GSK3β mediates the induced expression of synaptic acetylcholinesterase during apoptosis

Peng Jing, Qihuang Jin, Jun Wu and Xue-Jun Zhang

Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Shanghai, China

Alzheimer’s disease (AD) is a chronic neurodegenerative disorder characterized by a progressive loss of cholinergic neurons. Two of the characteristic neurochemical changes that occur in the AD brain are the loss of acetylcholinesterase (AChE; EC 3.1.1.7) and choline acetyltransferase activities (Davies and Maloney 1976). Despite the overall loss of AChE in the brain, AChE activity is increased in plaques and tangles very early in the process of amyloid deposition (Ulrich et al. 1990). Thus, AChE probably plays an important role in AD development.

Acetylcholinesterase is a critical enzyme that rapidly hydrolyzes and inactivates acetylcholine at cholinergic synapses and neuromuscular junctions (Taylor and Radic 1994). AChE is also involved in many non-cholinergic functions, such as cell adhesion, proliferation, and neurite outgrowth (Layer et al. 1993; Koenigsberger et al. 1997; Day and Greenfield 2002; Whyte and Greenfield 2003; Johnson and Moore 2004). Alternative splicing allows the production of three distinct AChE variants, each with a different carboxy-terminal sequence – the ‘synaptic’(S), ‘erythrocytic’(E), and ‘readthrough’(R) AChE isoforms (Massoulie et al. 1993; Soreq and Seidman 2001). In mammals, the AChE-R variant produces a soluble monomer that is up-regulated in the brain during stress (Pick et al. 2004); the AChE-E variant produces a glycoinositol phospholipid-anchored dimer that is expressed mainly in blood cells; and the AChE-S variant was once thought to be expressed only in the brain and muscle tissues and now has been found to express in tissues devoid of cholinergic responses and in several types of hematopoietic cells (Tranum-Jensen and Behnke 1981; Small et al. 1996; Grisaru et al. 1999).

In recent years increasing evidence has shown that AChE-S may be involved in apoptosis (Robitzki et al. 1998). We have reported that AChE-S expression was induced during apoptosis in various cell types by different apoptotic stimuli, as the blockage of AChE expression by antisense or siRNA led to activation of GSK3β. Two different inhibitors of GSK3β (lithium and GSK3β-specific inhibitor VIII) could block A23187- or TG-induced up-regulation of AChE activity, AChE-S mRNA level and protein expression. However, lithium could not inhibit the induction of AChE-R mRNA and protein under similar conditions. Taken together, our results show that GSK3β is specifically involved in the induction of AChE-S expression in PC12 cells during apoptosis.

Keywords: A23187, acetylcholinesterase, apoptosis, glycoinositol phospholipid, polyacrylamide gel electrophoresis, PAGE, polyvinylidene difluoride, PBS, phosphate-buffered saline, PI, propidium iodide; TG, thapsigargin.

Received May 27, 2007; revised manuscript received August 9, 2007; accepted September 8, 2007.

Address correspondence and reprint requests to Xue-Jun Zhang, Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 YueYang Road, Shanghai 200031, China.

E-mail: xjzhang@sibs.ac.cn

Abbreviations used: AChE, acetylcholinesterase; AD, Alzheimer’s disease; BCA, bicinchoninic acid; GSK3β, glycogen synthase kinase-3β; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI, propidium iodide; TG, thapsigargin.
can prevent apoptosis induction (Zhang et al. 2002). Also silencing of the ACHE gene by siRNA inhibits the interaction between apoptotic protease-activating factor-1 and cytochrome c, indicating that ACHE may play a critical role in the apoptosome (Park et al. 2004).

Glycogen synthase kinase-3 (GSK3) is an ubiquitous serine/threonine kinase that is present in mammals in two isoforms: α and β (Woodgett 1990). Although GSK3β was first identified as an enzyme capable of phosphorylating glycogen synthase to inhibit glycogen synthesis, since then it has been found to phosphorylate nearly 50 substrates (Jope and Johnson 2004). Phosphorylating these substrates allows GSK3 to modulate many fundamental processes including cell structure, metabolism, gene expression, and, in a seemingly paradoxical manner, apoptosis (Frame et al. 2001; Grimes and Jope 2001). As many cells undergo apoptosis in the plaques and tangles of the AD patients’ brain, we questioned whether a 2001; Grimes and Jope 2001).

In this study we used A23187 and thapsigargin (TG) to stimulate the calcium dyshomeostasis to simulate what occurs in AD. The calcium ionophore A23187 induces apoptosis by increasing cytosolic Ca2+ in various cell types (Azmi et al. 1996; Petersen et al. 2000). TG is the sarcoplasmic reticulum Ca2+-ATPase pump inhibitor and can deplete Ca2+ within the endoplasmic reticulum, resulting in a transient increase of cytoplasmic Ca2+ levels (Sagara and Inesi 1991). TG can induce perturbations in cellular Ca2+ homeostasis which has been reported to induce apoptosis in both normal and malignant cells (Krebs 1998).

Herein, we demonstrate that GSK3β regulates the induction of ACHE-S activity but not of ACHE-R during apoptosis caused by calcium dyshomeostasis. Our data provide evidence that both ACHE-R and ACHE-S were up-regulated by A23187 and TG. Our results also show that GSK3β can only regulate ACHE-S activity and expression but has no obvious effect on ACHE-R. So we propose a new mechanism of ACHE regulation in AD development, which may be useful in the treatment of neurodegenerative disorders associated with ACHE-S induction.

### Materials and methods

#### Expression vectors and constructs

The full-length rat-GSK3β was subcloned into pcDNA3.1A mammalian expression vector. GSK3β Ser9 was mutated to alanine with mutagenic oligonucleotide 5'-GAGAACCACC-GCATTTGGGAGAGCTGC-3' and 5'-TTCGCCAAATGGGGGTGGTTCTCGGTCG-3' using KOD-Plus (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer’s instructions. The 2.2 kb DNA fragment of the human ACHE promoter (Ben Aziz-Aloya et al. 1993) was subcloned into BglII and HindIII sites of the pGL3 basic vector (Promega, Madison, WI, USA) with a downstream tag firefly luciferase gene, pACHE-Luc (Wan et al. 2000). All plamids were confirmed by automated sequencing analysis.

#### Materials

Lithium chloride was purchased from Sigma Chemicals (St Louis, MO, USA). A23187, TG, and GSK3β-specific inhibitor VIII were obtained from Calbiochem (La Jolla, CA, USA). Anti-ACHE antibody (H-134) was purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-cleaved caspase 3 (D175) and phospho-GSK3β (Ser9) antibodies were from Cell Signaling (Beverly, MA, USA). Anti-GSK3β antibody was from Cell Signaling or BD Bioscience (San Jose, CA, USA).

#### Mammalian cell culture and treatments

Cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% heated inactivated fetal bovine serum (Gibco-BRL) and 10% horse serum (Gibco-BRL). 293T cells and SK cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium supplemented with 15% heated inactivated fetal bovine serum. Cells were grown at 37°C in 5% CO2.

#### Detection of cell death

PC12 cells were plated in 6-well dishes at 1 × 10⁶ cells/well 1 day prior to A23187 and TG treatment. The cells were treated with the agents at the indicated time. After treatment, cells were trypsinized and collected with the supernatants, and cell viability was determined by propidium iodide (PI, Sigma) using flow cytometry.

#### Determination of cellular ACHE activity

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in lysis buffer (50 mmol/L potassium phosphate, pH 7.4; 0.5% Tween 20; 1 mol/L NaCl; 1 µg/mL aprotinin). The homogenates were sonicated briefly on ice and then centrifuged at 13 400 g at 4°C for 5 min. Supernatant fractions were collected on ice and the protein concentration was determined by bicinchoninic acid (BCA) (Pierce, Rockford, IL, USA). ACHE activity was determined by the method of Ellman et al. (1961) (Ellman et al. 1961). All ACHE determinations were performed in the presence of 0.1 mmol/L tetrasopropyl pyrophosphoramide to inhibit butyryl cholinesterase activity. Specific ACHE activity was defined as the number of units of ACHE activity versus corresponding relative protein concentration. Control values were averaged and then normalized to one.
Semi-quantitative RT-PCR
Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For reverse transcription reaction, combined 2 μg RNA, 1 μl oligo(dT), and adjusted volume to 11 μl with diethyryrocarbonate-treated water, the mixture was incubated at 70°C for 5 min and placed on ice. Then we added 4 μl 5 × M-MLV reaction buffer, 2 μl 4 × deoxynucleotides (10 mmol/L), 1 μl M-MLV (Promega), 0.5 μl RNasei (Promega) and adjusted volume to 20 μl with diethyryrocarbonate-treated water, incubated the tube at 37°C for 60 min and terminated the reaction by incubating at 70°C for 10 min. The product of the reverse transcriptase reaction was used for amplification of AChE and actin cDNA (control), in the presence of Taq DNA polymerase, deoxynucleotide mix, PCR buffer (all from MBI Fermentas, Hanover, MD, USA), downstream and upstream primers. Amplification conditions were denaturation at 94°C 15 s (2 min for the first cycle), annealing at 60°C or 62°C for 30 s and extension at 72°C for 40 s (10 min for the last cycle) for 22–35 cycles. 5 μl of 30 μl-amplification reaction mixtures was separated on a 15% agarose gel stained with ethidium bromide. To detect the mRNA of the AChE variants, the PCR primers were employed as follows:

\[
\begin{align*}
\beta\text{-actin(+)} & : 5'\text{-CAACCGTGAAGAGATGACCCAGAT-3'} \\
\beta\text{-actin(–)} & : 5'\text{-CATGAGGTCTTTACGGATGTCAACG-3'} \\
1389(+)/1819(–) & : 5'\text{-CCGGAATTCCTCGGCCTCCACATTGACTTG-3'} \\
I4/R & : 5'\text{-CTTCCAACCTTGCGCCTGT-3'}. \\
\end{align*}
\]

Protein expression and immunoblotting
The cells were washed twice with ice-cold PBS, scraped the cells off the plate and centrifuged at 1000 g, the cells were lysed in 1 × sodium dodecyl sulfate sample buffer, sonicated to shear DNA and cleared of cellular debris by centrifugation (13 400 g, 4°C). The protein concentrations were measured using BCA (Pierce). Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE), and the proteins were detected immunologically after electrotransfer onto nitrocellulose membranes or polyvinylidene difluoride membranes. The membranes were blocked in Tris-Buffered Saline Tween-20 containing 5% non-fat dry milk for 1 h at 25°C. The membranes were then incubated with appropriate primary antibodies in blocking solution followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were visualized with chemiluminescence reagent.

Luciferase assays
Luciferase activity assays were performed with a Dual-Luciferase Reporter Assay System (Promega), and the activity was measured with a luminometer BGP (MGM Instruments, Hamden, CT, USA). Firefly luciferase activity was normalized to Renilla luciferase activity in each well (n = 3/transfection experiment).

Non-denaturing polyacrylamide gel electrophoresis
Cells were washed twice with ice-cold PBS and then lysed in lysis buffer (50 mmol/L potassium phosphate, pH 7.4; 0.5% Tween 20; 1 mol/L NaCl; 0.5% Triton X-100; 0.25% sodium deoxycholate; 10% glycerol; aprotinin). The homogenates were sonicated briefly on ice and then centrifuged at 13 400 g at 4°C for 5 min. Supernatant fractions were collected on ice and the protein concentration was determined by BCA (Pierce). Electrophoresis in 7.5% polyacrylamide slab gels was performed in the absence of detergent, in the presence of 0.5% Triton X-100, or in the presence of 0.5% Triton X-100 plus 0.25% sodium deoxycholate. Cholinesterase activity was visualized after the migration, by the method of Kamovsky and Roots (1964).

Statistical analysis
Data were presented as the mean ± SD. Significant differences in treatment groups were determined using the unpaired Student’s t-test. Differences were considered statistically significant at p < 0.05 or p < 0.01.

Results
Induction of apoptosis by A23187 and TG in PC12 cells
Neuroendocrine PC12 cells were derived from rat adrenal pheochromocytomas and have been widely used as an in vitro model for neural cells. PC12 cells were treated with A23187 and TG. To determine whether A23187 and TG could induce apoptosis, PI staining was employed for detecting apoptosis. As shown in Fig. 1b, PI-positive cells increased time dependently in PC12 cells treated with 4 μmol/L A23187, apoptotic rates also increased time dependently, but the apoptotic rate was only about 31% after 24 h (Fig. 1d). To further confirm apoptosis induced by A23187 or TG, cleaved caspase 3 was detected by western blot analysis. Our data showed that exposure of PC12 cells to A23187 or TG resulted in an increase of cleaved caspase 3 in a time-dependent manner (Fig. 2a and b).

After treatment with A23187 and TG, total AChE activity and expression were increased and GSK3β was activated in PC12 cells
Our previous studies, and those of other laboratories, have demonstrated that AChE-S can be up-regulated by various stimuli of apoptosis. To determine whether AChE expression would be up-regulated in PC12 cells exposed to A23187 or TG, the esterase activity, the protein and mRNA level of AChE-S were detected. First we investigated AChE activity (Fig. 3a and b). The cells treated with A23187 or TG significantly increased in AChE activity in a time-dependent manner. This observation is consistent with our previous findings (Zhang et al. 2002; Zhu et al. 2007b). With western blot analysis, it was shown that total AChE protein expression also time dependently increased in the cells exposed to both the agents (Fig. 3c and d).

It has been shown that many stimuli which cause apoptosis can activate GSK3β (Klein and Melton 1996). GSK3β...
activity is regulated by antagonistic serine and tyrosine phosphorylation. Phosphorylation at Ser9 inhibits GSK3β activity (Stambolic and Woodgett 1994). To determine if A23187 or TG can activate GSK3β, we examined the phosphorylation level of GSK3β at Ser9 in PC12 cells. PC12 cells were treated with A23187 or TG for the indicated times. The results revealed that the phosphorylated GSK3β at Ser9 time dependently decreased after treatments (Fig. 4a and b). The protein expression of total GSK3β did not significantly change in PC12 cells exposed to A23187 and TG (Fig. 4a and b). These results suggest that both A23187 and TG can induce the activation of GSK3β, which correlates with the changes of AChE activities, protein. With semi-quantitative RT-PCR assay, it was found that AChE-S mRNA increased in a time-dependent manner in PC12 cells after A23187 treatment too (Fig. 5a). The primer 1819(−)S locates in exon-6 (Fig. 8a).

By inhibiting GSK3β, AChE activities and AChE-S mRNA levels are blocked but have no obvious inhibition on AChE protein levels in PC12 cells treated with A23187 and TG. To determine whether the activation of GSK3β is required for AChE activity and expression in apoptosis, PC12 cells were pre-treated with lithium or GSK3β specific inhibitor VIII for 1 h and then exposed to A23187 or TG. As shown in Fig. 6a and c, the increased AChE activities were significantly inhibited by lithium and GSK3β specific inhibitor VIII. We also found that AChE-S mRNA levels were decreased by lithium (Fig. 5b). The above results indicated that GSK3β may increase AChEs, at least AChE-Ss, transcription. To confirm this prediction, a constitutive active
GSK3\(\beta\) mutant, GSK3\(\beta\)(S9A), was used. GSK3\(\beta\)(S9A) or pcDNA3.1A, pAChE-Luc and pRL-SV40 were transiently co-transfected to Hela cells and incubated in the presence or absence of A23187 or TG. Our data showed that GSK3\(\beta\)(S9A) can induce about a twofold induction of AChE promoter activity compared with pcDNA3.1A both in normal and apoptotic conditions triggered by A23187 or TG (Fig. 5c). The results of luciferase assay support our speculation that GSK3\(\beta\) can up-regulate AChE transcription in another way.

At the same time GSK3\(\beta\) activity was blocked by lithium, as the phosphorylation levels of GSK3\(\beta\) at Ser9 were increased by lithium (Fig. 6b and d). The GSK3\(\beta\)-specific inhibitor VIII is an inhibitor to compete with ATP and have no effect on the phosphorylation of GSK3\(\beta\). Furthermore AChE protein levels after pre-treatment of lithium were detected. To our surprise, the results showed that lithium did not inhibit AChE protein induction when apoptosis was triggered by A23187 and TG (Fig. 7a and b).

AChE-R is also increased, but cannot be blocked by lithium in apoptotic PC12 cells induced by A23187 and TG, while AChE-S can be inhibited.

Acetylcholinesterase-R mRNA levels increased considerably within 30 min of confined swim stress, or after exposure to anti-AChEs or acetylcholine analogues. Similar effects have been observed in mammalian brain neurons in vivo after stress, in cortical and hippocampal neurons in brain slices and in cultured HEK293 cells (Soreq and Seidman 2001). Furthermore it has been demonstrated that PC12 cells express both AChE-S and AChE-R (Ross et al. 2003). The anti-AChE antibodies we used in our experiments, H-134 and polyclonal antibodies made in our laboratory, can recognize both forms of AChE-S and AChE-R. To determine
if the above puzzling results were caused by AChE-R, we investigated the alterations of AChE-R. We designed a down-primer (I4/R) locates in intron-4 and directly assayed AChE-R mRNA levels by semi-quantitative RT-PCR (Fig. 8a). Results indicated that exposure to A23187 induces a time-dependently increase of mRNA levels of AChE-R (Fig. 8b). However, we have demonstrated that AChE-S mRNA stability was enhanced by A23187 (Zhu et al. 2007b), AChE-R mRNA stability was significantly less stable than AChE-S mRNA (Soreq and Seidman 2001). So we could understand the observation that AChE-R mRNA decreased after treatment with A23187 for 36 h (Fig. 8b). Pre-treatment of PC12 cells with lithium did not decrease AChE-R mRNA enhancement associated with exposure to A23187 (Fig. 8c). Together these results indicate that conditions of A23187 exposure also significantly increased AChE-R mRNA levels but lithium could not block it.

Acetylcholinesterase-R does not have any feature that allows for its attachment to other molecules and it remains monomeric (G1) and soluble. Differently AChE-S has a cysteine located three residues from the carboxyl terminus which allows it dimerization by disulphide bridging, two additional monomers can become associated by hydrophobic interactions and produce tetramers, so AChE-S subunits produce amphiphilic monomers (G1), homomeric dimers (G2) and tetramers (G4) (Soreq and Seidman 2001; Perrier et al. 2005). It has previously been reported that three forms of AChE, G1, G2, and G4, have different migration rates in non-denaturing PAGE (Bon et al. 1988). We directly separated AChE in the extracts of PC12 cells on non-denaturing PAGE and visualized the various forms by the method of Karnovsky and Roots (1964) (Fig. 8d). The data indicated an obvious increase in AChE activity of all of the three isoforms, G1, G2, and G4, upon treatment with A23187. But only G2 and G4 forms activities were inhibited after pre-treatment with lithium (Fig. 8d). It has been shown that only AChE-S has G2 and G4 forms in PC12 cells, so we demonstrated that only AChE-S was inhibited by lithium. These findings were consistent with our previous results. Our results also confirmed that GSK3β regulated only AChE-S when PC12 cells were triggered towards apoptosis but had no obvious effect on AChE-R.

Discussion

In retinospheroids during their proliferation period, antisense treatment of butyrylcholinesterase increased AChE expression and also apoptosis (Robitzki et al. 1998). We and others

© 2007 The Authors
have demonstrated that AChE expression was induced in various cell types by different apoptotic stimuli, and the blockage of AChE expression by antisense or siRNA can prevent apoptosis induction (Zhang et al. 2002; Park et al. 2004). Also, silencing of the \( \text{ACHE} \) gene by siRNA inhibits the interaction between apoptotic protease-activating factor-1 and cytochrome \( c \), indicating that AChE may play a critical role in apoptosome formation (Park et al. 2004). However, as AChE is a critical enzyme in AD development, its regulation in neurons during apoptosis remained to be elucidated. PC12

**Fig. 6** The LiCl and glycogen synthase kinase-3\( \beta \) (GSK3\( \beta \))-specific inhibitor VIII inhibit GSK3\( \beta \) activity, and block up-regulation of acetylcholinesterase (AChE) activity. (a and c) AChE activities were analyzed by Ellman method. PC12 cells were exposed to dimethylsulfoxide (DMSO), 4 \( \mu \)mol/L A23187 or 0.5 \( \mu \)mol/L thapsigargin (TG) for the indicated periods in the presence 20 mmol/L NaCl, 20 mmol/L LiCl or 10 \( \mu \)mol/L GSK3\( \beta \)-specific inhibitor VIII pre-treatment for 1 h. (b) The results from western blot of PC12 cells. The cells were treated with DMSO, 4 \( \mu \)mol/L A23187 or 0.5 \( \mu \)mol/L TG for 24 h in the absence or presence of NaCl or LiCl pre-treatment for 1 h. GSK3\( \beta \), phospho-GSK3\( \beta \)(Ser9) were analyzed by immunoblotting. Results were presented as the means \( \pm \) SD of three separate experiments. *\( p < 0.05 \) when compared with DMSO.

**Fig. 7** Western blot analysis of acetylcholinesterase (AChE) expression in absence or presence of NaCl or LiCl. PC12 cells were treated with dimethylsulfoxide (DMSO), 4 \( \mu \)mol/L A23187 (a) or 0.5 \( \mu \)mol/L thapsigargin (TG) (b) for 24 h in the absence or presence of NaCl or LiCl pre-treatment for 1 h. Then cells were collected and lysed, AChE protein was detected by immunoblotting. The graph shows the densitometric value of AChE versus \( \beta \)-actin. The densitometric graphs were presented as the means \( \pm \) SD of three separate experiments. *\( p < 0.05 \) when compared with DMSO.

© 2007 The Authors
cells, which have been used widely as an in vitro model for neural cells, were used in our present study. Dysregulation of intracellular calcium signaling has been linked to the pathogenesis of AD. Furthermore calcium dyshomeostasis can induce apoptosis. Our previous study have shown that intracellular calcium dyshomeostasis can increase AChE expression in Hela and MDA cells (Zhu et al. 2007b). Whether calcium dyshomeostasis can induce AChE in nerve cells, however, has not been confirmed. In the current study, we examined if AChE can be induced by calcium dyshomeostasis in PC12 cells and studied the regulation mechanism primarily.

First, we examined that AChE can be induced by calcium dyshomeostasis in PC12 cells. We used A23187 and TG which can both induce apoptosis by increasing cytosolic Ca\(^{2+}\) in various cell types (Sagara and Inesi 1991; Petersen et al. 2000). PC12 cells were challenged with A23187 and TG to perturb calcium homeostasis to partially simulate the events that occur in the cells of AD patients. After treatment with A23187 or TG for 24 h, PI-positive cells could reach about 50% or 30%, respectively (Fig. 1). The caspase 3 is activated by both A23187 and TG in PC12 cells (Fig. 2). Using this apoptotic model, we found that both A23187 and TG enhanced AChE activity and expression as demonstrated by western blot (Fig. 3). Furthermore, semi-quantitative RT-PCR assays indicated that the mRNA levels of AChE-S were induced by A23187 (4 \(\mu\)mol/L). (c) AChE-R mRNA but could not be decreased by lithium (analysis of AChE-R mRNA levels by semi-quantitative RT-PCR. The graph shows the densitometric value of AChE versus \(\beta\)-actin. The densitometric graphs were presented as the means \pm SD of three separate experiments. * \(p < 0.05\) when compared with dimethylsulfoxide (DMSO). (d) Non-denaturing polyacrylamide gel electrophoresis of cell extracts from PC12 cells. G1, G2, and G4 form of AChE can be separated by their migration in Triton-X 100 and then AChE activity on the gel was visualized by the method of Karnovsky and Roots (1964). PC12 cells were cultured for the indicated times in presence of DMSO or 4 \(\mu\)mol/L A23187.
total GSK3β protein expression levels were unchanged when cells were exposed to these agents. These results indicated that GSK3β was activated when PC12 cells were exposed to A23187 or TG.

However, the expression patterns of GSK3β and AChE in the AD brains are very similar. Previous studies have also shown that both AChE and GSK3β have association with Aβ and tau, but it is still unknown whether there is a direct relationship between AChE and GSK3β. To elucidate the association between GSK3β and AChE, lithium chloride and GSK3β-specific inhibitor VIII were used to inhibit GSK3β activity. Lithium is a direct and indirect inhibitor of GSK3β, which can enhance the phosphorylation level at Ser9 of GSK3β. After GSK3β activity was inhibited by lithium or GSK3β specific inhibitor VIII, up-regulation of AChE activities were significantly blocked in PC12 cells exposed to A23187 or TG (Fig. 6). The induction of AChE-S mRNA level was also decreased by lithium in PC12 cells exposed to A23187 (Fig. 5b). Furthermore, a constitutive active GSK3β mutant, GSK3β(S9A), can induce AChE promoter activity compared with pcDNA3.1A (Fig. 5c).

However, our results showed that lithium cannot inhibit the induction of AChE expression in PC12 cells during apoptosis obviously. It has been reported that AChE-R expression is also induced under stress (Kauf er et al. 1998; Mesheror et al. 2002) and during specific stages of murine brain development (Dori et al. 2005), two processes in which cholinergic neurons are subjected to considerable structural and functional changes. PC12 cells have been reported to express AChE-R (Ross et al. 2003), so we examined the mRNA level of AChE-R during apoptosis. By further examination, we found that AChE-R can also be induced by A23187, but lithium could not inhibit its up-regulation (Fig. 8b and c). We also found that only G2 and G4 forms of AChE activity were blocked by lithium, G1 forms activity did not appear to be decreased (Fig. 8c). To our knowledge, AChE-S can form monomer(G1), dimer(G2) and tetramer(G4) while AChE-R remains as monomer(G1). Taken together, these findings indicate that both AChE-S and AChE-R are up-regulated during apoptosis, but only AChE-S induction is dependent on GSK3β activation.

The possible mechanism by which GSK3β controls over AChE-S but not AChE-R is still not clear, but alternate promoter usage and 3’ alternative splicing may be involved. First, in the promoter region of ACHΕ gene there are many transcription factor binding sites that can be directly or indirectly regulated by GSK3β, such as Sp-1, CREB, AP-1, NF-kB, c-MYC, HSF-1, and NFAT (Mesheror et al. 2004). It has been demonstrated that AChE expression may be mediated by the activation of JNK pathway during apoptosis through a c-jun-dependent mechanism (Deng et al. 2006). AP-1 and Sp-1 are two of the transcription factors binding sites of JNK/c-jun pathway. We have demonstrated that NFAT is also involved in AChE-S up-regulation during apoptosis (Zhu et al. 2007a). Previous findings also indicate that CREB or c-fos are involved in AChE-R induction up-regulation under stress (Zimmerman and Soreq 2006). However, GSK3β can only phosphorylate much of the substrates which have been pre-phosphorylated by other kinases. So there are possible other kinases involved in the activation or inactivation of these transcription factors. It made the transcriptional regulation of ACHΕ gene confused. We suspected that there are transcription factors which are only involved in AChE-S or AChE-R transcriptional regulation respectively, but the mechanisms would be studied further.

Second, 3’ alternative splicing of AChE pre-mRNA maybe involved. RNA splicing is a complex mechanism wherein five different small nuclear ribonucleoproteins, components of the spliceosome, play a role together with other factors (Green 1991; Lamond 1991). Among these factors are a family of proteins called SR proteins because they contain an arginine- and serine-rich domain in their C-terminal portion that influences the selection of alternative splicing sites (Fu et al. 1992; Zahler et al. 1993). It has been recently demonstrated that GSK3 phosphorylates SC35 and that GSK3 activity affects SC35 localization in nuclear speckles. Inhibition of GSK3 by lithium results in increased exon 10+ tau transcription and increased nuclear localization of SC35 in speckles (Hernandez et al. 2004). SC35 and hnrNPA1 also have effect in the determination of amyloid precursor protein isoforms (Donev et al. 2007). It was identified recently that SC35 is a likely factor mediating long-lasting alternative splicing of ACHΕ gene expression in prefrontal cortical neurons following repeated stress and demonstrated close association of SC35 with AChE-R in cultured cells as well as in stressed, developing and transgenic animals (Mesheror et al. 2005). ASF/SF2 caused the opposite effect. We hypothesize that SC35 play a different role or GSK3 regulate ASF/SF2 during apoptosis. There are probably other factors involved in this mechanism, and further investigation would be needed.

In conclusion, A23187 and TG, which disturb calcium homeostasis, can induce the caspase 3-dependent apoptosis in PC12 cells. In these apoptotic models, it has been demonstrated that: (i) the two agents can time dependently induce the up-regulation of AChΕ activity and the activation of GSK3β and (ii) GSK3β can only regulate AChE-S transcription during apoptosis in PC12 cells but has no obvious effect on AChE-R up-regulation.

Acetylcholinesterase is the target of drugs designed to combat nerval disorders, such as myasthenia gravis and glaucoma, and most recently to alleviate the cholinergic deficiency associated with AD (Silman and Sussman 2005). Now GSK3β also is an important target for AD. Lithium is a widely used medication for bipolar mood disorder. Our findings can provide a novel mechanism of these drugs for AD.
Acknowledgements

This work was supported in part by grants from the National Natural Science Foundation of China (No. 30570920, 30623003), the third phase creative program of Chinese Academy of Sciences (No. KSCX1-YW-R-13), the National Key Basic Research Project 973 (No. 2005CB522602), and the Science and Technology Commission of Shanghai Municipality (No. 06JC14076 and No. 06DZ22032).

References


