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Aβ-induced Ca^{2+} influx regulates astrocytic BACE1 expression via calcineurin/NFAT4 signals

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ABSTRACT

The β-site APP cleaving enzyme (BACE1) is required for the production of β-amyloid peptides, which give rise to β-amyloid (Aβ) deposits in the brains of Alzheimer's disease (AD) patients. In brains, BACE1 is primarily expressed by neurons, however BACE1 expression has also been observed in reactive astrocytes in close proximity to β-amyloid plaques in the brains of aged Tg2576 AD model mice. To date, the direct effects of Aβ on BACE1 gene expression in astrocytes is unknown. We found that Aβ42 or Aβ25–35 treatment induced BACE1 expression in primary astrocytes as well as human astrocytoma cell line. Aβ neurotoxicity has been associated with the disruption of intracellular calcium homeostasis both in neurons and in glial cells. Here, we demonstrated that NFAT4, a transcription factor tightly regulated by the calcium/calmodulin-dependent phosphatase, calcineurin, was activated in astrocytes applied with calcium ionophore or Aβ. Aβ-activated NFAT4 proteins were associated with astrocytic BACE1 gene expression via direct interaction with the BACE1 promoter region.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder of senile dementia in elderly people and is characterized by two major morpho-pathological hallmarks: deposition of extracellular amyloid plaques accompanied by intracellular neurofibrillary tangles (NFT) in the brain [1]. Amyloid-beta peptide (Aβ), the major component of plaques, is produced from amyloid precursor protein (APP) by proteolytic cleavage at its N- and C-termini by β- and γ-secretase, respectively [2]. β-Secretase has been identified by several groups and was named β-site APP cleaving enzyme 1 (BACE1), Asp2 or memapsin [3]. BACE1 mRNA shows the highest level of expression in the brain and is especially detected in neurons but not in glial cells [3,4]. However, recent studies have observed BACE1 immunoreactivity in reactive astrocytes around senile plaques in old Tg2576 mice [5,6]. It has been reported that various inflammatory cytokines and oxidative stress can induce astrocytic BACE1 expression [7,8] and studies by Cho et al. have shown that IFN-γ, pro-inflammatory cytokine, generates BACE1 expression and activity in astrocytes [9].

Since BACE1 levels were observed in astrocytes surrounding Aβ plaques, we hypothesized that Aβ42, the critical component of plaques may induce BACE1 gene expression in astrocytes. Aβ42 neurotoxicity has been associated with the destabilization of intracellular calcium homeostasis both in neurons and in glial cells, although the mechanisms of Aβ-induced disruption of calcium homeostasis remain unclear. Disrupted calcium homeostasis has been observed in the brains of AD patients. The levels of intracellular calcium and calcium-related enzyme activity were found to be elevated in the brains from AD patients [10]. Furthermore, our previous study showed that neuronal BACE1 expression was up-regulated by disrupted intracellular calcium homeostasis that was mediated by nuclear factor of activated T-cells 1 (NFAT1) [11]. In this study, we found that Aβ42 treatment stimulated astrocytic BACE1 gene expression at transcription level via activation of calcineurin (CaN)/NFAT4 signaling cascade. Furthermore, we found that Aβ-induced BACE1 expression occurs via a calcium permeable channel formed by membrane-inserted Aβ in astrocytes. Treatment with Zn^{2+} or clioquinol, which has been known to inhibit channels formed by Aβ, blocked Aβ-induced astrocytic BACE1 gene expression.

Our findings suggest that stimulation with Aβ42 potentiates astrocytic BACE1 gene expression via disturbance of intracellular calcium homeostasis that is mediated by Aβ-induced activation of CaN/NFAT4 signaling pathway in astrocytes.

2. Materials and methods

2.1. Cell cultures

U373MG cells were maintained in Dulbecco's modified Eagle medium (DMEM; 4.5 g/ml glucose with pyruvate; HyClone) with

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10% fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin. Cortical astrocyte cultures were established from 2-day-old pups. Briefly, mouse brains were removed and the meningeal tissue was stripped off. Brains were dissociated mechanically and seeded in poly-D-lysin (Sigma)-coated dishes into medium consisting of DMEM with 10% FBS and then incubated at 37 °C in a humidified 5% CO_2 air atmosphere. After 2 weeks in culture, media were removed and the cells were trypsinized and replated at low density in 12-well plates for reagent treatment [5].

2.2. Peptides and treatment

Cells were treated with calcium ionophores, ionomycin or A23187 (Sigma) in DMEM supplemented with 1% FBS. Synthetic Aβ25–35 (U.S. Peptide, Inc.) and Aβ42 (Bachem) were dissolved in dimethylsulfoxide (DMSO; Amresco) and diluted to final concentration of 1 mM with water [11]. Prior to treatment with calcium ionophore or Aβ peptides, cells were treated with the following reagents as indicated in figures, cyclosporine A (CsA), BAPTA-AM, 2-amino-ethoxy diphenylborate (2-APB), dantrolene, nifedipine, EGTA, clioquinol, $ZnCl_2$ (all from Sigma) and U73122 (Calbiochem).

2.3. BACE1 promoter assay and cDNA construct

For analysis of BACE1 promoter activity, the following two constructs were used; uBACE-1Ka (−1 ∼ −994 bp) and uBACE-2K (+50 ∼ −2100 bp) of human BACE1 gene, as previously reported [9,11]. Numbering system followed previous report [12] (GenBank accession number AY542689). U373MG cells were transfected with the BACE1 promoter constructs using Lipofectamine reagent and Plus reagent (Invitrogen) [9]. For detection of the promoter activity, cell lysates were prepared in Passive Lysis Buffer (Promega) and luciferase assays were performed according to the manufacturer's instructions for Dual Luciferase Assay System (Promega). The signals were measured using auto microplate reader (Infinite M200, Tecan). Transfection efficiency was normalized as described previously [11]. For NFAT4 overexpression, cells were transiently transfected with cDNA constructs encoding NFAT4 (provided from Dr. HD Youn in Seoul National University, Seoul, Korea). DNA amounts were normalized with mock vector.

2.4. Western blot analysis

Cells were lyzed using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS and 50 mM Tris) supplemented with protease inhibitor cocktail and divalent cation chelators (EGTA 1 mM and EDTA 1 mM). Protein extracts were quantified using the bicinchoninic acid (BCA) protein assay solution (Amersham Pharmacia) and equally loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels [13]. For detection of BACE1 levels, anti-BACE1 polyclonal antibody (Calbiochem) or monoclonal antibodies against the C-terminus of BACE1 (Chemicon) were used. Anti-actin antibody (Sigma), anti-NFAT1 (Affinity BioReagents), anti-STAT1 (Upstate Biotechnology) and anti-NFAT4 monoclonal antibody (Santa Cruz Biotechnologies) were used.

2.5. Electrophoresis mobility shift assay (EMSA)

For binding reactions with nuclear extracts from U373MG cells, biotin-labeled probe against the NFAT-binding sequences (TGGAAAAAC) in the human BACE1 promoter region were generated [11]. The BACE1-NFAT probe (5′-biotin-TGCAGCCTG-GAAAAACTCTTC-3′). Nuclear extracts (5 μg) from U373MG cells treated with calcium ionophore were pre-incubated with poly (dI-dC) (1 μg; Sigma) and then reacted with biotin-labeled BACE1-NFAT probes for 30 min at room temperature. To confirm the specificity of interactions, competition assays using non-labeled cold probes were performed. A 100-fold molar excess of cold probes were pre-incubated with nuclear extracts. To detect the novel interaction between NFAT probes and NFAT4 proteins, anti-NFAT4 antibody (2 μg) was pre-added to the extracts for 30 min. Incubated mixtures were analyzed by 5% nondenaturing PAGE as previously described [9,11]. The signals were observed using a Light Super Shifted Module Kit (Pierce) in Bio-Imaging Analyzer (LAS-3000; Fuji).

2.6. Detection of sAPPβ levels

To identify BACE1 enzymatic function, the levels of sAPPβ in conditioned media (CM) were analyzed. For protein precipitation in CM, an equal volume of 20% trichloroacetic acid (Sigma) was subjected to CM and incubated for 24 h in 4 °C and then centrifuged at 10,000*g* for 5 min. The supernatant was removed and pellets were washed with ice-cold acetone (Sigma). Dried pellets were resuspended in RIPA buffer and the protein concentration was estimated using BCA solution (Pierce). The precipitated proteins were analyzed by 8% SDS–PAGE. To recognize the sAPPβ proteins, anti-sAPPβ polyclonal antibody (Signet Laboratories) was used.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software. Differences between groups were examined for statistical analysis using Tukey–Kramer Multiple Comparisons Tests.

3. Results

3.1. Aβ treatment induces BACE1 expression via calcium-related signaling in astrocytes

To test whether Aβ treatment regulates BACE1 promoter activity in astrocytes, the luciferase reporter assay for measuring BACE1 promoter activity was performed. We utilized the pGL3-Basic vector containing the promoter region of human BACE1 gene [9,11] and the human astrocytoma cell line U373MG. After 24 h, the BACE1 promoter was activated in U373MG cells that were stimulated with Aβ1–42 or Aβ25–35, a region of the Aβ peptide that is critically involved in neurotoxicity and aggregation [14] (Fig. 1A). BACE1 promoter activity was elevated by Aβ25–35 treatment in a dose-dependent manner in the concentration range tested (Fig. 1B). To investigate whether Aβ-induced BACE1 promoter activation is mediated by Aβ-stimulated increases in intracellular calcium levels, U373MG cells were pre-treated with BAPTA-AM to chelate intracellular calcium since Aβ has been shown to elevate intracellular calcium concentration in astrocytes [15]. As a result, pre-treatment with BAPTA-AM completely blocked Aβ-induced BACE1 promoter activity (Fig. 1C). We have previously shown that the CaN/NFAT1 signaling pathway, regulated by intracellular calcium levels, modulates BACE1 gene expression in neurons [11]. This led us to pre-treat cells with cyclosporine A (CsA), a calcineurin inhibitor. This treatment resulted in a blockage of Aβ-elicited BACE1 promoter activity (Fig. 1D). Next, to confirm whether Aβ treatment affects BACE1 protein levels in astrocytes, protein extracts from mouse primary astrocytes were analyzed using Western blot analysis. As expected, BACE1 protein expression was not detected in the absence of stimulation, however treatment with Aβ42 or Aβ25–35 potentiated BACE1 protein expression (arrow head, Fig. 1E) and CsA pre-treatment led to down-regulation of Aβ25–35-triggered BACE1 protein expression in astrocytes