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Activation of the endoplasmic reticulum stress response by the amyloid-beta 1–40 peptide in brain endothelial cells

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ABSTRACT

Neurovascular dysfunction arising from endothelial cell damage is an early pathogenic event that contributes to the neurodegenerative process occurring in Alzheimer's disease (AD). Since the mechanisms underlying endothelial dysfunction are not fully elucidated, this study was aimed to explore the hypothesis that brain endothelial cell death is induced upon the sustained activation of the endoplasmic reticulum (ER) stress response by amyloid-beta (Aβ) peptide, which deposits in the cerebral vessels in many AD patients and transgenic mice. Incubation of rat brain endothelial cells (RBE4 cell line) with $A\beta_{1-40}$ increased the levels of several markers of ER stress-induced unfolded protein response (UPR), in a time-dependent manner, and affected the Ca^{2+} homeostasis due to the release of Ca^{2+} from this intracellular store. Finally, $A\beta_{1-40}$ was shown to activate both mitochondria-dependent and -independent apoptotic cell death pathways. Enhanced release of cytochrome c from mitochondria and activation of the downstream caspase-9 were observed in cells treated with $A\beta_{1-40}$ concomitantly with caspase-12 activation. Furthermore, $A\beta_{1-40}$ activated the apoptosis effectors' caspase-3 and promoted the translocation of apoptosis-inducing factor (AIF) to the nucleus demonstrating the involvement of caspase-dependent and -independent mechanisms during Aβ-induced endothelial cell death. In conclusion, our data demonstrate that ER stress plays a significant role in $A\beta_{1-40}$-induced apoptotic cell death in brain endothelial cells suggesting that ER stress-targeted therapeutic strategies might be useful in AD to counteract vascular defects and ultimately neurodegeneration.

1. Introduction

According to the 'Amyloid Cascade Hypothesis', the accumulation of amyloid-beta (Aβ) in brain parenchyma is responsible for the neurodegenerative process in Alzheimer's disease (AD) [1]. Furthermore, numerous studies support that neurovascular dysfunction contributes to neurodegeneration and cognitive decline and thus have a major role in AD pathogenesis (reviewed in [2]). First, vascular risk factors such as diabetes, obesity, hypercholesterolemia, hypertension, atherosclerosis, and stroke significantly increase the risk to develop AD [3,4]. Second, combined evidences from neuroimaging and neuropathological studies show that signs of vascular pathology develop early in AD and occur before the disease becomes symptomatic [5,6]. Third, deficient clearance of Aβ across the blood–brain barrier (BBB) has been described in the brain of AD patients [2]. Finally, Aβ deposition was found in the cerebral microvasculature of AD transgenic mice and in cerebrovessels in many AD patients and contributes to the age-dependent degeneration of cerebral vasculature and development of cerebral amyloid angiopathy, characterized by dysfunction of brain capillary endothelium [7,8]. This dysfunction correlates with the toxic effects of Aβ on endothelial cells

Abbreviations: AD, Alzheimer's disease; AIF, apoptosis-inducing factor; AM, acetoxymethyl ester; APP, amyloid precursor protein; ATF, activating transcription factor; Aβ, amyloid-beta; BBB, blood–brain barrier; bFGF, basic fibroblast growth factor; CHOP, CAAT/enhancer binding protein homologous protein; DMSO, dimethyl sulfoxide; ECF, enhanced chemifluorescence; ECs, endothelial cells; eIF2α, α subunit of eukaryotic translation initiation factor 2; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, glucose-regulated protein of 78 kDa; IRE1, inositol-requiring protein-1; JNK, c-jun N-terminal kinase; LDH, lactate dehydrogenase; LRP, low-density lipoprotein receptor-related protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD+, oxidized nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; PARP1, poly(ADP-ribose) polymerase 1; PERK, protein kinase RNA-like ER kinase; PI, propidium iodide; PS, presenilinM; PVDF, polyvinylidene difluoride; RAGE, receptor for advanced glycation end products; RBE4, rat brain endothelial cell line; RT, room temperature; Ry, ryanodine; RyR, ryanodine receptor; SDS-PAGE, SDS-polyacrylamide gel; SERCA, sarco/ER Ca^{2+} ATPase; TBP, TATA-binding protein; TBS-T, TBS-Tween; ThS, thioflavine S; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; UPR, unfolded protein response; WB, Western blot; XBP-1, X-box binding protein-1; $\Delta\psi$mit, mitochondrial membrane potential

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(ECs) and has been extensively described in cultured cells, in isolated vessels as well as in whole animals [9–12]. Aβ induces irreversible morphological and functional changes of ECs resulting in suppression of their proliferative activity and reduced survival [13,14]. In addition, Aβ-induced apoptosis was demonstrated in cultured cerebral ECs [9], but the underlying mechanisms have not been fully elucidated. It was reported that endoplasmic reticulum (ER) stress is a stimulus that initiates apoptotic cell death pathways in vascular ECs [15,16], but its role in Aβ-induced endothelial dysfunction has not been addressed yet.

The ER is the principal organelle responsible for proper folding/processing of nascent proteins and Ca^{2+} homeostasis. Perturbed ER function leads to a state known as ER stress. To ameliorate this stress, mammalian cells possess a homeostatic set of protein signaling pathways and transcription factors termed the unfolded protein response (UPR). The UPR utilizes three types of ER-resident sensor proteins, inositol-requiring protein-1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6), that are activated after the ER-resident chaperone glucose-regulated protein of 78 kDa (GRP78, also known by immunoglobulin binding protein, BiP) dissociates from their ER luminal domains and initiates ER-to-nucleus signaling cascades to maintain the homeostasis [17,18]. However, persistent and severe ER stress triggers apoptotic cascades resulting in cell death [19]. Upon dimerization and autophosphorylation, PERK inactivates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) by phosphorylation at Ser51, required for 80S ribosome assembly, thus inhibiting general protein translation through a decrease in the GTP-bound form of eIF2α, but increasing the translation of the transcription factor ATF4. In the nucleus, ATF4 induces the transcription of several genes involved in controlling the UPR and growth arrest and DNA damage-inducible protein-34 (GADD34). GADD34 induces a negative feedback and dephosphorylates eIF2α [20,21]. IRE1α removes a 26-base intron of mRNA of X-box binding protein-1 (XBP-1) inducing a more efficient translation and a more stable protein. The resulting XBP-1 protein with 54 kDa instead of 33 kDa comprises the original N-terminal DNA binding domain and a transactivation domain in the C-terminal and is a transcription factor that regulates genes involved in the ER-associated degradation pathway and increases the expression of several ER resident chaperones, such as GRP78 [22]. Upon dissociation from GRP78, ATF6 migrates to the Golgi apparatus where it is cleaved to release a transcription factor that upregulates genes involved in amplification of folding capacity such as chaperones including GRP78 and also genes for protein disulfide isomerases, CAAT/enhancer binding protein homologous protein (CHOP, also termed GADD153), XBP-1 [21,23], and genes involved in angiogenesis and autophagy [20]. During prolonged ER stress, apoptotic cell death is induced by the c-jun N-terminal kinase (JNK) pathway, by caspases, including the ER membrane-associated caspase-12 (in murine or the homologue caspase-4 in humans) or upregulation of the transcription of CHOP [19,24]. CHOP functions to block cells transition from G1 to S phase during cell cycle progression and can directly activate GADD34 increasing oxidation reactions at the ER [25]. Furthermore, CHOP down-regulates Bcl-2 and induces the translocation of Bax to mitochondria and subsequent cytochrome c release and activation of the apoptosis-effector caspase-3 [26]. Besides, ER and mitochondria are physically close and in contact and communicate through Ca^{2+} signals [27]. In normal conditions, Ca^{2+} released from ER is taken up by mitochondria and increases ATP production. Nevertheless, an overload in Ca^{2+} uptake by mitochondria upon ER Ca^{2+} release promoted by apoptotic stimuli or ER stress could induce the release of cytochrome c and other caspase cofactors leading to apoptotic cell death [27]. In neurons, the mitochondrial uptake of Ca^{2+} released form ER is essential to activate the mitochondrial apoptotic cell death pathway under $A\beta_{1-40}$-induced ER stress conditions [28].

Reports are available describing the engagement of ER stress in AD. In brain post-mortem samples from early AD patients, but not in non-demented subjects, ER stress markers have been detected in the temporal cortex and hippocampus [29]. Moreover, in transgenic mice modeling AD, increased brain levels of several ER stress markers have been described [30,31]. The presenilins (PSs, components of the γ-secretase complex present in the ER membrane) function as low-conductance, passive ER Ca^{2+} leak channels and, consequently, familial AD-linked PS mutations disturb ER Ca^{2+} homeostasis leading to increased susceptibility to activation of UPR and caspase-4-induced apoptosis [19,32,33]. Nonetheless, mutant PS1 reduces global ER function since it suppresses the activation of IRE1α, ATF6, and PERK and, as a result, GRP78/BiP is downregulated in PS1 mutant AD patients [19]. GRP78/BiP is able to bind the amyloid precursor protein (APP) inhibiting Aβ generation [34,35]. Therefore, mutant PS1 may increase the generation of Aβ by reducing the levels of GRP78/BiP available to bind APP. Moreover, aberrant splicing of PS2, almost exclusively observed in the brains of sporadic AD patients, increases the production of Aβ and the vulnerability to ER stress [36] and, may thus be implicated in the pathogenesis of this form of the disease.

Our previous *in vitro* results highlighted the role of ER stress in neuronal dysfunction triggered by Aβ [37,38] but the impact of ER stress in Aβ-induced endothelial dysfunction has not been investigated yet. Therefore, the aim of this work was to analyze the molecular basis of cerebrovascular alterations in AD exploring the hypothesis that $A\beta_{1-40}$, which preferentially accumulates in brain vasculature [8,39], damages microvascular brain ECs through induction of ER stress-mediated cell death pathways.

2. Experimental procedures

2.1. Materials

Indo-1 acetoxymethyl ester (Indo-1/AM), Alexa Fluor 488 goat anti-rabbit IgG conjugate, Fura-2 acetoxymethyl ester (Fura-2/AM), Tetramethylrhodamine methyl ester (TMRM), and Hoechst 33342 were obtained from Molecular Probes (Leiden, The Netherlands). The synthetic $A\beta_{1-40}$ peptide was from Bachem (Bubendorf, Switzerland). Ionomycin, ProteoExtract® Subcellular Proteome Extraction Kit, and colorimetric substrates for caspase-3, -9 and -12 (Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-LEVD-pNA, respectively) 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 antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International Inc. (Temecula, CA, USA). The Glycergel Mounting Medium was purchased from DakoCytomation Inc. (Carpinteria, CA, USA). Bio-Rad protein dye assay reagent, acrylamide, and the prestained Precision Plus Protein All Blue Standard were purchased from Bio-Rad (Hercules, CA, USA). The *In Situ* Cell Death Detection Kit, Fluorescein (with terminal deoxynucleotidyl transferase dUTP nick-end labeling, TUNEL), and collagen were obtained from Roche Applied Science (Mannheim, Germany). Trypsin EDTA solution, thioflavin S (ThS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), anti-α-tubulin mouse monoclonal antibody, protease inhibitors (leupeptin, pepstatin A, chymostatin, and aprotinin), thapsigargin, recombinant human basic fibroblast growth factor (bFGF), oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), dantrolene sodium, and mouse monoclonal antibody reactive against ATF4 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal antibodies reactive against XBP1 and mouse monoclonal antibody reactive against TATA-binding protein (TBP) were acquired from Abcam plc (Cambridge, UK). Mouse monoclonal antibodies reactive against GRP78 and cytochrome c, and rabbit polyclonal antibody reactive against caspase-12 were from BD Biosciences (Heidelberg, Germany). Mouse monoclonal antibodies reactive against CHOP/GADD153 or apoptosis-inducing factor (AIF), and rabbit polyclonal antibodies reactive against ATF6α or Tom20 were obtained