Alpha-1-antichymotrypsin, an inflammatory protein overexpressed in Alzheimer’s disease brain, induces tau phosphorylation in neurons

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Amyloid plaques and neurofibrillary tangles are key pathological features of Alzheimer’s disease. Alzheimer’s disease pathology is also characterized by neuroinflammation and neuronal degeneration, with the proteins associated with inflammatory responses being found in tight association with the plaques. One such protein is the serine protease inhibitor α-1-antichymotrypsin (ACT). ACT has been shown to promote Aβ polymerization in vitro and in vivo, and levels of ACT protein in plasma and cerebrospinal fluid from Alzheimer’s patients have been found to correlate with progression of dementia. Here we investigated the possible involvement of ACT in tau phosphorylation and tangle formation. As was previously found for Alzheimer’s disease, brains from patients with non-Alzheimer’s tauopathies exhibited an enhanced expression of ACT, which correlated with the level of tau hyperphosphorylation. Transgenic mice expressing human ACT alone or ACT along with mutant human amyloid precursor protein (APP) showed a significant increase in tau phosphorylation, suggesting that this inflammatory protein can induce tau hyperphosphorylation. The increase in phosphorylation was observed at PHF-1 (P-Ser396/P-Thr404), P-Ser202 and P-Thr231 sites on tau, the P-tau epitopes that are associated with tangles in the patients. This result was further confirmed by the finding that addition of purified ACT induced the same Alzheimer’s disease-related tau hyperphosphorylation in cortical neurons cultured in vitro. This correlated with an increase in extracellular signal regulated kinase (ERK) and glycogen synthase kinase-3 activation, indicating their involvement in ACT-induced tau phosphorylation. The ACT-treated neurons showed neurite loss and subsequently underwent apoptosis. Approximately 40–50% of neurons were TUNEL positive by 6 and at 24 h >70% of the neurons showed staining suggesting that ACT was inducing apoptosis in these neurons. These findings indicate that inappropriate inflammatory responses are a potential threat to the brain and that intervention directed at inhibiting the expression or function of ACT could be of therapeutic value in neurodegenerative diseases such as Alzheimer’s and other tauopathies.

Keywords: alpha 1-antichymotrypsin; Alzheimer’s disease; apoptosis; inflammation; neuron; phosphorylation; tau

Abbreviations: AAT = α1-antitrypsin; ACT = α1-antichymotrypsin; ERK = early signal regulated kinase; GSK = glycogen synthase kinase; NFT = neurofibrillary tangle; PHF = paired helical filament

Received June 23, 2006. Revised August 19, 2006. Accepted August 21, 2006

Introduction

Alpha-1-antichymotrypsin (ACT) is an acute phase serum glycoprotein that belongs to a class of serine protease inhibitors named serpins and is an integral component of the amyloid plaques in Alzheimer’s disease patients (Abraham et al., 1988). ACT is overexpressed in the astrocytes surrounding the Alzheimer plaques (Abraham et al., 1988, 1990; Pasternack et al., 1989) and elevated levels of the protein have been reported in the cerebrospinal fluid and plasma of the patients (Harigaya et al., 1995; Licastro et al., 1995; DeKosky et al., 2003). The presence of inflammatory proteins, including ACT, apolipoprotein E (ApoE) and complement in plaques led to the hypothesis
that these proteins might serve to promote Aβ polymerization and amyloid formation (Abraham et al., 1988, 2000; McGeer et al., 1989; Rogers et al., 1992; Wisniewski and Frangione, 1992). Like ApoE, ACT binds to Aβ and promotes its assembly into neurotoxic amyloid filaments (Strittmatter et al., 1993b; Ma et al., 1994, 1996; Sanan et al., 1994; Wisniewski et al., 1994; Lukacs and Christianson, 1996; Webster and Rogers, 1996; Abraham et al., 2000). The contribution of ACT to both plaque formation and associated cognitive decline has been established in vivo in mice (Mucke et al., 2000; Nilsson et al., 2001a, 2004). The direct role of ACT in amyloid fibril formation in vitro and in transgenic mice suggests that its elevated levels in the human brain perhaps contribute to the amyloid pathology in Alzheimer’s disease. Genetic studies also support a role for ACT and ApoE in this disease. While inheritance of an ApoE4 allele is a strong risk factor for development of late-onset Alzheimer’s disease, studies of ACT polymorphisms have been positive but less conclusive (Corder et al., 1993; Strittmatter et al., 1993a; Kamboh et al., 1995, 2006; Poirier et al., 1995; Licastro et al., 1999, 2004; Wang et al., 2002).

In addition to plaques, Alzheimer’s disease is also characterized by neurofibrillary tangles (NFT). NFTs are mainly comprised of hyperphosphorylated forms of the microtubule binding protein tau (Delacourte and Defossez, 1986; Kosik et al., 1986, 1988; Lee et al., 1989, 1990; Mandelkow and Mandelkow, 1993; Goedert, 1996). Hyperphosphorylation of tau promotes its dissociation from microtubules and its polymerization into paired helical filaments (PHFs), resulting in disorganization of the microtubule cytoskeleton and blocking of axonal transport (Bramblett et al., 1993; Busciglio et al., 1995; Terwel et al., 2002; Mandelkow et al., 2003).

Because ACT is greatly overexpressed in the areas of Alzheimer’s disease brain showing amyloid and tangle pathology, and because it enhances Aβ polymerization, we examined whether ACT might also affect tau and its phosphorylation. Here we tested the hypothesis that ACT expression promotes the hyperphosphorylation and aggregation of tau leading to neurodegeneration. We found increased levels of ACT in the brain samples from tauopathy patients, suggesting that ACT could be inducing tau hyperphosphorylation. Furthermore, ACT directly induced tau hyperphosphorylation, neurite degeneration and apoptosis in cultured neurons. These results raise the possibility that in addition to promoting amyloid deposition, ACT overexpression also contributes to tau hyperphosphorylation and tangle formation, thus enhancing neurodegeneration.

Material and methods

Material

The tissue culture reagents and electrophoresis supplies used in this study were purchased from Gibco/Invitrogen, Carlsbad, CA. α1-ACT (RDI-ACT or ACT) and α1-antitrypsin (α1-AAT or AAT) were from Research Diagnostic Inc., Flanders, NJ and RDI Division of Fitzgerald Industries, Intl, Concord MA. We chose to use the 90% pure ACT since in the 98% pure ACT its PSA binding epitope was missing; the AAT was 96% pure. Poly-d-lysine (PDL) was from Sigma, St Louis, MO. Human ACT antibody was purchased from Accurate Chemicals, Westbury, NY; P-Thr231 and P-Ser202 phospho-specific tau antibodies were from Biosource International, Camarillo, CA. Antibodies to PHF-1, P-Ser202tau (CP13), total tau (TGG) and to the conformation specific tau (MC1), were a kind gift from Dr Peter Davies, Albert Einstein College of Medicine, Bronx, NY. HT-7 monoclonal antibody towards the total tau was from Innogenetics, Inc. Alexa 488 and 594 secondary antibodies were purchased from Invitrogen/Molecular Probes. Enhanced chemiluminescence (ECL) reagent was from Pierce Biotechnology Inc., Rockford, IL. The nitrocellulose membrane was from Schleicher and Schuell (Keene, NH) and the TUNEL assay kit was purchased from Roche Diagnostics Corporation, Indianapolis, IN. Paraffin-embedded sections of human brain [FTDP, corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) as well as normal tissue] samples were from the brain bank of the Florida Alzheimer’s Disease Research Center and the Florida Alzheimer’s Disease Initiative.

Transgenic mice

Construction of the ACT and APP/ACT transgenic mice was described previously (Nilsson et al., 2001a). In the current study we used ACT, APP ACT/APP and non-transgenic mice to examine the changes in tau phosphorylation induced by APP and ACT. Mice were anaesthetized using Nembutal and perfused with saline solution. The brains were dissected and half of the brain was immersion fixed with 4% para-formaldehyde and the other half was used for brain protein extraction. For protein extraction, brains were homogenized in lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM MgCl2, 1 mM EGTA, 20 mM NaF, 2 mM Na3VO4 and protease inhibitors (1 tablet mini-complete/10 ml lysis buffer, Roche). Samples were centrifuged at 14,000 r.p.m. for 30 min and equal amounts of proteins were used for western blot analysis. For immunohistochemical analysis, after 24 h of immersion fixation the brains were immersed in 10, 20 and 30% sucrose solution sequentially for cryoprotection. Brain sections were made using a freezing stage sliding microtome and stored in phosphate-buffered saline (PBS) containing sodium azide (0.02%) for immunohistochemical analysis with P-tau antibodies.

Cortical neuronal cultures

Timed pregnant C57/black mice were obtained from Harlan. Cortical neurons were cultured from embryonic day 18 mice as described previously (Park et al., 1998). The animals were anaesthetized with Nembutal and the embryos were dissected out, and their brains triturated in 0.25% trypsin. The dissociated cortex was centrifuged and the cells resuspended in neurobasal media with B27 supplement and plated onto PDL-coated culture plates. Non-neuronal cells were removed by treatment with fluoro-deoxy-uridine (EDU) after 18 h of culture. The cells were replenished with fresh media every fourth day. The experiments were done using 1-week-old cultures.

The ACT and AAT were reconstituted according to the manufacturer’s protocol and used at a concentration of 0.5 or 1 mg/ml in the culture media, an ACT level similar to that found in Alzheimer’s disease brain (Abraham et al., 1988; Lieberman et al., 1995).
Human neurons were purchased from ScienCell Research Labs (San Diego, CA) and cultured according to the manufacturer's protocol.

**Immunocytochemistry and immunohistochemistry**

Cortical neurons were plated onto 8-chamber slides pre-coated with PDL for immunocytochemical analysis. Cells were cultured as indicated above for 1 week. ACT was added to the wells at a concentration of 0.5–1 mg/ml. At the end of the treatment, cells were fixed with 4% para-formaldehyde for 10 min, washed with PBS and incubated for 1 h at room temperature with 10% normal goat serum (NGS) in Tris-buffered saline (TBS) containing 0.2% Triton-X-100 (TBST) to inhibit non-specific binding. Primary antibody diluted in 1% bovine serum albumin (BSA)/TBST was added to the cells and incubated overnight at 4°C. Cells were washed several times with PBS and incubated with secondary antibodies (Alexa 488 and Alexa 594) for 1 h at room temperature. Slides were washed in PBS and mounted using aquamount. The staining was visualized and analysed under a Nikon Eclipse E1000 microscope using Image Proplus software.

For immunohistochemical analysis of the brain, the sections were mounted on Superfrost slides and non-specific binding was blocked by incubating the sections with 10% NGS/TBST for 2 h at room temperature. Sections were then incubated with appropriate dilutions of the primary antibody (PHF-1, P-Ser202, P-Thr231 and TG5 antibodies) in 1% BSA/TBST overnight at 4°C in a humidified chamber. After thorough washing, the sections were incubated with appropriate dilutions of biotinylated mouse or rabbit secondary antibodies for 1 h at room temperature. Slides were incubated with ABC reagent (Vectastain kit) and developed with the DAB kit from Vector Laboratories. The staining was visualized and analysed using a Nikon Eclipse E1000 fluorescence microscope using the Genus 2.81 software from Applied Imaging.

For paraffin-embedded human brain sections from tauopathies, the slides were de-paraffinized by warming at 60°C for 1 h and immersing in xylene. The slides were re-hydrated and the antigen recovery was performed by warming the slides for 10 min at 95°C in 10 mM citrate buffer, pH 6.0. Endogenous peroxidase activity was inhibited by incubation in 0.5% H2O2 in methanol for 20 min, after which slides were washed in PBS and the staining was followed as above using the appropriate antibodies.

**Western blot analysis**

Cortical neurons plated onto PDL-coated 6-well plates were treated with ACT for different time periods. At the end of the incubation, cells were washed with PBS and cell extracts were made in Tricine sample buffer (Invitrogen). Samples were boiled and aliquots of equal amounts of protein were separated on a 10–20% Tris–Tricine gradient gel. The proteins were electro-blotted to a nitrocellulose membrane. Non-specific binding was blocked by incubation with 5% non-fat dry milk in TBS for 2 h at room temperature and incubated over night at 4°C with primary antibodies diluted in 3% BSA/TBS. The blots were washed thoroughly with PBS containing 0.05% Tween-20, and incubated with peroxidase-conjugated secondary antibodies diluted in blocking buffer for 2 h at room temperature and developed using the super signal ECL reagent. For analysis of brain extract, equal amounts of protein from the extracts made in HEPES lysis buffer were boiled with Tricine sample buffer and PAGE and western immunoblot analysis was performed as described above.

**TUNEL assay in cortical neurons treated with ACT**

In order to determine whether the ACT treatment results in apoptosis in cortical neurons, we performed TUNEL assays. Cells were treated with ACT as described in the immunocytochemistry protocol, and fixed with para-formaldehyde. After washing the cells with PBS, the TUNEL assay was performed according to the manufacturer’s protocol using the fluorescein conjugated probe. Cells were counter stained with Hoechst to assess the nuclear morphology. The Hoechst staining was performed by incubating the slides for 5 min at room temperature with 1 μg/ml Hoechst no. 33342 (Sigma) diluted in PBS. The slides were washed and mounted using aquamount for analysis with the fluorescent microscope.

Statistical analysis was performed using Student’s t-tests.

**Results**

**Increased level of ACT Immunoreactivity in FTDP, CBD and PSP brain samples**

It has been shown that ACT expression is significantly increased in the brains of Alzheimer’s disease patients and ACT is an integral component of the amyloid plaques (Abraham et al., 1988, 1990; Pasternack et al., 1989). Although the involvement ACT in Aβ polymerization is established, nothing much is known about its role in tangle formation. In order to determine the correlation between tau hyperphosphorylation and ACT expression, we first examined the brains of a variety of tauopathies in which tau is hyperphosphorylated and forms NFT as in Alzheimer’s disease. Specifically, we examined brains from frontotemoral dementia and parkinsonism linked to chromosome 17 (FTDP-17), PSP and CBD as well as normal controls for ACT expression using a polyclonal human ACT antibody. Immunostaining analysis of the sections was also performed using the PHF-1 tau antibody to confirm the presence of tau pathology (Fig. 1A). Our analysis revealed that under all the conditions where tau hyperphosphorylation was present (PHF-1 positive), an increase in the level of ACT immunoreactivity was also visible (Fig. 1B and D). We analysed brains from three independent individuals with or without disease in each case and the histogram represents the quantification from those samples. It appeared that the ACT immunoreactivity was increased in both neurons as well as astrocytes in the tauopathy samples (Fig. 1C).

**Increased tau phosphorylation in mice expressing the human ACT gene alone and in combination with mutant APP**

A striking difference between Alzheimer’s disease and normal brains is the presence of gliosis in Alzheimer’s disease and the overexpression of inflammatory proteins such as ACT in the activated astrocytes surrounding amyloid
plaques (Abraham et al., 1988, 1990; Pasternack et al., 1989; Alzheimer et al., 1995). Hyperphosphorylation of tau is believed to be necessary and sufficient for PHF formation in Alzheimer’s disease (Delacourte and Defossez, 1986; Kosik et al., 1986, 1988; Lee et al., 1989, 1990; Mandelkow and Mandelkow, 1993; Goedert, 1996). Since we observed increased ACT staining in tauopathy brain samples, we hypothesized that ACT overexpression contributes to tau hyperphosphorylation. To test this hypothesis we used antibodies raised against Alzheimer’s PHFs that recognize tau phosphorylation on the major sites and analysed P-tau in age-matched mice expressing human ACT, human mutant APP (V717F), a combination of ACT and APP and non-transgenic normal control mice. The brains of these mice were also analysed for the presence of plaques using the APP specific 6E10 antibody. The mice expressing APP alone and in combination with ACT showed significant levels of plaque staining (Fig. 2A, row 1: columns 3 and 4). Immunohistochemical analysis revealed that there were also increased levels of P-Ser396/P-Thr404 (PHF-1) (Fig. 2A, row 2), and P-Ser202 (CP13) (Fig. 2A, row 3) phosphorylated tau in mice expressing human ACT, mutant APP as well as ACT and APP together compared with the non-transgenic (NTG) mice. PHF-1 staining and P-Ser202 staining appeared to be cellular in the mice expressing human ACT (Fig. 2A insets in column 2 row 2, and column 2 row 3). In the case of APP mice, the P-tau staining appeared to be present in the processes surrounding the plaques (Fig. 2A insets in column 3 row 2, and column 3 row 3). When both transgenes were present, the P-tau staining around the plaque was visibly increased (Fig. 2A insets in column 4 row 2, and column 4 row 3). Analysis with the conformation
specific antibody MC1 showed an increased staining in the brains of mice overexpressing APP alone (inset, column 3 row 4) and in combination with ACT (inset, column 4 row 4). When ACT was co-expressed with APP the staining was increased around the plaques. These results suggested that the Aβ generated by APP overexpressing mice was associating with tau and altering its conformation. The level of total tau was not altered in these mice as evident from the staining with TG5 antibody that detects the total amount of tau (data not shown). The increase in PHF-1 and P-Ser202 tau hyperphosphorylation was confirmed by western blot analysis of the brain samples from these mice (Fig. 2B). We believe that in the case of mice expressing APP and ACT/APP, the hyperphosphorylated tau accumulates around the plaques making it insoluble and affecting the actual level detected by western blot analysis. The histogram in 2B shows the ratio of phosphorylated tau in transgenic mouse to that in the NTG. The western blot shown is representative of three independent experiments using brains from three independent mice. Taken together (Fig. 2A and B) it is clear

Fig. 2 Phospho-tau analysis in transgenic mice expressing human ACT, human mutant APP (V717F) and ACT/APP: Brains from one year old transgenic mice expressing human ACT or APP alone or together were analysed by immunohistochemistry (IHC) and western blot analysis. (A) Immunostaining in the mice brain sections using 6E10 to assess APP expression and, PHF-1, P-Ser202 (CP13), MC1 (conformation specific) antibodies to assess changes in tau. The inset shows ×100 magnification of the plaque or cell staining in each section. B represents the levels of P-Ser202 and the PHF-1 tau in the brains of different transgenic mice assessed by western blot analysis. (*P < 0.05).
that the overexpression of the inflammatory protein ACT correlated with the induction of tau hyperphosphorylation and suggested the possible involvement of ACT in this process.

**Induction of tau phosphorylation in cortical neurons after ACT treatment**

Although we observed an increase in tau phosphorylation in brains of human tauopathies and in human ACT and mutant APP overexpressing transgenic mouse brains, it was not clear whether ACT was actually inducing tau hyperphosphorylation. In order to test this possibility, we studied the effect of purified ACT in *in vitro* cultured cortical neurons. Towards this purpose cortical neurons were cultured from brains of E18 mouse embryos and treated with ACT for 1, 2, 4, 6 and 24 h. Samples were prepared and subjected to electrophoresis and western blot analysis at the end of the incubation periods. We found that the treatment of cortical neurons with ACT induced tau hyperphosphorylation at PHF-1 (Ser396/Thr404), P-Ser202 and P-Thr231. Phosphorylation was significantly higher at the times indicated (Fig. 3A, B and C), with maximum at
around 2 h and the level had begun to decline by 24 h (data not shown). The blot above each histogram is representative of three independent experiments with the specified P-tau antibody. The blots were re-probed with HT-7 antibody to determine the total levels of the different tau isoforms (Fig. 3D) and data showed that the ACT treatment specifically affected the tau hyperphosphorylation without changing the level of total tau. The increase in tau hyperphosphorylation observed in in vitro ACT-treated neurons was relatively higher than that observed in vivo in brains from mice expressing ACT transgene. We believe that this is due to the difference in the level of ACT used in vitro compared with that expressed in vivo. In our future studies we will try to induce increased expression of ACT in the human ACT mice by injury (Nilsson et al., 2001a) and determine the tau hyperphosphorylation under this condition.

In order to prove that the effect on tau hyperphosphorylation observed with the purified ACT was specific to ACT and not due to an impurity in the preparation, tau phosphorylation in cortical neurons treated with similarly-purified AAT was determined. Cortical neurons were treated with same concentrations (0.25–1.0 mg/ml) of AAT or ACT for 3 h and extracts were analysed by western immunoblot. AAT treatment did not show even a slight increase in the level of tau hyperphosphorylation, whereas ACT treatment resulted in enhanced PHF-1 (P-Ser396/P-Thr404), P-Ser202 and P-Thr231 tau phosphorylation (Fig. 4). While P-Ser202 and P-Thr231 were induced by ACT concentrations above 0.5 mg/ml, an increase in PHF-1 phosphorylation was visible at 0.25 mg/ml. The result shown is a representation of two independent experiments performed.

**Immunocytochemical analysis of PHF-1 and P-ser202 tau in ACT-treated cortical neurons**

The phosphorylation of tau in mouse cortical neurons treated with ACT was further examined by immunocytochemistry. Cells plated onto 8-chamber slides were treated with ACT for different time periods and co-immunostained with PHF-1 monoclonal antibody and P-Ser202 polyclonal antibody. Alexa 488 and 594 fluorophores were used to detect the signals as described in the Material and methods. We observed an increase in the PHF-1 and P-Ser202 tau staining in cortical neurons after ACT treatment for 3 and 6 h (Fig. 5A). The wells that received ACT treatment for 24 h showed many cells with condensed nuclei, suggesting that ACT induces cell death in cortical neurons upon prolonged treatment. This result may explain the decrease in the level of phospho-tau observed at 24 h by western blot analysis (data not shown). After 24 h of ACT treatment, the cells showed PHF staining mainly in the base of the short, thick neurite, whereas at 3 and 6 h after ACT treatment, more staining was visible in the neurites.

We also examined cultures of human brain neurons treated with ACT for changes in tau phosphorylation by immunostaining using P-Ser202 and PHF-1 antibodies. These cells also showed an increase in P-Ser202 and PHF-1 tau phospho-epitopes, further confirming that ACT indeed induced tau hyperphosphorylation (Fig. 5B). As in the case of mouse cortical neurons, the ACT-induced tau hyperphosphorylation was detectable at 3 and by 24 h the cells appeared to have beaded neurites. Since these cultures were a mixed population of neurons and glia, we did not use them for western blot analysis.

**Immunostaining of the neurons with NeuN and ACT antibodies**

Immunostaining experiments have shown that ACT is found not only in astrocytes where it is produced, but also within neurons in Alzheimer’s disease brain (Abraham et al., 1988). To determine whether the immunostaining of ACT observed in vivo in neurons could be due to the internalization of ACT secreted from astrocytes, we examined whether the in vitro added ACT was taken up by cultured neurons. The mouse neurons were treated with 0.5 mg/ml ACT for different time periods and analysed for ACT immunoreactivity using a polyclonal human ACT antibody. The cells were co-stained with a neuronal marker, NeuN antibody. The results showed that neurons treated with ACT became strongly ACT immunoreactive (Fig. 6A and B). The number of NeuN positive cells was significantly higher in the untreated wells compared with that in the wells treated with...
Fig. 5 Detection of PHF-1 and P-Ser202 in cortical neurons by immunostaining. (A) Mouse cortical neurons were cultured in 8-chamber slides and treated with or without 0.5 mg/ml ACT for 3, 6 or 24 h and probed with PHF-1 monoclonal and P-Ser202 tau polyclonal antibodies. The PHF-1 antibodies stained the neurites whereas the P-Ser202 antibody stained the cell body. (B) P-Ser202 and PHF-1 tau staining in human neurons treated with ACT.
ACT (60–80% less compared with the number in untreated), suggesting that ACT might have been inducing apoptosis in the neurons. By 24 h, the cells that were most strongly positive for ACT showed shrinkage of the cell body and were negative for NeuN staining. The strong immunoreactivity of the cells towards ACT antibody suggests that the neurons were internalizing ACT and undergoing neurodegeneration. Similar results were obtained with human neurons treated with ACT; the neurons that endocytosed ACT showed degeneration and loss of neurites as observed by co-staining with ACT and β-tubulin III antibodies (Fig. 6C). By 24 h the neurons were left with almost no neurites and aggregated tubulin was present in the cell body. Unlike NeuN antibody, the β-tubulin III antibody retained the immunoreactivity towards the dying neurons after ACT treatment. We examined this with the mouse neurons as well and the data were identical (data not shown) suggesting that NeuN is a good marker for only healthy mature neurons. Contrary to NeuN, β-tubulin III is a marker for mature and immature neurons and thus the results suggest that ACT could be inducing cell cycle activation in the neurons leading to apoptosis.

**ACT induces cell death in neurons**

The above experiments suggested that *in vitro* treatment of cortical neurons with ACT resulted in ACT internalization and the induction of tau phosphorylation. The tau
phosphorylation decreased by 24 h, the time at which the maximum number of ACT positive cells was observed. This finding, along with the observation that cells strongly positive for ACT showed nuclear condensation suggested that ACT was inducing apoptosis in the neurons, a hypothesis that was tested by TUNEL staining. Neurons were treated with 0.5 mg/ml ACT for different time periods, and the TUNEL staining was performed according to the manufacturer’s protocol using the FITC labelled probe. Co-staining with Hoechst was performed to analyse the nuclear morphology of the cells after the treatment. Our results showed that there was a significant increase in the number of TUNEL positive cells after ACT treatment, and that the number reached a maximum at 24 h (Fig. 7A and B). The Hoechst staining showed condensed nuclei or fragmented DNA after ACT treatment of the neurons, also suggesting that these cells were undergoing apoptosis.

**ACT induces ERK and glycogen synthase kinase-3 (GSK-3) activation in cortical neurons**

The observation that ACT treatment of cortical neurons led to an increase in phosphorylation of tau protein prompted us to examine the kinase(s) responsible for this phosphorylation. We first examined the early extracellular signal regulated kinase, ERK, (MAP kinase) since it is one of the kinases known to phosphorylate tau and ERK has been shown to be associated with NFT and senile plaques (Trojanowski et al., 1993; Pei et al., 2002; Zhu et al., 2002). We treated the mouse cortical neurons for different time intervals with 1 mg/ml ACT; cells were fixed and probed with a phospho-specific ERK antibody (Thr202/Tyr204, Cell Signaling) and Alexafluor 568 secondary antibody. The results showed that ACT treatment induced an increase in the phosphorylation of ERK within 30 min (panel C, Fig. 8) of treatment. By 24 h there was a reduction in the number of P-ERK positive neurons (data not shown). This P-ERK activation was confirmed by western blot analysis as well. ACT-induced a dose-dependent increase in phosphorylation of ERK in cortical neurons (with 0.75 and 1.0 mg/ml ACT inducing maximum P-ERK) compared with that treated with AAT (Fig. 9A) for 3 h. This again clearly shows that the effect observed with ACT is specifically due to this inflammatory protein and not due to any impurities in the preparation. The result shown is a representation of two independent experiments.

Another major kinase shown to be associated with neurodegenerative diseases and to induce tau hyperphosphorylation is the glycogen synthase kinase 3β (GSK-3β). Since ACT is an acute phase protein expressed under inflammatory conditions and since GSK-3 phosphorylates tau at Thr231 (Cho and Johnson, 2004), we decided to examine this kinase in the neuronal extracts after ACT treatment. Mouse cortical neurons were treated with ACT for 1, 2, 4 and 6 h and western immunoblot analysis was performed using a phospho-specific antibody that detects both alpha and beta isoforms of GSK-3 phosphorylated at Ser21 (S21) or Ser9 (S9) on alpha and beta, respectively. We found a significant decrease in the phosphorylation state of both α and β GSK-3 at 1 h continued to 6 h (Fig. 9B) after ACT treatment. Dephosphorylation of GSK-3 at S21
and S9 has been shown to be associated with its activation and the result we observed with ACT suggests that this could be one of the possible mechanisms involved in ACT-induced tau hyperphosphorylation.

**Discussion**

Alzheimer’s disease is a neurodegenerative disease associated with neuronal loss in hippocampus and cortex (Ball, 1977; Arendt et al., 1985; Bobinski et al., 1998; Busser et al., 1998;
It is characterized by the amyloid deposition, as well as by tau hyperphosphorylation and tangle formation (Wilcock and Esiri, 1982; Miller et al., 1984; Alzheimer et al., 1995; Trojanowski et al., 1995; Fukutani et al., 1997). Several studies have shown that Aβ generation is increased by mutations in APP and the presenilin 1 and 2 genes (Hardy, 1997). The excess Aβ produced by neurons affects surrounding astrocytes and microglia, resulting in their activation and expression of proteins normally associated with inflammation. A number of such inflammatory proteins have been found to be overexpressed in affected areas of Alzheimer’s disease brain, including complement, IL-1β, TNF-α, ApoE, IL-6 and ACT (Abraham et al., 1988; McGeer et al., 1989; Pasternack et al., 1989; Rogers et al., 1992; Wisniewski and Frangione, 1992; Mrak et al., 1995; Nilsson et al., 1998; Potter et al., 2001). For example, the microglia around the plaques in Alzheimer’s disease brain have been shown to express a high level of the cytokine IL-1β that correlate with the extent of the disease pathology (Griffin et al., 1995; Sheng et al., 1995; Mrak and Griffin, 2001). This suggests that the elevated levels of Aβ may be causing an inflammatory response, leading to an increase in the expression of proteins associated with inflammation. IL-1β has been shown to enhance the expression of ACT in human astrocytes and to increase the translation of APP (Das and Potter, 1995; Lieb et al., 1996; Rogers et al., 1999; Kordula et al., 2000; Nilsson et al., 2001b; Kiss et al., 2005). The finding that ACT is associated with amyloid and is expressed only in regions where there is overexpression of IL-1β in Alzheimer’s disease suggests that this coupled expression may be of significant importance to the pathogenesis of plaques and tangles associated with the disease. Indeed, mixed cultures of human glia from amyloid prone cortical tissue activate and express IL-1 and ACT, while no such inflammatory activation arises in mixed glial cultures from cerebellum, suggesting that the regional specificities of amyloid deposition in Alzheimer’s disease may reflect on basic differences in inflammatory capacity between different brain regions (Das and Potter, 1995). Accordingly, the previous finding that ACT binds Aβ and enhances its fibrillation along with the current finding that ACT induces Alzheimer’s disease specific tau phosphorylation leads to a new insight: that inflammatory molecules affect the entire spectrum of pathology associated with this disease.

The mechanism by which tau becomes hyperphosphorylated in Alzheimer’s disease is not clear. In vitro studies analysing phosphorylation of tau have shown that a number of kinases can induce phosphorylation of tau at specific sites that are similar to those observed to be hyperphosphorylated in Alzheimer’s disease brains (Arriagada et al., 1992; Vincent and Davies, 1992; Iqbal et al., 1993; Vincent et al., 1994; Goedert, 1996). Furthermore, inhibition of GSK-3 has been shown to be associated with a decrease in tau hyperphosphorylation and neurodegeneration (Lucas et al., 2001; Hernandez et al., 2002; Noble et al., 2005; Engel et al., 2006). These results, along with the finding that some of these kinases are elevated in the brains of Alzheimer patients, suggest that proteins associated with inflammation could activate one or more kinases contributing to tau hyperphosphorylation and tangle formation. Based on the results reported above, we hypothesize that the increased Aβ level in Alzheimer’s disease brain activates glia to express inflammatory proteins such as IL-1 and thus ACT, which in turn increases expression or activation of certain kinases, such as ERK and GSK-3, resulting in tau hyperphosphorylation and neuronal degeneration. Therefore our results suggest that inflammatory proteins can activate a cascade of signalling pathways leading to neurodegeneration. Interestingly, a correlation between inflammation and GSK-3 activation is supported by the work from different groups (Dugo et al., 2005; Cuzzocrea et al., 2006; Dugo et al., 2006). Thus our current data open up a new pathway by which Aβ may bring about the pathology observed in Alzheimer’s disease through induction of an inflammatory cascade.

Studies done in vivo and in vitro have shown that non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of Alzheimer’s disease possibly through the suppression of neuroinflammation (McGeer et al., 1990, 1994a; McGeer and Rogers, 1992; Rogers et al., 1993, 1996; Weggen et al., 2001, 2003a, b; Klegeris and McGeer, 2005). For example patients who took NSAIDs regularly showed a lower risk of Alzheimer’s than those who did not. The fact that diagnosed patients treated with NSAIDs showed no improvement suggests that the inflammatory proteins, like ACT, perhaps influence the early stages of Alzheimer’s disease and an intervention at this stage with the anti-inflammatory drugs is important in achieving a beneficial effect. This interpretation is further supported by the studies from Cole’s group (Morihara et al., 2005) who showed that anti-inflammatory drug ibuprofen reduces the expression of IL-1β as well as ACT in transgenic mice expressing the Swedish mutant of APP. They suggest that the positive effect of NSAIDs observed in reducing plaque pathology and Aβ oligomerization may not be due to a direct effect of the anti-inflammatory drug on Aβ, but could be due to its inhibitory effect on the proinflammatory cytokine IL-1β and the acute phase reactant ACT. Indeed, there is evidence suggesting that activated microglia and astrocytes facilitate neurodegeneration in Alzheimer’s disease by not only accelerating amyloid pathology, but also by enhancing the NFT formation (McGeer et al., 1994b; DiPatre and Gelman, 1997; Griffin and Mrak, 2002; Kitazawa et al., 2004; Licastro et al., 2004). In vitro ACT treatment led to hyperphosphorylation of tau and neurite degeneration and neuronal apoptosis in cortical neurons. Thus, our finding that ACT can induce tau hyperphosphorylation and apoptosis in neurons provides a new link between Alzheimer’s pathology and inflammation and is consistent with the previous findings.

In summary, our studies reveal a novel mechanism by which an imbalance in the proteins associated with
inflammation can lead to one of the characteristic pathologies observed in Alzheimer’s disease, specifically tangle formation. The role of inflammation in plaque formation has been well studied with important implications for therapy. These new data now suggest that a similarly thorough analysis of the mechanisms by which ACT induces tau hyperphosphorylation and neuronal apoptosis will also open up new avenues to the development of novel Alzheimer’s disease therapies.

Acknowledgements

We would like to thank Dr Peter Davies for the generous supply of tau antibodies, and Tiffany Hughes for genotyping and other technical assistance. This work was supported by grants AG09665 and 1P50AG025711-01 from the NIA to H.P.

References


