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Effects of amyloid-β peptide on glutamine transporter mRNA expression and cell viability in cultured rat cortical cells

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ABSTRACT: Alzheimer's disease is a major neurodegenerative disorder in which there is an overproduction and accumulation of amyloid-β (Aβ) peptides. During the initial stages of the disease, glutamate receptors are dysregulated by Aβ accumulation resulting in the disruption of glutamatergic synaptic transmission. We used rat cortical cell cultures to examine the effects of Aβ(25–35)-induced neurotoxicity on glutamine transporters involved in the glutamate cycle. In primary mixed cell cultures prepared from cerebral cortex, incubation with 10 μM Aβ(25–35) for 12 h, but not for 24 h, markedly suppressed system A transporter 1 (SAT1) mRNA expression. On the other hand, Aβ(25–35) had no effect on SAT1 mRNA level in neuronal cell cultures. Treatment of both types of cell cultures with Aβ(25–35) resulted in a significant decrease in cell survival in a concentration and time-dependent manner, as determined by MTT assay. These results indicated that Aβ may impair neuronal function and transmitter synthesis and perhaps reduce excitotoxicity through a reduction in neuronal glutamine uptake.

KEYWORDS: Alzheimer's disease, amyloid-β peptide, mRNA glutamine transporter

INTRODUCTION

Alzheimer's disease is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles, neuritic plaques, synaptic depletion, and neuronal cell loss in specific cortical and subcortical areas of the brain[1]. Senile plaques contain amyloid-β (Aβ) peptide, produced by cleavage of the amyloid precursor protein (APP) by β- and γ-secretases[2]. Aβ is a peptide of 39–43 amino acids that forms insoluble aggregates surrounded by degenerating neuritis and activated glial cells[3]. Numerous experiments with synthetic Aβ(1–42) suggest that this peptide is toxic to cultured neurons by inducing protein oxidation, lipid peroxidation, and oxidative stress in the cellular environment in ways that are inhibited by antioxidants of free-radicals[4]. In culture, Aβ can directly induce neuronal cell death[5] and can render neurons vulnerable to excitotoxicity[6] and oxidative insults[7].

Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system that contributes not only to fast synaptic neurotransmission but also to complex physiological processes, such as memory, learning, plasticity, and neuronal cell death[8]. The neurons use glutamine as the main substrate for synthesis of glutamate and γ-aminobutyric acid[9,10]. The release of glutamine from astrocytes and the uptake of glutamine by neurons are integral steps in the glutamate-glutamine cycle, a major pathway for the replenishment of neuronal glutamate[11].

Recently, cDNA encoding three distinct system A amino acid transporters (SAT1 to SAT3) have been cloned and functionally identified[12]. SAT1 isoform is preferentially expressed on the plasma membrane of glutamatergic neurons and encodes for a highly efficient glutamine transporter[13,14]. Dolinska et al[15] reported that SAT1 mRNA is transcribed in neurons but not in cerebral cortical astrocytes or cerebellar astrocytes. A malfunction in the glutamate transport system can lead to accumulation of excessive glutamate in the synapse, which is harmful to neurons and could result in neurodegeneration[16,17]. However, the causal relationship between Aβ and glutamine transporters in Alzheimer's disease is not yet well

defined.

A small 11-amino acid fragment of the full-length peptide, Aβ(25–35), is a convenient alternative in Alzheimer's disease investigations as the peptide mimics several toxicological and oxidative stress properties of the native full-length peptide[4,18,19]. Recently, our group demonstrated that treatment of mixed cell cultures with 10 μM Aβ(25–35) for 12 h and 24 h reduces SAT1 protein level as demonstrated by immunocytochemical and quantitative immunoreactivity analysis[20]. We now report the effects of Aβ(25–35) on SAT1 expression in rat cortical primary cell cultures.

MATERIALS AND METHODS

Enriched primary neuronal cell cultures were prepared from cerebral cortices of 10 embryonic day-18 (E18) Wistar rat foetuses as described previously[21,22]. Cells plated at 5×10^5 cells/ml were cultured in poly-L-lysine coated multiwell plates and maintained in serum-free Dulbecco's modified Eagles medium (DMEM) supplemented with B27 under a humidified atmosphere of 5% CO_2/95% air at 37 °C for 5 days before incubating with peptide.

Primary mixed cell cultures were prepared from cerebral cortices of 10 1-day old Wistar rat neonates as previously described[23]. Cells plated at 5×10^5 cells/ml were cultured in poly-L-lysine coated multiwell plates. The dissociated cortical cells were suspended in Neurobasal-A medium containing 2% (v/v) B27 and 1% (v/v) GlutaMAX I and maintained as described for the E18 cells.

The B27-containing medium was removed from neuronal and mixed cell cultures at day 6. Cells were then washed twice with DMEM or Neurobasal-A medium and then incubated for 12 h and 24 h in the absence or presence of 3 and 10 μM Aβ(25–35) (Sigma) to produce a sub-lethal neurotoxicity.

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and the yield was determined by spectrophotometry at 260 nm. The Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using the Qiagen one step RT-PCR kit. PCR amplifications of SAT1 employed primers 5′-ACAGGCGACATTCTCATCCT-3′ (forward) and 5′-GTTTCAGTGGCCTTCACCAT-3′ (reverse), giving rise to a 426 base pair amplicon[24], and β-actin (housekeeping gene) primers 5′-CCCAGAGCAAGAGAGGCATC-3′ (forward) and 5′-CTCAGGAGGAGCAATGATCT-3′ (reverse), giving rise to a 830 base pair amplicon[25]. Primers were obtained from the Bioservice Unit of the National Science and Technology Development Agency, Thailand. Thermocycling conditions, optimized in order to allow amplicon synthesis within the linear-log phase of amplification, were as follows: reverse-transcription at 50 °C for 30 min; inactivation of reverse transcriptase and activation of Hot start *Taq* DNA polymerase at 95 °C for 15 min; 30 cycles of 30 sec at 94 °C, 1 min at 55 °C (for STAT1) or 1 min at 72 °C (for β-actin); and a final extension step at 72 °C for 10 min. Amplicons were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide. Densitometric analysis was performed using QUANTITY ONE 4.5.1 (Bio-Rad).

This method is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt to a crystalline blue formazan product by cellular oxidoreductase[26]. Therefore, the amount of formazan produced is proportional to the number of viable cells. The culture medium was removed from the treated cells and replaced with a solution of MTT (0.5 mg/ml) in phosphate-buffered saline, pH 7.4. After incubation for 4 h at 37 °C, the solution was removed and the resulting blue formazan solubilized in 0.1 ml of 0.04 M HCl in isopropanol. Absorbance at 570 nm was measured at 630 nm using a microplate reader (Bio-Tex EL 311).

Data were analysed by one-way ANOVA using LSD for post-hoc comparisons. Differences were considered statistically significant if $P < 0.05$. Each experiment was repeated 3–4 times and results from a representative experiment are shown.

RESULTS

To determine the effect of Aβ(25–35) on expression of SAT1 in both neuronal and mixed cell cultures, cells were cultured for 5 days and then incubated with Aβ(25–35) for 12 h and 24 h. SAT1 mRNA levels were determined relative to those of β-actin[27].

The RT-PCR of the mixed cell culture showed that 10 μM Aβ(25–35) significantly suppressed SAT1 mRNA expression after incubation for 12 h ($P < 0.001$) (Fig. 1). However, SAT1 expression at 24 h showed no variation after exposure to 3 and 10 μM of Aβ(25–35). In neuronal cell culture, expression of SAT1 mRNA did not change after exposure to 3 and 10 μM of Aβ(25–35) for 12 and 24 h when compared with the control (Fig. 2).

Neuronal cell cultures showed a significant loss of cell viability, as determined by MTT method, when treated for 24 h with Aβ(25–35) in a dose-dependent manner ($P < 0.05$) (Fig. 3). A significant cell loss was also observed after treatment with 10 μM Aβ(25–35) for 12 h ($P < 0.05$). Viability of mixed cell cultures was reduced when exposed to 3 and 10 μM

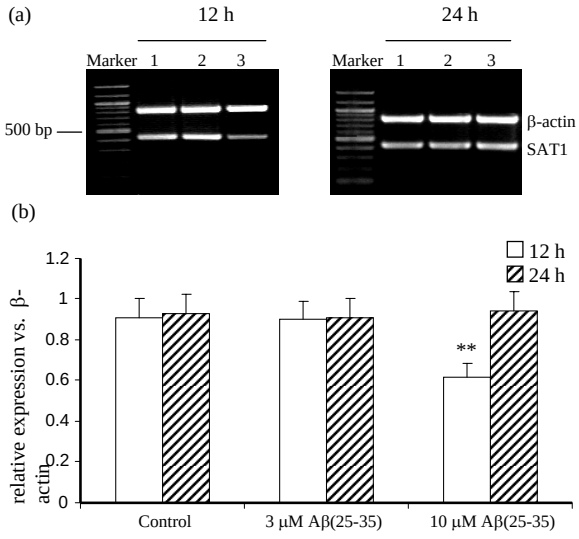

Fig. 1 Expression of SAT1 mRNA in mixed cell culture exposed to Aβ(25–35). Mixed cell cultures were incubated with Aβ(25–35) for 12 and 24 h and subjected to RT-PCR for amplification of SAT1 and β-actin mRNA. (a) Amplicons separated by gel-electrophoresis and stained with ethidium-bromide (b) quantitated bands. Lane 1: control; lane 2: exposed to 3 μM Aβ(25–35); lane 3: exposed to 10 μM Aβ(25–35). $^{**}P < 0.001$ compared with control. Columns show mean ± SEM from three independent experiments.

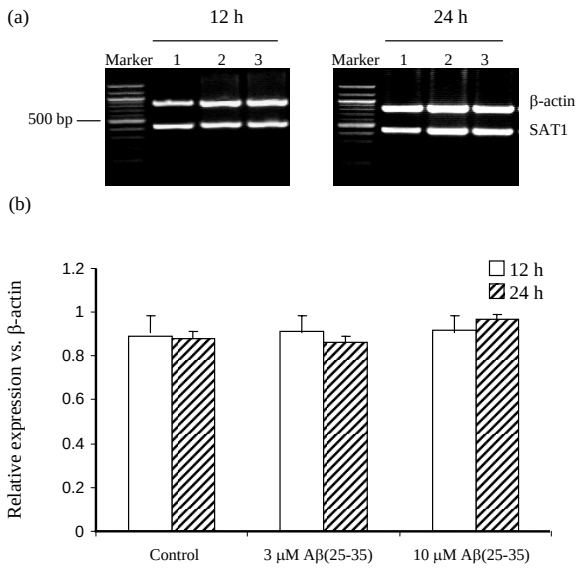

Fig. 2 Expression of SAT1 mRNA in the neuronal cell culture exposed to Aβ(25–35). Neuronal cell cultures were incubated with Aβ(25–35) for 12 and 24 h and subjected to RT-PCR for amplification of SAT1 and β-actin mRNA. (a) Amplicons separated by gel-electrophoresis and stained with ethidium-bromide (b) quantitated bands. Lane 1: control; lane 2: exposed to 3 μM Aβ(25–35); lane 3: exposed to 10 μM Aβ(25–35). Columns show mean ± SEM from three independent experiments.

Aβ(25–35) for 12 and 24 h ($P < 0.05$ and $P < 0.001$ respectively).

DISCUSSION

Results from this study indicated a significant decrease of SAT1 mRNA level after 12 h exposure to 10 μM Aβ(25–35) in mixed but not in neuronal cell cultures. Baron et al[28] suggested that Aβ exerts its toxic effect via activation of transcription factors. Aβ(25–35) induces expression of the growth arrest and DNA damage-inducible gene (gadd 45) implicated in the DNA excision-repair process. Accumulation and oligomerization of Aβ is also thought to play a central role in Alzheimer's disease pathogenesis. Aβ can produce oxidative damage by stimulating neuroinflammation, as well as generating reactive oxygen species as a result of binding to mitochondrial proteins[29]. Studies of neuron co-cultures showed that astrocyte formation of nitric oxide and other reactive oxygen species does occur in a number of neurodegenerative disorders[30,31]. Thus a mixed cell culture will be more sensitive to toxic effects than the neuronal cell culture when exposed to Aβ. Our data imply that a decrease of glutamine uptake may play an important role in Alzheimer's disease progression. However, Aβ(25–35) had no effect on SAT1 mRNA expression at 24 h in both mixed and neuronal cell cultures. Tian et al[32] showed that SAT2 mRNA level does not decrease after exposure to a low dose of Aβ(25–35). This effect may act as a cellular defence against neurodegeneration in pathogenesis of Alzheimer's disease.

Viability of mixed and neuronal cell cultures, measured by MTT method, was reduced when exposed to Aβ(25–35) in both a time- and concentration-dependent manner. In neuron-enriched cultures, Aβ is known to increase vulnerability to excitotoxicity[33]. Wei et al[34] showed that Aβ(1–42), in a time (1–48 h) and concentration (0.01–20 μM)-dependent manner, induces toxicity in cultured neurons. Similarly, Domenici et al[35] demonstrated that while pure neuronal culture shows a significant cell loss only at the highest concentration of Aβ(25–35), mixed cell culture manifests a toxic effect in a dose-dependent manner which was significant down to the lowest concentration. As observed by Pfrieger and Barres[36], neurons co-cultured with astrocytes develop

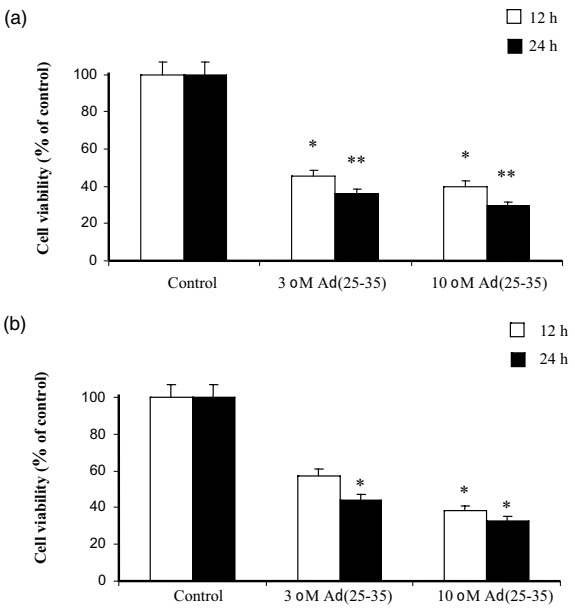

Fig. 3 Effect of Aβ(25–35) on neuronal cell viability. (a) Mixed and (b) neuronal cell cultures were treated with 3 and 10 µM Aβ(25–35) for 12 and 24 h and cell viability was determined using MTT assay. $*P < 0.05$ and $**P < 0.001$ compared to control. Data show mean ± SEM of four independent experiments.

approximately 7-fold more synapses and have a 7-fold increase in synaptic efficacy compared with neurons raised in the absence of astrocytes. Astrocytes could contribute to neurodegeneration, as Aβ-activated astrocytes overexpress factors such as interleukin 1β, nitric oxide, and S100β[37–39], which are all potentially neurotoxic[35]. In summary, our findings show that the reduction of cell viability is more prominent in mixed cell cultures ($P < 0.001$) than neuronal cell cultures ($P < 0.05$) when exposed to Aβ. Additionally, in mixed cell cultures, Aβ markedly suppressed SAT1 mRNA expression ($P < 0.001$) but not in cultured neuronal cells. This observation supports our previous report[20] that the presence of astroglia enhances Aβ induced neurotoxicity in cultured cortical cells. A possible explanation is that neurons can be damaged indirectly by the effect of Aβ-astrocyte interaction. In addition, short-time exposure to 0.1–50 µM Aβ(25–35) is not toxic to neurons, while longer exposures to the peptide are increasingly toxic[40]. Recently, Geci et al[41] showed that incubation of neuronal cell cultures with lower concentrations of Aβ(25–35) (5 and 10 µM) trigger apoptosis within 24 h of treatment. Our study suggests that Aβ may impair neuronal function and transmitter synthesis, and perhaps reduce excitotoxicity through a reduction in neuronal glutamine uptake.

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