Inhibition of Aβ production and APP maturation by a specific PKA inhibitor

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Received 26 May 2003; accepted 30 May 2003

First published online 13 June 2003

Edited by Jesus Avila

Abstract Alzheimer's disease is characterized pathologically by extracellular amyloid β protein (Aβ) deposition in the brain. The Aβ peptide, a 39–42 amino acid fragment, is derived from defined proteolysis of the amyloid precursor protein (APP) [Glenner et al., Appl. Pathol. 2 (1984) 357–369; Selkoe, Neuron 6 (1991) 487–498] and is the primary component of senile plaques. Although it is known that intracellular APP is subjected to posttranslational modification, the molecular mechanism that regulates the APP processing is not completely clear. In the present study, we demonstrates that H89, a specific inhibitor for cAMP dependent protein kinase A (PKA), inhibits Aβ production and APP secretion in a dose dependent manner in cells stably transfected with human APP bearing a 'Swedish mutation'. Concurrent with the effect, H89 inhibits C-terminal fragment of the APP. We also found that the PKA inhibitor abolishes the mature form of intracellular APP and accumulates the immature form. Finally, direct administration of H89 into brains of transgenic mice overexpressing human APP shows that the compound inhibits Aβ production in the hippocampal region. Our data suggests that PKA plays an important role in the maturation of APP associated with APP processing.

Key words: Amyloid β protein; Protein kinase A; Amyloid precursor protein; Protein kinase A inhibitor

1. Introduction

The hallmarks of Alzheimer's disease (AD) neuropathology are senile plaques and neurofibrillary tangles. Plaques consist primarily of β-amyloid (Aβ), a 39–42 amino acid fragment derived from defined proteolysis of amyloid precursor protein (APP) [1–4], while the neurofibrillary tangles are composed largely of hyperphosphorylated tau [5,6]. APP is a transmembrane glycoprotein and is thought to be cleaved by secretases (α, β and γ) in the secretory pathway generating Aβ peptide and secreted APP. Several lines of evidence have suggested that posttranslational modification of APP plays an important role in intracellular sorting, secretion, axon transport as well as processing of APP protein. Western blot analysis of APP from the human cerebrospinal fluid treated with *N*-glycanase and *O*-glycanase showed that APP is both *N*- and *O*-glycosylated [7,8]. The immature form of APP (imAPP) is predominantly *N*-glycosylated and localized in the endoplasmic reticulum/*cis*-Golgi. The mature form (mAPP) is an *N*,*O*-glycosylated species concentrated in the *trans*-Golgi/plasma membrane [9]. Treatment of transfected hippocampal neurons with the *N*-glycosylation inhibitor tunicamycin induces missorting of wild-type APP, suggesting the importance of glycosylation in axonal sorting of APP [10]. In addition, studies with site-directed mutagenesis at two potential *N*-linked glycosylation sites of APP also demonstrated that expression of the mutant APP led to aberrant APP processing and suggested that the glycosylation may modulate the intracellular sorting of APP in COS-1 cells [11]. Moreover, studies with mutated *N*- and *O*-glycosylation sites in CHO cells showed that APP secretion was diminished when core *N*-glycosylation or *N*-glycan processing was blocked [8]. Cells expressing the defective *O*-glycosylation of APP noticeably decreased the generation of the intracellular APP carboxy-terminal fragment (CTF), a product of α-secretase, and both $A\beta_{40}$ and $A\beta_{42}$ in medium, β- and γ-secretase products. Consequently, APP was accumulated in intracellular reticular compartments such as the endoplasmic reticulum. These results demonstrated that the majority of APP cleavage by α-, β-, and γ-secretases occurs after *O*-glycosylation during APP transport through the Golgi complex or in compartments subsequent to *trans*-Golgi of the APP secretory pathway [12].

The cAMP dependent protein kinase (PKA) plays a key role in many signal transduction processes, mediating the majority of the known effects of cAMP in eukaryotic cells. PKA has been implicated in the AD pathology of abnormal tau phosphorylation leading to neurofibrillary tangle formation. A recent study by Jicha et al. [13] demonstrated that PKA was tightly associated with the neurofibrillary pathology, positioning PKA to participate directly in the pathological hyperphosphorylation of tau on Ser214 and Ser409 seen in brains with AD. PKA phosphorylations on tau precede or are coincident with the initial appearance of filamentous aggregates of tau. However, the role of PKA on APP biology such as APP secretion and processing is not clear. In this report, we attempt to elucidate the molecular mechanism underlying APP secretion and processing using the selective PKA inhibitor H89 and demonstrate that PKA activity is required for APP maturation (glycosylation), secretion as well as APP processing.

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00645-8

2. Materials and methods

2.1. Cell lines and treatments

All cell lines were cultured at 37°C and 5% CO_2. Swedish (sw) APP_{751} and wild type (wt) APP_{751} stable HEK293 cells clones were cultured in Dulbecco's modified Eagle's medium (DMEM-F12 3:1) (Life Technologies, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum, 20 mM HEPES, 50 μg/ml tobramycin, and 300 μg/ml G418. Parental 293 cells were grown in identical medium without the addition of G418. Each cell line was plated to 50–80% confluence 1 day prior to analysis. The wtAPP_{751} and swAPP_{751} stable HEK293 cells were treated with the PKA inhibitors H89 dihydrochloride and myristoylated protein kinase A inhibitor (PKI, 14–22 amide) (Calbiochem, San Diego, CA, USA) in six well culture dishes at 0–20 μM for 7 h prior to harvesting. At appropriate time, supernatants (medium) were removed and diluted for Aβ enzyme-linked immunosorbent assay (ELISA) and cells were harvested, using trypsin–EDTA, washed once in phosphate buffered saline (PBS) and then Dounce homogenized (Kontes Dounce) in lysis buffer A (10 mM K_2HPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 2 mM dithiothreitol, 1 μM microcystin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, pH 7.2) and incubated on ice for 30 min. The cytosolic protein fraction was clarified by microcentrifuging at 14 000 rpm for 30 min at 4°C. Total protein concentration of the supernatants was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Confluent HEK293 cells were fed with methionine-free DMEM for 1 h followed by a medium change with methionine-free DMEM ^{35}S for 2 h. After incubation, cell medium was collected for Aβ/swAPP analysis. Adherent cells were rinsed three times with PBS followed by cell lysis. Aβ was then immunoprecipitated from cell medium using monoclonal antibodies 4G8 and 6E10 (Senetek, Maryland Heights, MO, USA) against Aβ, and APP from cell lysis using a rabbit antibody (BX6) against APP overnight at 4°C. The following day, protein G Sepharose (Amersham Pharmacia, Piscataway, NJ, USA) was added and immune complexes collected at 4°C rotating for 1–3 h. Antigen–antibody–protein G pellets were washed five times with STEN (STEN wash buffer: 50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 0.2% NP-40), resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, air dried, and exposed to film to assess ^{35}S labeling.

2.2. MTT assays

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) reagent (Sigma, St. Louis, MO, USA) was used in a colorimetric assay to determine cell viability and assess cytotoxicity. Assays were performed by adding 10 mg/ml MTT directly to culture wells, incubating for 1–4 h and then following removal of medium, adding 1 ml 0.05 N HCl and incubating for 10–20 min at room temperature. After incubation, absorbance was read at 596 nm using a Bio-Rad model 3550 96 well microplate reader. The quantity of formazan is directly proportional to the number of living cells in culture.

2.3. Aβ ELISA

Determination of total Aβ and $Aβ_{1–42}$ was quantified by a sandwich ELISA described previously [14]. Briefly, cell supernatants were diluted in specimen buffer (0.6% bovine serum albumin, 8 mM $Na_2HPO_4·7H_2O$, 1.5 mM $NaH_2PO_4·H_2O$, 145 mM NaCl, 0.05% thimerosal, 0.05% Triton X-405). Samples were loaded onto Immunlon-4 plates, along with a set of $Aβ_{1–42}$ standards diluted in specimen buffer (0–1000 pg/ml) (Bachem, Torrance, CA, USA), and incubated overnight at 4°C. Plates were then washed with PBS/0.05% Tween 20, pH 7.4, followed by a 1 h incubation at room temperature with 3D6 biotinylated reporter antibody specific for $Aβ_{1–5}$ (diluted 1:2000 in 0.25% casein buffer). Plates were again washed, followed by a 1 h room temperature incubation with streptavidin–horseradish peroxidase conjugate (Amersham Life Sciences, Arlington Heights, IL, USA) diluted 1:1000 in 0.25% casein buffer. Following a final series of washes, TMB substrate (Pierce) was added for 15 min, after which time the enzymatic reaction was stopped with 2 N H_2SO_4. Reaction products were quantified by measuring the absorbance difference at 450 nm and 650 nm.

2.4. PKA kinase assays

PKA kinase activity was assessed using the Upstate Biotechnology (UBI, Lake Placid, NY, USA) PKA Kinase Assay kit. Either 10 μl of whole cell lysate or 10 μl of bovine heart purified PKA catalytic subunit (UBI) representing 34.48 ng of enzyme was used in kinase assays. In assays using purified enzyme, PKA inhibitors were included at concentrations specified above. PKA activity was measured in a total reaction volume of 60 μl containing 83.3 μM kemptide substrate (LRRASLG), 1–2 μM cAMP, 0.3 μM PKC inhibitor peptide, 3.3 μM compound R24571, and 5–10 μCi [$γ$-^{32}P]ATP in 1×assay dilution buffer (UBI). PKA assays were incubated at 30°C for 10 min. Reactions were stopped by spotting onto p81 phosphocellulose filter paper, washed three times in 0.75% H_3PO_4, and rinsed in acetone prior to scintillation counting. Phosphate incorporation in kemptide substrate peptide was used as a readout of kinase activity. For whole cell lysate assays, sample cpm were normalized based on total protein concentration.

2.5. Intracerebral ventricle injection of PKA inhibitor, H89

PDAPP ($APP_{717V–F}$) transgenic mice were allowed to acclimate in a clear 4×4×4 inch observation cage with free access to food pellets. Eight week old animals ($n = 10$) were injected i.c.v. according to a method developed by Laursen and Belknap [15]. Briefly, unanesthetized animals were gently restrained and injected with H89 103 μM. Injections were made using a 50 μl Hamilton syringe fitted with a 26 gauge needle including a small piece of PE20 tubing slipped over the needle allowing a 3.7 mm projection. Holding the needle at a 45° angle, the bregma was located by feeling, the needle moved laterally about 2 mm and then pushed into the ventricle. Five microliter of inhibitor solution was rapidly injected and the animals were returned to their cage for a 3 h incubation. Three hours post inhibitor injection, mice were CO_2 anesthetized, decapitated and specific brain regions were isolated following dissection. Hippocampus and cortex tissue was immediately homogenized in a guanidine HCl solution and assessed using the Aβ sandwich ELISA.

3. Results and discussion

H89 and PKI are both well-known selective inhibitors of cAMP dependent protein kinase. H89 is an isoquinolinesulfonamide that acts as a competitive inhibitor against ATP for binding to the catalytic subunit of PKA [16]. PKI is a heat-stable protein kinase inhibitor peptide that, like the R subunits, mimics the protein substrate providing a pseudophosphorylation site and functions as a competitive inhibitor by binding to the catalytic site of PKA [17,18]. We sought to determine if inhibition of the PKA signal pathway would affect APP processing. In this study, we used well-characterized HEK293 cells stably transfected with wtAPP_{751} or swAPP_{751} [19], producing elevated Aβ and secreted APP in the medium. Our data demonstrate that both PKI and H89 treatment lower total Aβ and $Aβ_{1–42}$ production in the swAPP cell medium as determined by Aβ sandwich ELISA analysis (Fig. 1A,B). Further, we demonstrated that treatment of swAPP cells with either inhibitor at the highest concentration (20 μM) does not cause cytotoxicity to the swAPP cells based on MTT viability analysis (data not shown), suggesting that an effect on PKA and not reduced cell viability is the cause of the observed reduction in Aβ formation. The fact that PKI delivers less potency could be due to its poorer cell permeability. In addition to the dose dependent inhibition of Aβ in the HEK293 swAPP_{751} stable cell line, H89 also demonstrated a strong inhibition of PKA activity in both swAPP and wtAPP cell lines following H89 addition to the culture medium (data not shown). This inhibitory effect was produced at inhibitor concentrations reported in the literature to be selective for PKA inhibition. Our data further imply

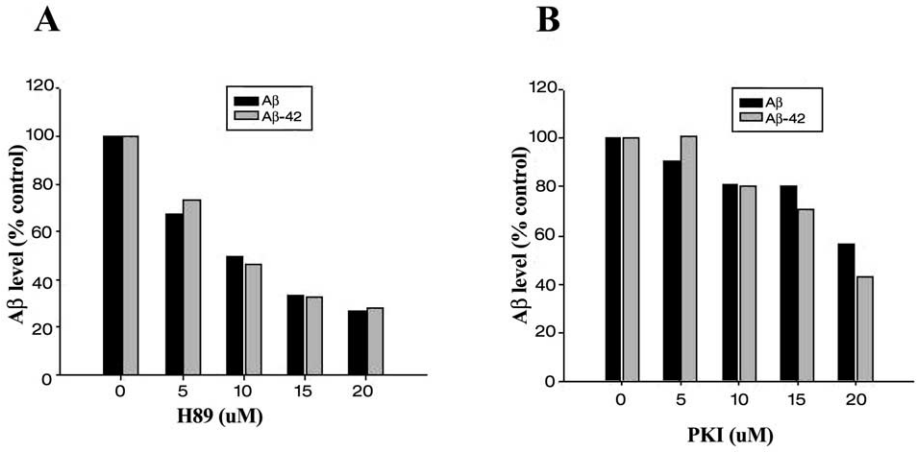

Fig. 1. H89 (A) or PKI (B) treatment lowers total Aβ and $Aβ_{1–42}$ production in swAPP cells. H89 (20 μM) or PKI (20 μM) does not cause cytotoxicity to the swAPP cells (data not shown).

that PKA enzyme activity plays an important role in the APP processing.

To confirm whether the H89 inhibitor was indeed having an effect on APP processing, sAPP and Aβ were specifically immunoprecipitated from harvested $swAPP_{751}$ cell medium following [^{35}S]methionine labeling (Fig. 2). The Aβ was specifically detected in the supernatant by two different monoclonal antibodies (6E10/4G8), both recognizing sAPP, Aβ and P_3. In addition, a rabbit polyclonal antibody (BX6) was used for detection of APP and CTF in cell lysate. The amount of Aβ in cell supernatant was determined by immunoprecipitation. Complementing our already described data, a reduced level of Aβ formation was associated with a decline in APP to almost zero as H89 concentration was increased (Fig. 2A,B). Further, in the cell lysate immunoprecipitations, decreased CTF levels were observed also in response to increasing concentrations of H89 (Fig. 2C).

APP is subject to glycosylation and phosphorylation following protein synthesis, and is then thought to be cleaved in an intracellular secretory pathway after or during these posttranslational modifications [20–22]. One of the posttranslational modifications of APP is *N*-, *O*-glycosylation or both. Following the modifications, cellular APP exists in two isoforms, the immature isoform (imAPP), which is normally associated with *N*-glycosylation, and the mature isoform (mAPP), which is *N*- and *O*-glycosylated. We therefore wanted to determine if the posttranslational modifications of APP play an important role in H89 mediated inhibition of Aβ production. Following treatment of $swAPP_{751}$ cells with H89 at increasing doses, the secretion of sAPP is significantly reduced (Fig. 3A) and the mature form of APP is notably abolished (Fig. 3B). However, the immature form of APP level increases, suggesting a possible defect in *O*-glycosylation but intact *N*-glycosylation. Additional studies to specifically distinguish *O*- from *N*-glycosylation inhibition by PKA inhibitor are under way.

PDAPP ($APP_{717V–F}$) transgenic mice over-expressing FAD $APP_{717V–F}$ mutant show elevated Aβ levels in the brain in an age dependent manner and progressively develop some of the pathological hallmarks of AD including numerous extracellular thioflavin S-positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis and microgliosis [14,23]. In order to assess in vivo effects of PKA inhibitors on Aβ deposition, we directly administered H89 through the lateral ventricle adjacent to the bregma of brains of PDAPP ($APP_{717V–F}$) transgenic mice. Following injection in H89, we were able to show that the PKA inhibitor resulted in a reduction of Aβ deposition in the hippocampus (Fig. 4), a prominent brain region affected by AD pathology, suggesting an in vivo role for PKA in APP metabolism.

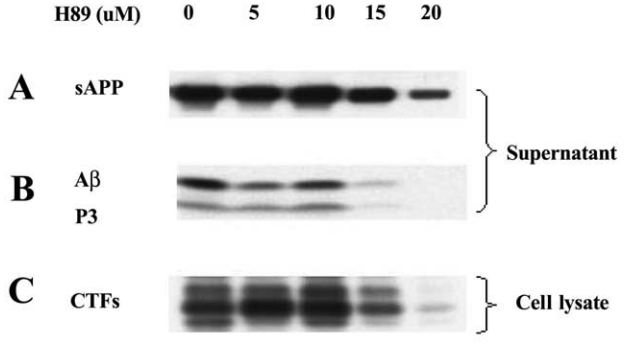

Fig. 2. H89 inhibits sAPP (A), Aβ production (B) and CTF formation (C) in the $swAPP_{751}$ stable cell line as determined by immunoprecipitation analysis. The Aβ could be specifically detected in supernatant by two different monoclonal antibodies (6E10/4G8). Antibody BX6 recognizes the APP and CTF in cell lysate. The levels of the secreted APP (sAPP), Aβ peptides and CTFs are notably reduced following H89 treatment.

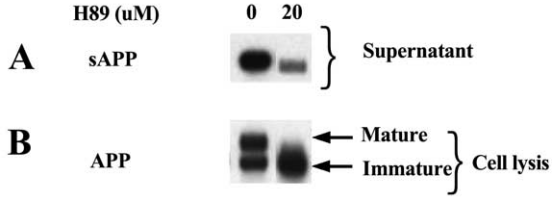

Fig. 3. A: sAPP was immuno-precipitated from supernatants and the sAPP levels were inhibited by the treatment of H89. B: The mature isoform of APP was completely abolished and the immature APP isoform level was increased by H89. The mature isoform is *N*- and *O*-glycosylated (sAPP), whereas the immature isoform is normally associated with *N*-glycosylation. Our data suggest that H89 conceivably blocks the maturation of APP by inhibiting the *O*-glycosylation during APP processing.

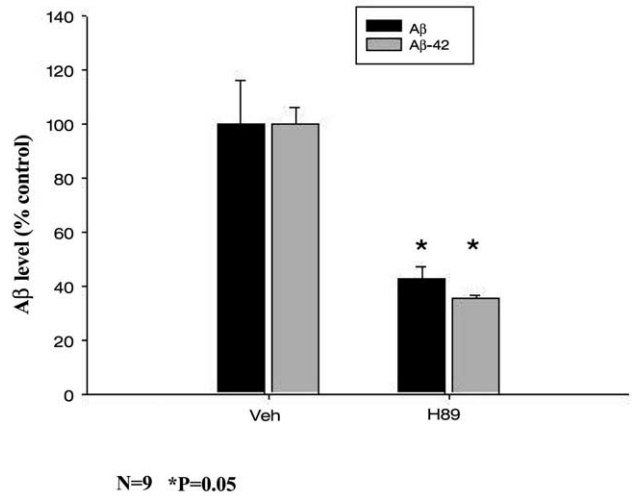

Fig. 4. H89 inhibits Aβ production in brains of transgenic mice overexpressing the APP gene bearing the Indiana mutation (APP_{717V-F}). Following direct injection of H89 into the brains of APP_{717V-F} mice, the Aβ level of hippocampus was measured by ELISA analysis and was shown to be inhibited by H89. (Veh, DMSO vehicle).

H89, a specific inhibitor of PKA, potently inhibited Aβ production in $swAPP_{751}$ HEK293 cell and brains of transgenic mice overexpressing APP_{717V-F} and the secretion of APP. Inhibition of PKA blocks the maturation of APP, possibly via blocking the *O*-glycosylation of APP, as evidenced by abrogation of the mature form and accumulation of the immature form of APP by H89. It is conceivable that imAPP, unlike mAPP, fails to be transported to the proper compartments (e.g. in the *trans*-Golgi network) in the secretory pathway for processing or to be recognized by secretases. Our hypothesis is consistent with the observation that APP processing by α-, β- and γ-secretases occurs after *O*-glycosylation [12].

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