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ACTIVATION OF EUKARYOTIC INITIATION FACTOR-2α-KINASES IN OKADAIC ACID-TREATED NEURONS

S. M. KIM,[a,b] S. Y. YOON,[a,b]* J. E. CHOI,[a,b] J. S. PARK,[a,b] J. M. CHOI,[a,b] T. NGUYEN[a,b] AND D. H. KIM[a,b]*

[a]*Department of Anatomy and Cell Biology, University of Ulsan College of Medicine, Seoul, Korea*

[b]*Research Institute for Biomacromolecules, University of Ulsan College of Medicine, Seoul, Korea*

Abstract—Phosphorylation of eukaryotic initiation factor-2α (eIF2α) is increased in Alzheimer's disease (AD) and this protein can be phosphorylated by several kinases, including double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), amino acids-regulated eIF2α kinase (GCN2) and heme-regulated eIF2α kinase (HRI). PKR and PERK especially are activated in the AD brain, and GCN2 is reported to increase presenilin-1 (PS1) activity. Okadaic acid (OA), a protein phosphatase-2A (PP2A) inhibitor, is known to increase tau phosphorylation, β-amyloid (Aβ) deposition and neuronal death, which are the pathological characteristics of AD. Here, we show that the phosphorylation of eIF2α is increased and its kinases, PKR, PERK and GCN2 are activated in rat neurons by OA. Activating transcription factor (ATF4) which induces apoptosis in response to eIF2α phosphorylation was increased and translocated to nuclei in OA-treated neurons. These results suggest that the successive events of activation of eIF2α kinases and eIF2α phosphorylation leading to ATF4 nuclear translocation may contribute to neuronal death. However, PKR inhibitors did not reduce eIF2α phosphorylation or neuronal toxicity despite inhibiting PKR activity. These results suggest that PKR might not be the most responsible kinase for eIF2α phosphorylation or cell death in PP2A-inhibited conditions such as AD. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: eukaryotic initiation factor-2α, Alzheimer's disease, double-stranded RNA-dependent protein kinase, PKR-like endoplasmic reticulum kinase, amino acids-regulated eIF2α kinase.

Eukaryotic initiation factor-2α (eIF2α) is involved in the protein translation initiation process and is inhibited by phosphorylation (reviewed in (Gebauer and Hentze, 2004)). eIF2α phosphorylation is increased in association with neuronal degeneration and cognitive decline in Alzheimer's disease (AD) (Chang et al., 2002b; Paccalin et al., 2006; Unterberger et al., 2006; O'Connor et al., 2008). AD is characterized by the progressive loss of neurons and synapses, and exhibits two distinctive pathologic characteristics: neurofibrillary tangles (NFT) and senile plaques (Gomez-Isla et al., 1997). NFTs are formed by intraneuronal accumulation of paired helical filaments composed of abnormally hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986). Senile plaques are primarily composed of β-amyloid peptides (Aβ) derived from amyloid precursor protein (APP) that has undergone proteolytic processing by β-secretase (BACE-1) and γ-secretase. Recently, BACE-1 levels were found to be translationally increased by phosphorylation of eIF2α (O'Connor et al., 2008). Moreover, eIF2α phosphorylation is increased in various cell and animal models of AD (Chang et al., 2002a; Page et al., 2006) and is positively correlated with BACE1 levels and β-amyloid loads (O'Connor et al., 2008). Thus, eIF2α phosphorylation may play a significant role in the pathogenesis of AD.

eIF2α can be phosphorylated by several kinases, including double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), amino acids-regulated eIF2α kinase (GCN2) and heme-regulated eIF2α kinase (HRI). PKR was originally characterized as a kinase activated by auto- or trans-phosphorylation upon binding to double-stranded viral RNA, and was shown to be involved in the interferon-induced antiviral defense response through induction of a general shutdown of translation via eIF2α phosphorylation (Williams, 1999). Besides viral infection, PKR can also be activated by a number of cellular stresses to stimulate cell death programs (Williams, 1999; Gil and Esteban, 2000). Most notably, PKR is activated in AD and is closely associated with NFTs and senile plaques (Peel and Bredesen, 2003; Onuki et al., 2004; Paccalin et al., 2006; Bullido et al., 2008).

PERK is an endoplasmic reticulum (ER)-resident transmembrane protein kinase (Shi et al., 1998; Harding et al., 1999). In response to ER stress, PERK is activated by autophosphorylation and phosphorylates eIF2α, leading to suppression of protein translation (Harding et al., 1999). In AD neurons, the unfolded protein response (UPR) and ER stress response are increased, together with PERK activation and neurofilament phosphorylation (Hoozemans et al., 2005, 2009; Unterberger et al., 2006). Another eIF2α kinase, GCN2, is activated by autophosphorylation in response to amino acid deprivation (Wek et al., 1995; Garcia-Barrio et al., 2002). GCN2 regulates synaptic plasticity,

*Correspondence to: S. Y. Yoon or D. H. Kim, Department of Anatomy and Cell Biology, University of Ulsan College of Medicine, 388-1 PoongNap-Dong, SongPa-Gu, Seoul 138-736, Korea. Tel: +82-2-3010-4241 or +82-2-3010-4272; fax: +82-2-3010-8038.
E-mail addresses: ysy@amc.seoul.kr (S. Y. Yoon) or dhkim@amc.seoul.kr (D. H. Kim).

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; ATF4, activating transcription factor 4; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; GCN2, amino acids-regulated eIF2α kinase; HRI, heme-regulated eIF2α kinase; NFT, neurofibrillary tangles; OA, okadaic acid; PERK, PKR-like endoplasmic reticulum kinase; PKR, double-stranded RNA-dependent protein kinase; PKRi, PKR inhibitor; PP2A, protein phosphatase-2A; UPR, unfolded protein response.

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as well as learning and memory, through modulation of the activating transcription factor 4 (ATF4/CREB2) pathway (Costa-Mattioli et al., 2005), and is also involved in regulating γ-secretase activity during amino acid imbalance, suggesting a role in AD (Mitsuda et al., 2007).

Several studies have suggested that the number of NFTs is strongly correlated with the degree of dementia in AD (Arriagada et al., 1992; Gomez-Isla et al., 1997; Mackay et al., 1997). Aberrant phosphorylation of tau is therefore a critical step in the progress of neurodegeneration in AD. Although the specific cellular events that result in tau phosphorylation have not yet been determined, the involvement of decreased protein phosphatase activity has been hypothesized to result in the neurofibrillary pathology of AD (Gong et al., 1995, 2000; Vogelsberg-Ragaglia et al., 2001; Sontag et al., 2004a,b; Tanimukai et al., 2005). Okadaic acid (OA), a protein phosphatase-2A (PP2A) inhibitor, induces hyperphosphorylation of tau and cell death in cultured neurons (Kim et al., 1999; Wang et al., 2001). *In vivo*, OA induces an AD-like pathology, stimulating Aβ deposition and subsequent neuronal degeneration, synaptic loss and memory impairment (Arendt et al., 1998; Sun et al., 2003).

To investigate the role of eIF2α phosphorylation in AD, we assessed the levels of phosphorylated eIF2α (p-eIF2α) and activation of its upstream kinases in OA-treated neurons by immunostaining for p-eIF2α, p-PKR, p-PERK and p-GCN. We found that the levels of p-eIF2α and phosphorylated (activated) forms of its kinases were increased in OA-treated neurons and were predominantly increased in neurites and cell bodies, compared to control neurons. ATF4 which induces apoptosis in response to eIF2α phosphorylation was increased and translocated to nuclei in OA-treated neurons. These results suggest that all the events of activation of eIF2α kinases, eIF2α phosphorylation and ATF4 nuclear translocation may be involved in PP2A-inhibited neurodegenerative conditions such as AD.

EXPERIMENTAL PROCEDURES

All procedures were intended to minimizing the number of animals used and their suffering and approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences in Seoul, Korea.

Neurons and cell culture

Primary cultures of rat cortical neurons were prepared from brains of embryonic day 16 pups, as described previously (Yoon et al., 2005). Briefly, cerebral cortices were dissected in calcium- and magnesium-free Hank's balanced salt solution, and incubated with a 0.125% trypsin solution for 10 min at 37 °C. Trypsin was inactivated with Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, and cortical tissue was further dissociated by repeated trituration using a Pasteur pipette. The resulting cell suspensions were diluted in neurobasal medium supplemented with B27 components (Gibco–BRL, Grand Island, NY, USA.), and seeded onto plates or coverslips coated with poly-D-lysine (Sigma, 50 μg/mL) and laminin (1 μg/mL, Gibco–BRL, Grand Island, NY, USA). Neurons were maintained at 37 °C in 5% CO_2 for 12 days prior to the addition of drugs. Chinese hamster ovary (CHO) cells and SH-SY5Y cells were cultured in F12 (Invitrogen) or DMEM/F12 (Invitrogen) medium, respectively, supplemented with L-glutamine and 10% (v/v) heat-inactivated fetal bovine serum.

Drug treatment

OA (Boehringer Mannheim) was administerd at 10 nM. For control experiments, DMSO was added at the final concentration present in OA-treated cultures (0.001%). Where used, 2-aminopurine (2-AP; Sigma) and PKR inhibitor (PKRi; Calbiochem), were added 30 min prior to OA treatment at the final concentrations.

Western blot analysis

For Western blot analyses, cell monolayers were removed using a cell scraper, and cells were centrifuged at 2000 rpm for 5 min. The cell pellet was resuspended in protein extraction solution (PRO-Prep, Intron), according to the manufacturer's guide, and incubated at −20 °C for 20 min. The suspension was centrifuged at 13000 rpm (4 °C) for 5 min, and the supernatant was transferred to a 1.5 ml tube. Protein concentrations were determined by the Bradford method. Equal amounts of protein were mixed with sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate [SDS], 2.5% glycerol, 0.5% 2-β-mercaptoethanol, Bromophenol Blue), boiled for 5 min and stored at −20 °C until ready for use. Proteins were resolved by SDS-polyacrylamide gel electrophoresis at a constant voltage (130 V), and subsequently transferred to polyvinylidene difluoride membranes (0.2-μm pore size; BioRad) at 110 V for 2 h. After a 1-h incubation in blocking TTBS buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20) containing 2% bovine serum albumin and 2% normal horse serum, blots were incubated for 16 h at 4 °C with one or more of the following primary antibodies: rabbit eIF2α-pS51 (1:1000; Cell Signaling), rabbit PKR-pT451 (1:1000; Biosource), rabbit PERK-pT981 (1:200; Santacruz), rabbit GCN-pT898 (1:1000; Cell Signaling), mouse APP-NT (1:1000; Chemicon), mouse PHF-1 (a kind gift from Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY, USA), rabbit tau-pS396 (1:1000; Biosource) and rabbit BACE-1 (1:1000; Abcam). Blots were washed in TTBS buffer, incubated with horseradish peroxidase-conjugated anti-IgG (1:5000; Pierce) and visualized using enhanced chemiluminescence reagents (Amersham) and X-ray film.

Immunocytochemistry

For immunofluorescence analyses, cells grown on glass coverslips were fixed for 30 min at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer, and cell membranes were permeabilized by incubating for 30 min in 0.05 M Tris buffer (pH 7.4) containing 0.1% Triton X-100, 2% BSA and 2% normal horse serum. Cultures were incubated overnight at 4 °C with one or more of the following primary antibodies: rabbit eIF2α-pS51 (1:100; Cell Signaling), rabbit PKR-pT451 (1:100; Biosource), rabbit PERK-pT981 (1:100; Santacruz) and rabbit GCN-pT898 (1:100; Cell Signaling), mouse APP-NT (1:1000; Chemicon), mouse PHF-1 (source indicated above). Thereafter, cells were washed and incubated with FITC- or Cy3-labeled secondary antibodies (1:300; Jackson Laboratories). Images were obtained with a Leica DMIRB microscope and CoolSNAP-cf CCD-camera (Roper Scientific). For double immunostaining, two antibodies originating from different species were selected, and a bandpass fluorescence filter was used. Virtually no staining was detected in negative controls in which primary antibodies were omitted.

Statistical analysis

Data are presented as mean±SEM from three independent experiments performed in duplicate. Results were evaluated with the Student's *t*-test, with statistical significance at $P<0.05$.