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Loss of proteostasis induced by amyloid beta peptide in brain endothelial cells

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ABSTRACT

Abnormal accumulation of amyloid-β (Aβ) peptide in the brain is a pathological hallmark of Alzheimer's disease (AD). In addition to neurotoxic effects, Aβ also damages brain endothelial cells (ECs) and may thus contribute to the degeneration of cerebral vasculature, which has been proposed as an early pathogenic event in the course of AD and is able to trigger and/or potentiate the neurodegenerative process and cognitive decline. However, the mechanisms underlying Aβ-induced endothelial dysfunction are not completely understood. Here we hypothesized that Aβ impairs protein quality control mechanisms both in the secretory pathway and in the cytosol in brain ECs, leading cells to death. In rat brain RBE4 cells, we demonstrated that $A\beta_{1-40}$ induces the failure of the ER stress-adaptive unfolded protein response (UPR), deregulates the ubiquitin–proteasome system (UPS) decreasing overall proteasome activity with accumulation of ubiquitinated proteins and impairs the autophagic protein degradation pathway due to failure in the autophagic flux, which culminates in cell demise. In conclusion, Aβ deregulates proteostasis in brain ECs and, as a consequence, these cells die by apoptosis.

1. Introduction

Alzheimer's disease (AD) is the most frequent cause of dementia in the elderly. One neuropathological hallmark of AD is the extracellular accumulation of amyloid-beta (Aβ) peptide in brain parenchyma and, according to the 'Amyloid Cascade Hypothesis', Aβ triggers a cascade of toxic events leading to selective synaptic and neuronal loss [1,2]. In addition, the majority of patients with AD have cerebral amyloid angiopathy (CAA), thus showing vascular deposition of Aβ [3] as a consequence of impaired transport of Aβ across the blood–brain barrier (BBB) [4,5]. Moreover, Aβ contributes to BBB leakage in humans with CAA and in transgenic mice [6]. A growing body of evidences indicate that neurovascular dysfunction occurs before the first symptoms of the disease and plays an important role in the neurodegenerative process and consequent cognitive decline in AD [4,7]. Recent findings in mice modeling AD demonstrated that microvascular impairment directly correlates with Aβ accumulation [8]. Indeed, results obtained in human and animal cultured cells and animal isolated vessels suggest that the age-dependent degeneration of brain vasculature and dysfunction of brain capillary endothelium is due to the toxic effects of Aβ peptide on endothelial cells (ECs) [9–12]. In human ECs, Aβ causes irreversible morphological and functional changes that result in inhibition of their proliferative activity through accumulation of autophagic vesicles, reduces survival and induces apoptosis [13–15]. However, the molecular mechanisms underlying Aβ-induced endothelial dysfunction have not been fully elucidated yet.

The endoplasmic reticulum (ER) is an organelle involved in folding and processing of proteins in the secretory pathway and in Ca^{2+} homeostasis. When the amount of proteins with abnormal conformation exceeds the repair capacity of the ER, the signaling pathways of the unfolded protein response (UPR), initiated by the ER stress sensors PERK (protein kinase RNA-like ER kinase), IRE1 (inositol-requiring protein-1) and ATF6 (activating transcription factor 6), are activated in order to re-establish ER homeostasis and avoid cell damage. The UPR mediators lead to the transcription of genes encoding ER-resident chaperones, such as GRP78, genes involved in ER and Golgi biogenesis, and also genes implicated in ER-associated degradation (ERAD), attenuating general translation to decrease the demand of ER and activate the ERAD to degrade the aberrant proteins [16,17]. During ERAD, an ubiquitin chain is added to misfolded proteins that are then retrotranslocated to the cytosol for degradation in the proteasome [18]. However, if UPR and ERAD fail, ER stress can also trigger the lysosome-mediated autophagic protein degradation pathway to preserve cell survival [19]. In the event of chronic or unmitigated ER stress, the UPR activates apoptotic cascades [20].

The aim of this work was to explore the hypothesis that Aβ damages microvascular brain ECs through the impairment of protein quality

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control mechanisms in the secretory pathway and in the cytosol leading to activation of apoptotic cell death pathways. Our results show that the $A\beta_{1-40}$ isoform, which accumulates in brain vasculature [21], activates ER stress, promotes the accumulation of ubiquitinated proteins and reduces the proteasome activity, inhibits macroautophagy and, in the absence of compensatory mechanisms, activates apoptosis in ECs derived from rat brain microvessels. This knowledge could contribute to the development of novel therapeutic strategies to prevent or delay the progression of AD.

2. Materials and methods

2.1. Materials

Collagen was obtained from Roche Applied Science (Mannheim, Germany). Mem-Alpha medium with Glutamax-1, Nut Mix F-10 W/GLUTAMAX-1, fetal bovine serum (FBS), and geneticin were acquired from Invitrogen Life Science (Paisley, UK). The synthetic $A\beta_{1-40}$ peptide and the Suc-Leu-Leu-Val-Tyr-AMC substrate were from Bachem (Bubendorf, Switzerland). Boc-Leu-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC substrates were acquired from Peptide Institute (Osaka, Japan). Hoechst 33342 and Alexa Fluor 594 goat anti-rabbit IgG conjugate were obtained from Molecular Probes (Leiden, The Netherlands). Glycergel Mounting Medium was obtained from DakoCytomation Inc. (Carpinteria, CA, USA). Colorimetric substrates for caspase-2, -3, -9, and -12 Ac-VDAVD-pNA, Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-LEVD-pNA were purchased from Calbiochem (Darmstadt, Germany). Polyvinylidene difluoride (PVDF) membrane, goat alkaline phosphatase-linked anti-rabbit and anti-mouse secondary antibodies, and the enhanced chemifluorescence (ECF) reagent were acquired from Amersham Pharmacia Biotech (Buckinghamshire, UK). Mouse monoclonal antibodies reactive against GRP78 and Beclin-1 were from BD Biosciences (Heidelberg, Germany). Rabbit polyclonal antibodies reactive against HDAC6 and XBP-1 (spliced and unspliced form) were from Abcam plc (Cambridge, UK). Rabbit monoclonal antibodies reactive against Bcl-2, LC3B-XP, Aβ and rabbit polyclonal antibodies reactive against LC3B and p62 were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International Inc. (Temecula, CA, USA). Mouse monoclonal anti-lysosomal-associated membrane protein (LAMP)-1 (clone H4A3) was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). Bio-Rad protein dye assay reagent, acrylamide and the pre-stained Precision Plus Protein All Blue Standard were purchased from Bio-Rad (Hercules, CA, USA). Anti-α-tubulin mouse monoclonal antibody, Trypsin-EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), protease inhibitors (leupeptin, pepstatin A, chymostatin, and aprotinin), phenylmethylsulfonyl fluoride (PMSF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), 1,4-dithiothreitol (DTT), ethylene glycol tetraacetic acid (EGTA), glycerol, bromophenol blue, NaH_2PO_4, ethylenediamine tetraacetic acid (EDTA), recombinant human basic fibroblast growth factor (bFGF), Tween 20, glucose, CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate), paraformaldehyde, phosphate-buffered saline (PBS), Coomassie G-250, thapsigargin, NH_4Cl, 3-methyladenine (3-MA), rapamycin and lactacystin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), NaCl, KCl, $MgCl_2$, $CaCl_2$, 2-propanol, HCl, Triton X-100, and methanol were purchased from Merck (Darmstadt, Germany).

2.2. Cell culture and treatments

The RBE4 cell line from rat brain microvasculature was provided by Dr. Jon Holy (University of Minnesota, Duluth, USA). It is a continuous, immortalized cell line that retains a stable phenotype reminiscent of BBB endothelium in vitro [22]. RBE4 cells at passages 10–35 were grown on 75 cm^2 tissue culture flasks coated with 4.15 μg/cm^2 collagen in MEM-Alpha medium with Glutamax-1 and Nut Mix F-10 W/GLUTAMAX-1 (1:1 vol/vol), supplemented with 10% heat inactivated FBS, 1 ng/ml bFGF and 0.3 mg/ml geneticin. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

The cells were plated on collagen-coated (4.15 μg/cm^2) multiwells for cell viability, western blotting (WB) analysis or measurement of proteasome, and caspase activities or coverslips coated with poly-L-lysine (0.1 mg/ml) plus collagen (4.15 μg/cm^2) for LC3B sub-cellular localization or analysis of necrotic/apoptotic cell death markers, at a density of approximately 22,000 cells/cm^2, and treated with 20 mM NH_4Cl, 10 mM 3-MA, 0.1 μM rapamycin, 2.5 μM $A\beta_{1-40}$, 2 μM thapsigargin, or 2 μM lactacystin.

Synthetic $A\beta_{1-40}$-HCl was dissolved in sterile water at a concentration of 6 mg/ml and then diluted to 1 mg/ml (231 μM) in PBS and stored at −20 °C. Thapsigargin (non-competitive inhibitor of ER Ca^{2+}-ATPase that depletes Ca^{2+} in the ER), rapamycin (macroautophagy inducer) and lactacystin (inhibitor of 20S proteasome) were diluted in DMSO and stored at −20 °C until use. 3-MA (macroautophagy inhibitor) was prepared in culture medium and NH_4Cl (inhibitor of lysosomal function by increasing the intra-lysosomal pH) was dissolved in sterile water both immediately before use.

2.3. Measurement of protein levels by western blotting analysis

After treatments, RBE4 cells were washed 2 times with PBS (pH 7.4), lysed and scrapped in ice cold lysis buffer (in mM): 25 HEPES-Na, 2 $MgCl_2$, 1 EDTA, 1 EGTA, supplemented with 0.1% Triton X-100, 100 μM PMSF, 2 mM DTT, and 1:1000 of a protease inhibitor cocktail (1 μg/ml leupeptin, pepstatin A, chymostatin, and antipain). The cellular extracts were then rapidly frozen in liquid N_2 and thawed three times and centrifuged for 1 min at 106 ×*g* at 4 °C. The supernatant was collected and the protein content was measured using the Bio-Rad protein dye assay reagent. Total cellular extracts containing 15 μg (for the ER stress markers GRP78 and XBP-1) or 50 μg (for LC3B, p62, Beclin-1, Bcl-2, LAMP-1, HDAC6 and ubiquitin) of protein were separated by electrophoresis on 15% (for LC3B) or 10% (for other proteins) (w/v) SDS-polyacrylamide gel (SDS-PAGE) after dilution (1:5) and denaturation at 95 °C for 5 min in sample buffer (in mM): 100 Tris, 100 DTT, 4% (v/v) SDS, 0.2% (w/v) bromophenol blue, and 20% (v/v) glycerol. For Aβ aggregation analysis, 40 μg of extracted cellular proteins or an 8.66 μg aliquot of the $A\beta_{1-40}$ stock was separated by electrophoresis on 4–16% Tris–Tricine SDS-PAGE [23] after dilution (1:2) in sample buffer: 40% (w/v) glycerol, 2% (w/v) SDS, 0.2 M Tris–HCl, pH 6.8, and 0.005% (w/v) Coomassie G-250. To facilitate the identification of proteins of interest, the pre-stained Precision Plus Protein All Blue Standards was used. Proteins were then transferred to PVDF membranes, which were further blocked for 1 h at room temperature (RT) with 5% (w/v) BSA in Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6) with 0.1% (w/v) Tween 20 (TBS-T). The membranes were next incubated overnight at 4 °C with primary mouse monoclonal antibodies against GRP78 (1:1000 dilution in TBS-T), Beclin-1 (1:1000 dilution in TBS-T), LAMP-1 (1:1000 dilution in TBS-T), with a primary rabbit monoclonal antibody against Bcl-2 (1:1000 dilution in TBS-T) or β-Amyloid (D54D2) XP® (1:200 dilution in TBS-T); or with primary rabbit polyclonal antibodies against LC3B (1:1000 dilution in TBS-T), p62 (1:1000 dilution in TBS-T), HDAC6 (1:1000 dilution in TBS-T), or ubiquitin (1:500 dilution in TBS-T). Control of protein loading was performed using primary mouse monoclonal antibodies against α-tubulin (1:20,000 dilution in TBS-T) or GAPDH (1:10,000 dilution in TBS-T). After washing, membranes were incubated for 1 h at RT with a goat alkaline phosphatase conjugated secondary anti-mouse or anti-rabbit antibodies (1:20,000 dilution in TBS-T). Bands of immunoreactive proteins were visualized after membrane incubation with ECF reagent for about 5 min, on a Versa Doc 3000 Imaging System (Bio-Rad, Hercules,