathway, phosphorylation and cleavage of both the PKR and eIF2α were studied in the neuronal cultures. Fig. 5c shows that both the Aβ peptide-induced phosphorylation and cleavage of PKR and eIF2α were attenuated by pretreating neurons with 40 μM IETD-CHO for 1 h prior to the application of Aβ peptide.

Aβ1–42 Peptide-induced Phosphorylation and Cleavage of PKR and eIF2α Were Inhibited by Both the Caspase-3 and Caspase-8 Inhibitors—Despite the similarity of neurotoxicity between Aβ peptides (46), we also examined whether PKR-eIF2α pathway activated by Aβ1–42 Peptide can be inhibited by the selective caspase-3 (DEVD-CHO) and caspase-8 (IETD-CHO) inhibitors. Similar to Aβ peptide, 25 μM Aβ1–42 peptide induced phosphorylation and cleavage of PKR and eIF2α after 4 h of the treatment (Fig. 6). Pretreatment of neurons with 100 μM DEVD-CHO or 40 μM IETD-CHO for 1 h attenuated both the Aβ peptide-induced phosphorylation and cleavage of PKR and eIF2α (Fig. 6).

HSP90 Was Not Involved in the Activation of PKR in Aβ Peptide Neurotoxicity—Apart from the cleavage of PKR, we demonstrated that phosphorylation of PKR was also mediated by caspase-3. Therefore, we attempted to further investigate how caspase-3 affects the phosphorylation of PKR. Because the biological nature of caspase-3 is a protease, it cannot function as a kinase to activate PKR by phosphorylating it. We speculated that caspase-3 might mediate PKR phosphorylation through a PKR repressor. HSP90 has been reported recently (47) to interact with PKR and to repress the activity of PKR, because an inhibitor of HSP90 geldanamycin has been shown to induce the activation of PKR by triggering dissociation of HSP90 from PKR. Using immunoprecipitation, we found that HSP90 was co-immunoprecipitated with PKR (Fig. 7). Treatment with Aβ peptide in neurons did not decrease the association of HSP90 from PKR (Fig. 7). Therefore, HSP90 may not be the candidate for caspase-3 to modulate neuronal PKR activity following the exposure of Aβ peptide.

DISCUSSION

We have demonstrated that PKR plays significant roles in Aβ peptide neurotoxicity (22) and in AD (21). Inhibition of caspase-3 activity reduces phosphorylation of PKR in neurons exposed to Aβ peptide. Early and mild activation of caspase-3 is triggered by an increase in [Ca2+]i, released from the ER and the activation of caspase-8. Although activation of caspase-3 is usually followed by the cleavage of its substrates, a PKR repressor HSP90 is unlikely to be the substrate for caspase-3 in Aβ peptide-induced apoptosis in neurons.

Neurotoxicity of Aβ Peptide in Neurons—It is generally believed that Aβ peptide plays an important role in the patho-
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Fig. 5. Blockade of caspase-8 activity by IETD-CHO reduces caspase-3 activity and both the phosphorylation and cleavage of PKR and eIF2α. a, a time course study of caspase-8 activity was performed in cultured cortical neurons exposed to 25 μM Aβ25-35 peptide. Results expressed as -fold of control are shown as the mean ± S.E. from three independent experiments. Significant difference is indicated by *, p < 0.05 versus the Aβ25-35 Peptide-treated group at time = 0 h (t test). With or without 1-h pretreatment of a cell-permeable inhibitor of caspase-8 IETD-CHO at 40 μM, cellular proteins from primary cortical neurons in the presence or absence of 25 μM Aβ25-35 peptide for 4 h were utilized different analysis. b, 50 μg of cellular proteins were subject to caspase-3 activity assays. Results expressed as -fold of control are shown as the mean ± S.E. from three independent experiments. Significant difference is indicated by *, p < 0.05 versus control (one-way ANOVA with Tukey's test). c, cellular proteins were also utilized for Western blot analysis. Representative gels from three independent experiments show the immunoreactivity toward phosphorylated (threonine 446 and 451) PKR, phosphorylated (serine 51) eIF2α, and both the intact and cleaved forms of eIF2α and PKR. β-Actin was used as the control for the Western blot experiments.

Fig. 6. Inhibition of Aβ1-42 peptide-induced caspase-3 or caspase-8 activity reduces both the phosphorylation and cleavage of PKR and eIF2α. Representative gels show that pretreatment with the DEVD-CHO at 100 μM or the IETD-CHO at 40 μM for 1 h reduced both the phosphorylation and cleavage of eIF2α and PKR in the primary cortical neurons challenged with Aβ1-42 peptide at 25 μM for 4 h. β-Actin was used as the control for the Western blot experiments.

Fig. 7. HSP90 was co-immunoprecipitated with PKR. With or without the exposure to 25 μM Aβ25-35 peptide for 6 h, cellular proteins were immunoprecipitated (IP) with the monoclonal PKR antibody. The immunoprecipitate was then subject to Western blot (WB) analysis on HSP90. Proteins subject to all procedures for immunoprecipitation without incubation with the monoclonal PKR antibody served as the negative control (NO IP).

In the brains of AD patients, Aβ1-42 peptide is the primary species of aggregated Aβ peptide found in amyloid plaques (1). Aβ25-35 peptide, a fragment of Aβ1-42 peptide, has been widely used as a toxic agent to induce neuronal cell death in culture, because it shows similar neurotoxicity as Aβ1-40 peptide (46). Treatment of neurons with Aβ1-42 or Aβ25-35 peptide can induce neuronal apoptosis (22). Despite the similarity of neurotoxicity between Aβ25-35 and Aβ1-42 peptides, both the peptides are investigated in the present study. We have shown previously that PKR plays an important role in mediating Aβ1-42 or Aβ25-35 Peptide-induced neuronal cell death (22). Aβ25-35 peptide is shown to induce caspase-3-mediated phosphorylation and cleavage of PKR and eIF2α, which is mediated by upstream signals such as caspase-8 and intracellular calcium release from the ER (see Figs. 2–5). Similar effects can also be observed in neurons exposed to Aβ1-42 peptide (Fig. 6). These lines of evidence suggest that activation of PKR is a common signaling event in neurons exposed to Aβ1-42 and Aβ25-35 peptide-induced neurotoxicity.

Both the synthetic Aβ1-42 and Aβ25-35 peptides are able to form fibrils in vitro (50). Fibril formation has been suggested to be required for Aβ peptide neurotoxicity (1, 2, 50–52). Treatment of neurons with Aβ peptide can induce activation of caspase-3 (20). Recent studies have indicated that other amyloidogenic peptides such as human amylin can also trigger activation of caspase-3 in RINm5F cells (53), suggesting that activation of caspase-3 may not be specific for Aβ peptide toxicity. It is also known that human amylin acts in a similar way as Aβ peptide in inducing calcium changes and that antagonists of human amylin can block Aβ peptide toxicity, implying that Aβ peptide and human amylin may have a combined site of action (54, 55). Yet, human amylin does not accumulate in AD brains, and nothing is known about PKR and human amy-
lin. More importantly, human amylin induces a relatively late activation of caspase-3 (12 h after the treatment) (53) whereas our results show that Aβ peptide significantly induces caspase-3 activation as early as 1 h after the treatment. Early activation of caspase-3 is important for the activation of PKR (as discussed below).

A Novel Role of Caspase-3: Activator of a Cellular Signaling Pathway—Caspase-3 has long been considered as an executioner in apoptosis (10, 11, 56, 57). Our present results may add a new concept on the functions of caspase-3; early activation of caspase-3 is an important event in mediating cellular signaling pathways such as PKR rather than only participating in the execution phase of apoptosis. There are a few recent studies indicating the importance of early activation of caspase-3 in physiological responses; caspase-3 is activated 2 min after a learning process in adult male zebra finch songbirds (58), and early activation of caspases including caspase-3 is involved in the early steps of lymphocyte activation (59). In the present study, caspase-3 is activated significantly in neurons 1 h after the treatment with Aβ peptide. Blockade of this early activation of caspase-3 can reduce phosphorylation of PKR and eIF2α, implying that caspase-3 is an important candidate in mediating early cellular signaling events associated with Aβ peptide neurotoxicity.

Apart from mediating phosphorylation of PKR, we demonstrate here that caspase-3 can also cleave PKR and eIF2α. For PKR cleavage, Western blot analysis using an antibody against the C-terminal domain of PKR reveals that the molecular mass of cleaved PKR was nearly 43 kDa, which is similar to the previous findings (36). For eIF2α cleavage, Western blot analysis reveals that the difference in molecular mass between the full-length and cleaved forms of eIF2α proteins is 2–3 kDa, which is also in agreement with the previous reports (36–38). Other studies have shown that PKR and eIF2α can be cleaved by caspases in other cell types during different apoptotic insults including treatment with poly(I)—poly(C) (38), tumor necrosis factor-α plus cycloheximide (36–38), cisplatin (37, 38), etoposide (37), and anti-Fas (36). Cleaved PKR is fully capable of phosphorylating eIF2α to the same extent as the full-length form of PKR, because the proteolysis of PKR by caspases releases the kinase domain from the N-terminal regulatory N-terminal domain (36). The N-terminal part of PKR fragment has been proposed to be the repressor of the PKR itself (60). Although PKR can be activated by cleavage, our results show that only a small portion of cleaved PKR was observed. This implies that the activation mode of PKR is not solely dependent on the cleavage of PKR. For the cleavage of eIF2α, it has been suggested that its cleavage causes functional changes of the eIF2α complex, which can no longer stimulate global protein translation (37). There was a large portion of cleaved eIF2α observed in Aβ peptide-treated neurons, suggesting that cleaved eIF2α can be an additional way to inhibit global protein translation. Taken together, although cleavage of eIF2α can augment the effects of its phosphorylation in protein translation, cleavage of PKR by activated caspase-3 is not the major mode of activation for PKR. Phosphorylation of PKR remains to be the major way for the activation of PKR.

Activation of caspase-3 has been reported previously (20) in neurons exposed to Aβ peptide. Cleavage of poly(ADP-ribose) polymerase and α-fodrin have also been found to be endogenous substrates for caspase-3 (20). In the present study, eIF2α and PKR are not only the substrates for caspase-3, their activation is also mediated by this caspase. As mentioned before, our results show that Aβ peptide induces early activation of caspase-3 in neurons (as early as 1 h after the treatment of Aβ peptide) whereas Harada and Sugimoto (20) focused on the late activation of caspase-3. Because early and mild activation of caspase-3 plays an important role in mediating PKR activation, we further show that calcium release from the ER and caspase-8 activation are upstream of caspase-3-mediated PKR activation in Aβ peptide neurotoxicity. Therefore, our present results provide novel information on how Aβ peptide mediates toxicity in neurons through caspase-3 and PKR.

Caspase-3-mediated PKR Activation by Aβ Peptide Is Partially Regulated by ER Calcium Release—Caspase-3 is early activated and plays important roles in mediating the activation of PKR in Aβ peptide neurotoxicity. We further study the upstream signals leading to the caspase-3-mediated activation of PKR. Aβ peptide has been shown to induce release of calcium from the ER to the cytosol, which may result in disturbance in intracellular calcium homeostasis (61). We have reported recently (49) that both the Aβ1–42 and Aβ25–35 peptides can induce calcium release from the ER, which is in consistent with the previous findings showing that both the peptides can trigger an elevation in [Ca2+]. (61, 62). Therefore, we used XeC to modulate Aβ peptide-triggered ER calcium release possibly via inositol 1,4,5-trisphosphate receptor (42). Our results show that attenuation of Aβ peptide-triggered ER calcium release by XeC significantly reduces caspase-3 activity, indicating that ER calcium release is likely to be a source for the caspase-3 activation. Indeed, activation of caspase-3 by an increase in calcium concentration has been reported (63). Therefore, Aβ peptide-triggered calcium release from the ER may account for the early activation of caspase-3 and the caspase-3-mediated activation of PKR. We have shown previously (22) that an intracellular calcium chelator BAPTA-AM can reduce Aβ peptide-triggered PKR activation in neurons, suggesting that changes in [Ca2+]i can stimulate PKR. We therefore cannot neglect the direct activation of PKR by calcium in Aβ peptide neurotoxicity. Mutations in PS-1 (grogenilin-1) gene enhance calcium release from the ER (64), sensitizing neurons to calcium insults triggered by Aβ peptide (65, 66). Recently, the activity of PERK (PKR-like ER kinase), which is activated by ER stress, has been found not to be altered by a PS-1 knock-in mutation (67). We speculate that PKR might play a more important role in PS-1-mediated ER calcium signaling as calcium release from the ER can activate PKR.

Caspase-8 Is an Upstream Modulator of Caspase-3-mediated PKR Activation—Apart from intracellular calcium, our results also indicate that caspase-8 can modulate caspase-3 activation in our experimental model, because inhibition of caspase-8 activity can significantly reduce Aβ peptide-triggered caspase-3 activation. In fact, caspase-3 can be activated or processed in vitro by caspase-8 (44, 45). We show that reduction of caspase-3 activity by inhibiting caspase-8 attenuates Aβ peptide-induced phosphorylation of PKR and eIF2α, suggesting that caspase-8 is most likely upstream of the caspase-3-mediated activation of PKR. Activation of caspase-8 has been shown in Aβ peptide-induced neuronal apoptosis, because inhibition of caspase-8 activity and expression of dominant negative Fas-associated death domain protect neurons from Aβ peptide neurotoxicity (12). Fas and Fas ligand have also been shown to be involved in Aβ peptide toxicity in cultured hippocampal neurons (43). In addition, neutralization of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) by neutralizing antibody can protect human neuroblastoma SH-SY5Y cells from Aβ peptide neurotoxicity (68). Besides, caspase-8 can be cleaved by calcium-activated protease calpain (69), which has been shown to be activated in Aβ-peptide-treated neurons (70). Although a recent study using fluorescent resonance energy transfer in COS-7 and NIH3T3 cells did not find any activation of caspase-8 stimulated by Aβ peptide (71), ours, along with those
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**Fig. 8. Representation of the activation of PKR-eIF2α pathway by Aβ peptide.** Aβ peptide can induce the activation of caspase-8. Caspase-3 can be activated by caspase-8 and by calcium release from the ER. PKR and eIF2α can be cleaved by caspase-3 in neurons exposed to Aβ peptide. Caspase-3 might cleave to activate PKR activators or to repress PKR repressors to modulate the activation of PKR. Both the phosphorylated PKR and cleaved PKR can phosphorylate eIF2α. Cleaved eIF2α might mimic the effects of eIF2α phosphorylation, leading to inhibition of global protein synthesis. PKR-eIF2α pathway has been shown to play important roles in mediating apoptosis.

from other laboratories, demonstrate the activation of caspase-8. In fact, our results show that caspase-8 activity in primary cultured neurons exposed to Aβ peptide was significantly increased even 1 h after the treatment. The discrepancy between different findings may be because of different types of cells used in the studies. For the activation of caspase-8 in neurons by Aβ peptide, it has been indicated to be linked with death receptors of the Fas/tumor necrosis factor family (12, 43). More importantly, the present results demonstrate that caspase-3-mediated activation of PKR can be mediated by caspase-8.

**HSP90 Is unlikely to Modulate Caspase-3-mediated PKR Activation in Aβ Peptide-induced Neuronal Death—Inhibition of caspase-3 activity by DEVD-CHO can block both the Aβ peptide-induced phosphorylation and cleavage of PKR and eIF2α. It appears that application of this caspase-3 inhibitor may alter the activity of multiple downstream pathways of caspase-3. Yet, the relationship between caspase-3 and PKR-eIF2α pathway is still evident, because attenuation of caspase-3 activity by either reduction of ER calcium release or inhibition of caspase-8 activity can modulate the PKR-eIF2α pathway. As caspase-3 is not a kinase, its action on activating PKR would not involve direct phosphorylation. Blockade of caspase-3 activity may therefore alter some PKR repressors or activators for PKR activation.

Having shown that early and mild activation of caspase-3 plays significant roles in PKR activation, we then attempt to investigate how caspase-3 mediates PKR activity in response to Aβ peptide. As stated above, our results show that cleavage of PKR by caspase-3 is not the major mode for the activation of PKR. Therefore, we searched for any PKR repressor that may be affected by caspase-3. Because the biological nature of caspase-3 is to cleave proteins but not to phosphorylate proteins, a PKR-activating protein, PACT, which is activated by phosphorylation (72), is unlikely to be the target for caspase-3-mediated PKR activation. HSP90 has been reported recently (47) to interact with PKR and to repress the expression of PKR, because geldanamycin, an inhibitor of HSP90, has been shown to induce activation of PKR by triggering dissociation of HSP90 from PKR. Our results indicate that HSP90 interacts with PKR in neurons as HSP90 is co-immunoprecipitated with PKR. However, Aβ peptide does not induce dissociation of HSP90 from PKR, suggesting that Aβ peptide-induced PKR activation is not mediated by the dissociation of HSP90 from PKR. Therefore, HSP90 might not be the target for caspase-3-mediated PKR activation in Aβ peptide neurotoxicity.

There have been some reports showing how PKR could regulate downstream apoptotic pathways, such as the activation of caspase-8 and caspase-9 (25, 31, 32). In the present study, we highlight how caspase-3, caspase-8, and ER calcium release at an upstream position modulate the PKR pathway in Aβ peptide-treated neurons (see the summarized diagram in Fig. 8). Taken together, our present results demonstrate a new mode of PKR activation and explore a new upstream signaling pathway of Aβ peptide neurotoxicity. Because phosphorylated PKR and eIF2α are observed in degenerating neurons of AD patients (21), understanding the molecular signaling mechanisms of neuronal apoptosis may pave the way for future therapeutic intervention against neuronal death in AD.

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