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Up-regulation of calsyntenin-3 by β-amyloid increases vulnerability of cortical neurons

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ABSTRACT

β-Amyloid (Aβ) may play an important role in the pathogenesis of Alzheimer's disease. However, a causal relationship between Aβ oligomers and layer-specific neurodegeneration has not been clarified. Here we show up-regulation of calsyntenin (Cst)-3 in cultured neurons treated with Aβ oligomers and in Tg2576 mice. Cst-3 is distributed in large neurons in layers 2–3 and 5 of the cerebral cortex, and accumulated in dystrophic neurites surrounding Aβ-plaques. Overexpression of Cst-3 accelerates neuronal death. These results indicate that up-regulation of Cst-3 in cortical neurons in layers 2–3 and 5 by Aβ oligomers may lead to increase in vulnerability of neurons.

1. Introduction

Alzheimer's disease (AD) is characterized by the accumulation of β-amyloid (Aβ) plaques and neurofibrillary tangles, and neuronal loss in the neocortex. Genetic, neuropathological, and biological evidence indicates that Aβ plays an important role in the early pathogenesis of AD: cognitive impairment caused by synaptic dysfunction [1–3]. Despite a poor correlation between Aβ-plaque density and cognitive decline in very mild AD patients, the amount of soluble Aβ oligomer in neocortex correlates well with cognitive decline or synaptic loss in humans and transgenic mice expressing mutant human amyloid precursor protein (APP) [4–8]. However, the molecular mechanisms of synaptic dysfunction by soluble Aβ oligomers remain to be fully elucidated. The identification of Aβ-induced genes that mediate synaptic dysfunction would provide considerable insight into the mechanisms of AD.

Calsyntenins (Cst, also termed alcadeins), type-1 transmembrane proteins of the cadherin superfamily, are found in the synaptic membrane in the adult brain [9]. Three Csts, Cst-1, Cst-2, and Cst-3, identified in human, mouse and rat [10,11], have the WDDS motif, which binds to the light chain of kinesin-1. In the past few years, biological evidence restricted to Cst-1 has been accumulated; Cst-1 acts as a cargo-docking protein for kinesin-1-mediated vesicular transport [12,13]. CASY-1, an ortholog of Csts in *Caenorhabditis elegans*, plays an important role in learning [14,15]. The expression of the Cst-1 gene is reduced in amyloid-rich areas in APP + PS1 Tg mouse brain [16]. In contrast, little is known about the regulation and the function of other Csts, especially Cst-3, except their distribution in cerebral cortex [11].

To address these issues, we examined the expression of Cst genes in Aβ-treated neuronal culture, and in the cerebral cortex from an AD mouse model (APP_{SW}, Tg2576). We also examined whether altered gene expression causes enhanced vulnerability of neurons to stimuli leading to neurodegeneration.

2. Materials and methods

2.1. Neuronal cell culture

Cerebral cortices dissected from day E17 embryonic rats were dissociated by incubation with 0.08% trypsin/0.008% DNase I at 37 °C for 10 min, and passed through a 62-μm nylon mesh. The cells (10^5 cells/dish for immunofluorescence or 4.5×10^6 cells/dish for preparation of RNA or protein) were seeded in gelatin–polyornithine-coated dishes with 3.5- or 6-cm diameter, respectively, and were cultured in MEM–5% fetal bovine serum–10 μM β-mercaptoethanol.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; Cst, calsyntenin; MEM-N2, minimum essential medium with N2 supplement.

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2.2. Treatment with Aβ

Peptides Aβ1-42 and Aβ42-1 (Bachem Inc.) were treated with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma), and then dried under vacuum in a SpeedVac. Freshly prepared Aβ1-42 or Aβ42-1 was prepared by dissolving the HFIP-treated peptides at 250 μM in 0.05 N HCl, filtering through a 0.45-μm membrane filter, and diluting to 5 μM with minimum essential medium with N2 supplement (MEM-N2). The peptide solution (2 or 5 μM) was added to the 7 DIV cultures immediately after preparation. Aβ1-42 oligomers and fibrils were prepared according to the methods of Stine et al. [17]. Briefly, Aβ1-42 oligomers were prepared by diluting 5 mM Aβ1-42 in DMSO to 100 μM in ice-cold Ham's F12, and incubating at 4 °C for 4 days. Aβ1-42 fibrils were prepared by diluting 5 mM Aβ1-42 in DMSO to 100 μM in 10 mM HCl, and incubating at 37 °C for 4 days. The peptide solution (2 μM) was added to the 7 DIV cultures.

2.3. Mice

Heterozygous Tg2576 (APP_{SW}) mice and wild-type littermates were purchased from Taconic Farms Inc. Mice were killed by anesthesia overdose and perfused transcardially with saline followed by ice-cold phosphate-buffered 4% paraformaldehyde. After post-fixation in phosphate-buffered 4% paraformaldehyde for 24 h at 4 °C and cryoprotection with 20% sucrose, brains were sectioned coronally at 10 μm through the entire hippocampus on a cryostat.

2.4. Suppressive subtractive hybridization

Suppressive subtractive hybridization screening was performed according to the manufacturer's instructions (Clontech). Briefly, poly-$(A)^+$RNA from cultured cortical neurons treated with 5 μM Aβ1-42 for 15 h was used as a "tester" and poly-$(A)^+$RNA from cultured cortical neurons treated with MEM-N2 was used as a "driver". From this screening, we identified a partial cDNA sequence (590 bp) corresponding to rat Cst-3 (accession number AJ431642).

2.5. Northern blot analysis

Poly-$(A)^+$RNA from cultured cortical neurons or mouse cerebral cortex was isolated using a Micro-FastTrack 2.0 Kit (Invitrogen). Aliquots of 2 μg of poly-$(A)^+$RNA were denatured, electrophoretically fractionated on a 1.4% agarose/formaldehyde gel, and transferred to a nylon membrane. Hybridization was performed in a solution containing cloned cDNA labeled with ^{32}P-dCTP using a random labeling kit (Roche Applied Science). Radioactivities of the bands were measured using a Bioimage analyzer BAS 2500.

2.6. Quantitative real-time PCR (QRT-PCR)

First-strand cDNA was synthesized from poly-$(A)^+$RNA of cultured cortical neurons using SuperScript II and oligo (dT) primers (Invitrogen). Quantitative RT-PCR analysis was performed using an iCycler iQ Detection System (Bio-Rad) with ELONGase Enzyme Mix (Invitrogen), SYBR Green I (1/50 000 dilution, Takara), and 400 nM gene-specific primers (nucleotides 37–60 and 242–219 for Cst-3; nucleotides 201–231 and 284–261 for ACTB). Results were evaluated with the ICYCLER IQ REAL-TIME DETECTION SYSTEM software (Bio-Rad).

2.7. Plasmid constructs

Cst-3 and Cst-2 were constructed by inserting PCR amplified fragments into pCMV-Tag5 containing a c-myc epitope (Stratagene). Detailed construct information is available upon request. Mouse Cst-1 cDNA clone (clone ID 6315355) was purchased from OPEN BIOSYSTEMS. The coding fragment of Cst-1 was cloned into pCMV-Tag5 containing a c-myc epitope (Stratagene).

2.8. Transfection

The constructs were transfected into 5 DIV cortical neurons with Lipofectamine 2000 according to the manufacturer's manual (Invitrogen). For serum withdrawal experiments, the culture medium was replaced with MEN-N2 18 h after transfection and the cells were cultured for an additional 6, 12, or 24 h.

2.9. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, treated with 0.3% triton X-100 for 10 min, blocked with 2% skim milk for 30 min, and reacted with the primary antibodies for 1 h, followed by a 1 h reaction with the secondary antibodies. Cell nuclei were stained with 10 μM Hoechst 33342 in PBS (−) for 10 min. Apoptotic neurons were counted in at least 60 transfected cells for each construct and in each transfection experiment. Immunofluorescence of frozen mouse brain was performed in the same manner as for cultured cells but followed by treatment with 10 mM $CuSO_4$ in 50 mM ammonium acetate buffer (pH 5.0) for 10 min for quenching autofluorescence of lipofuscin pigments inside neurons in aged mouse brain [18]. Immunofluorescence was visualized with an Olympus epifluorescence microscope.

2.10. Immunoblotting

Cultured cells were homogenized in 1% NP-40 containing 2 mM EDTA and protease inhibitors, and centrifuged for 20 min (14 000 rpm, 4 °C). Lysates were analyzed by SDS–PAGE (a 5–15% acrylamide linear gradient gel). After transferring to Immobilon, proteins were detected with specific antibodies using the enhanced chemiluminescence method.

2.11. Antibodies

Anti-Cst-3 polyclonal rabbit antibodies were raised against the peptide SSDERRIIETPPHRY (Cst-3 931–956) conjugated with KLH at the N-terminus of the peptide. The peptide antibodies were purified on the ligand-immobilized EAH Sepharose 4B (GE Healthcare). The polyclonal anti-myc tag antibodies (MBL), polyclonal anti-APP T668 (Cell Signaling), and a monoclonal anti-PHF antibody (AT8, Innogenetics) were purchased. Secondary antibodies were Texas red-conjugated anti-rabbit IgG (Vector), Alexa 488-conjugated goat anti-rabbit IgG, and Alexa 594-conjugated goat anti-mouse IgG_1 (Molecular Probe).

3. Results

3.1. Differential regulation of Csts by Aβ1-42

The cell viability of rat cortical neurons treated with freshly prepared Aβ1-42 assessed by trypan blue exclusion were 91%, 87%, 85%, and 66% of the untreated control after 6, 15, 24, and 48 h of treatment, respectively [19]. RNA isolated from neuronal cultures 15 h (slight neurodegeneration) after treatment with freshly prepared Aβ1-42 was used to generate an Aβ-inducing gene library using PCR-based suppression subtraction hybridization. We identified Cst-3 as a gene up-regulated by Aβ (Fig. 1A and B). Next, to determine whether other Csts are also up-regulated by Aβ, we assessed the expression of Cst-1 and Cst-2 in cortical neurons treated with freshly prepared Aβ1-42 for 15 h. Northern blot analyses