Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer’s disease pathology

Rebecca J. Griffin,* Aileen Moloney,* Mary Kelliher,* Janet A Johnston,† Rivka Ravid,‡ Peter Dockery,§ Rosemary O’Connor* and Cora O’Neill*

*Department of Biochemistry, BioSciences Institute, University College Cork, Cork, Ireland
†School of Biology and Biochemistry, Queen’s University Belfast, Medical Biology Centre, Belfast, UK
‡The Netherlands Brain Bank, Amsterdam, the Netherlands
§Department of Anatomy and BioSciences Institute, University College Cork, Cork, Ireland

Abstract

Studies suggest that activation of phosphoinositide 3-kinase-Akt may protect against neuronal cell death in Alzheimer’s disease (AD). Here, however, we provide evidence of increased Akt activation, and hyperphosphorylation of critical Akt substrates in AD brain, which link to AD pathogenesis, suggesting that treatments aiming to activate the pathway in AD need to be considered carefully. A different distribution of Akt and phospho-Akt was detected in AD temporal cortex neurons compared with control neurons, with increased levels of active phosphorylated-Akt in particulate fractions, and significant decreases in Akt levels in AD cytosolic fractions, causing increased activation of Akt (phosphorylated-Akt/total Akt ratio) in AD. In concordance, significant increases in the levels of phosphorylation of total Akt substrates, including: GSK3β(Ser9), tau(Ser214), mTOR(Ser2448), and decreased levels of the Akt target, p27kip1, were found in AD temporal cortex compared with controls. A significant loss and altered distribution of the major negative regulator of Akt, PTEN (phosphatase and tensin homologue deleted on chromosome 10), was also detected in AD neurons. Loss of phosphorylated-Akt and PTEN-containing neurons were found in hippocampal CA1 at end stages of AD. Taken together, these results support a potential role for aberrant control of Akt and PTEN signalling in AD.

Keywords: Akt/PKB, Alzheimer’s disease, glycogen synthase kinase 3β, neurofibrillary tangles, PTEN.


Alzheimer’s disease (AD) is characterised by the excessive accumulation of extracellular amyloid-β (Aβ)-containing plaques, and intraneuronal neurofibrillary tangles (NFTs), composed predominantly of abnormally hyper-phosphorylated tau protein. The accumulation of these lesions is believed to be the driving force for AD neurodegeneration, however, the signal transduction events that enable the pathologies are not well understood. Recently, attention has been drawn to signalling through phosphoinositide 3-kinase (PI3-kinase), and its downstream serine-threonine kinase, Akt (also known as protein kinase B, PKB), as a system that may link Aβ, NFTs and neuronal loss in AD. PI3-kinase Akt is activated by a wide variety of stimuli, including insulin, growth factors, cytokines and cellular stresses, to regulate diverse biological processes such as cellular survival, proliferation, growth, motility and metabolic functions (Lawlor and Alessi 2001; Brazil et al. 2004). Downstream targets of Akt include, GSK3, Bad, mTOR, mammalian target of rapamycin; NFT, neurofibrillary tangle; PBS-T, phosphate-buffered saline, Tween; PHF, paired helical filament; PI3-K, phosphoinositide-3-kinase; PIPES, piperazine-N,N’-bis-(2-ethanesulfonic-acid); PMSF, phenylmethylsulfonyl fluoride; PtdIns(3,4,5)P3, phosphoinositide-3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; TBS-T, Tris buffered saline, Tween.
mTOR, the Forkhead family of transcription factors, p27kip and NF-xB.

All Akt isoforms are highly expressed in the nervous system and Akt is a vital promoter of survival and neuroprotection in embryonic neurons (Brunet et al. 2001). Studies supporting the link between Akt signalling and AD have shown that PI3-kinase-Akt activation protects against Aβ neurotoxicity in vitro (Martin et al. 2001; Wei et al. 2002) and possibly in vivo in mouse models of AD (Stein and Johnson 2002). It has also been suggested that familial AD causing mutations in presenilin 1 may promote AD pathology by inhibiting the PI3-kinase-Akt pathway (Baki et al. 2004). The Akt-GSK3 axis is of importance in AD, as the constitutive activation of GSK3α/β is inactivated by Akt phosphorylation at GSK3α Ser21 and GSK3β Ser9, respectively. GSK3α can promote Aβ production in vitro (Phiel et al. 2003), and GSK3β is a major candidate kinase for tau hyperphosphorylation (Lovestone and Reynolds 1997; Bhat et al. 2004), thus Akt activation would be predicted to block both events. In line with this, Akt induced phosphorylation of GSK3β Ser9 (Hong and Lee 1997), by insulin/IGF-1, inhibits tau phosphorylation in vitro.

The above studies have led to the suggestion that PI3-kinase-Akt signalling is impaired in AD brain and that treatments activating this pathway should enhance neuronal survival and block AD pathology. However, contrary to expectations, a recent study of human post-mortem brain reported increased, rather than decreased, accumulation of hyperphosphorylation in AD associated with the development of neurofibrillary changes (I–VI) was performed according to the NINCDS–ADRDA criteria, and severity of dementia rated by the Global Deterioration Scale. Non-disease controls (n = 19) had no history or symptoms of neurological or psychiatric disorders. All cases were neuropathologically confirmed, using conventional histopathological techniques, and diagnosis performed using the CERAD criteria. Neuropathological staging of neurofibrillary changes (I–VI) was performed according informed consent from the donors or the next of kin was obtained in all cases. Clinical diagnosis of ‘probable Alzheimer’s disease’ (n = 19) was made according to the NINCDS–ADRDA criteria, and severity of dementia rated by the Global Deterioration Scale. Non-disease controls (n = 17) had no history or symptoms of neurological or psychiatric disorders. All cases were neuropathologically confirmed, using conventional histopathological techniques, and diagnosis performed using the CERAD criteria. Neuropathological staging of neurofibrillary changes (I–VI) was performed according

### Materials and methods

#### Brain tissue

Brain tissue was provided by the Netherlands Brain Bank (see Tables 1 and 2 for case details). Ethical approval and written consent of the donors or the next of kin was obtained in all cases. Clinical diagnosis of ‘probable Alzheimer’s disease’ (n = 19) was made according to the NINCDS–ADRDA criteria, and severity of dementia rated by the Global Deterioration Scale. Non-disease controls (n = 17) had no history or symptoms of neurological or psychiatric disorders. All cases were neuropathologically confirmed, using conventional histopathological techniques, and diagnosis performed using the CERAD criteria. Neuropathological staging of neurofibrillary changes (I–VI) was performed according
Table 2 Subject and post-mortem details of cases used for immunocytochemical analysis

<table>
<thead>
<tr>
<th>NFT stage</th>
<th>Senile plaques (years)</th>
<th>Age</th>
<th>Gender</th>
<th>Tissue pH</th>
<th>Tissue weight (g)</th>
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<td>1392 T</td>
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PMD, post-mortem delay; M, male; F, female; H, hippocampus; T, temporal cortex. See Materials and methods for further details.

to Braak and Braak (1991). The degree of Aβ deposition in neuritic senile plaques was assessed in the temporal cortex, indicated as 0, +, ++ and +++, for no, mild, moderate and high levels of senile plaques, respectively. One case C7LB (Table 1) was classified originally as a non-AD control case with stage IV pathology, but discovered subsequently to have some Lewy bodies, and is included in the control group. One case C6, had high levels of senile plaques, and was also Braak stage C for amyloid deposits. However, this patient did not have clinical dementia.

Preparation of tissue fractions

For western immunoblot analysis, medial temporal cortex was dissected, and meninges and white matter removed, prior to snap-freezing, and storage at ~70°C. Tissue fractions for western immunoblot analysis were prepared from two separate groups of AD and matched control temporal cortex samples in the course of this study (group 1; control n = 7, AD n = 7; group 2; control n = 7, AD n = 9, Table 1). Tissue fractions from group 2 were prepared subsequent to the preparation of fractions from group 1, as insufficient material remained in group 1 cases to continue with further analysis. In each group, AD and control tissue were very well matched and no significant difference existed in post-mortem delay, tissue pH (as an index of agonal status and tissue quality) or age between the AD and control cases (Table 1). Tissue fractions were prepared as described previously (Kellihir et al. 1999). Briefly, frozen brain tissue was thawed rapidly at 37°C in homogenisation buffer, 20 mM PIPES-KOH (pH 7.4), 0.32 M sucrose, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Tissue was homogenised using 10 volumes (w/v) of homogenisation buffer and centrifuged in a Beckman Ultracentrifuge at 100 000 g for 60 min at 4°C (Beckman Type 42.1 rotor). Particulate fractions (100 000 g pellet) were separated from cytosolic fractions (100 000 g supernatant) and suspended in 5 volumes (w/v) of homogenisation buffer, prior to storage of fractions at ~70°C. Protein concentration was determined using a modification of the Lowry procedure (Markwell et al. 1978).

Immunoblotting

Proteins were separated by SDS–PAGE (20 μg/lane), and transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell 0.2 mm) as previously described (Kellihir et al. 1999). Nitrocellulose membranes were blocked for 1 h in 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.1% Tween 20 (TBS-T) and 5% non-fat milk, and incubated overnight at 4°C with primary antibody diluted in TBS-T with 5% non-fat milk. Antiserum dilutions and sources were as follows: Akt (1 : 1000); phosphoSer473-Akt (1 : 500); PTEN (1 : 1000); phosphoSer9-GSK-3β (1 : 500); phosphoSer2448-mTOR (1 : 800); mTOR (1 : 1000); and phospho-Akt substrate (1 : 1000); all obtained from Cell Signalling Technology, Hitchin, UK; GSK3β (1 : 2500, BD Transduction Laboratories, Lexington, KY, USA); AT100 (1 : 100, Innogenetics, Ghent, Belgium); phosphoSer214-tau (1 : 500, Biosource International, Nivelles, Belgium); p7Nck1 (1 : 2500, BD Transduction Laboratories). Nitrocellulose membranes were washed three times in TBS-T, and incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (1 : 1000), depending on primary antiseria used, in TBS-T for 1 h, followed by washing in TBS-T. Immunoreactive proteins were detected with enhanced chemiluminescence (ECL) (Amersham Biosciences, Little Chalfont, UK). Blots were stripped in stripping buffer (2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol) and then probed with antibody against β-actin. Quantification of results was performed by densitometry (GeneGenius gel documentation and analysis system/Syngene, Cambridge, UK), quantifying the density of identically sized areas (corresponding to immunoreactive bands) and results analysed as total integrated densitometric volume values (arbitrary units). Results are reported as mean values ± SEM. The significance of differences between control and AD group was assessed by two-tailed Student’s t-test with p < 0.05 considered statistically significant.

Immunohistochemistry

Brain tissue was formalin fixed and paraffin embedded, and 8-μm-thick consecutive sections prepared on Superfrost slides (BDH Laboratory Supplies, Poole, UK). Control and AD tissue was matched for post-mortem delay, tissue pH and age (Table 2). Classical immunohistochemical procedures based on the avidin-biotin horseradish peroxidase method were applied. Sections (8 μm) were de-paraffinised and hydrated prior to microwave pre-treatment (15 min in 10 mM citrate buffer pH 6.4) to reveal phospho-Akt and PTEN immunoreactivity. Sections were incubated in 0.3% hydrogen peroxide in ethanol for 20 min, and blocked in 10% normal goat serum (Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (PBS) pH 7.4, followed by overnight incubation with primary antibody (phosphoSer473-Akt: 1 : 35, Cell Signalling Technology New England Biolabs Freemont, CA, USA; PTEN: 1 : 75, Neomarkers, Freemont, CA, USA) at 4°C. For PHF-1 (1 : 500, a generous gift from Dr Peter Davies, Albert Einstein College, Bronx, NY), antigen unmasking was not necessary and sections were blocked in 1.5% normal goat serum in TBS pH 7.4. Primary antibody was detected using isotype-specific biotin-labelled secondary antibody (Vector Laboratories, Burlingame, CA, USA), with
HRP conjugated avidin-biotin complex (ABC kits, Vector Laboratories). The presence of HRP was detected using 0.5 mg/mL 3’-diaminobenzidine (DAB) (Vector Laboratories). Sections were counterstained with Harris’ haematoxylin (BDH Laboratory Supplies). Negative control reactions, excluding primary or secondary antibody, were included in each analysis. Absorption experiments were performed to ensure the specificity of the reactivities observed.

Immunofluorescence in association with confocal microscopy was also used to visualise phospho-Akt immunoreactivity in formalin fixed paraffin embedded AD and control sections (Table 2). Following microwave treatment (15 min in 10 mM citrate buffer pH 4.0), sections were blocked in 10% normal donkey serum (Sigma-Aldrich) in PBS pH 7.4, followed by overnight incubation (4°C) with phosphoSer473-Akt (1 : 25, Cell Signalling Technology New England Biolabs). Slides were then incubated in Cy3 conjugated donkey anti-rabbit IgG secondary antibody (1 : 2000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at 22°C in the dark. In order to lower the intensity of lipofuscin autofluorescence, the slides were incubated for 5 min in 0.1% Sudan black B (Sigma-Aldrich) in 70% alcohol. Slides were then washed thoroughly in PBS, and mounted in Mowiol (Calbiochem, La Jolla, CA, USA). Images were captured using an MRC 1024 laser scanning confocal system (Bio-Rad Microscience Ltd, Hertfordshire, UK), mounted on a Zeiss Axioskop microscope (Germany). The sections were visualised using a water immersion lens (40×), and analysed at normal scan speed, with the step size between each plane of focus set at 0.36 μm. The Confocal Assistant processing package (Version 3.10, Todd Clarke Brelje) was used for image analysis and processing.

Morphometric analysis and co-localisation studies
Morphometric analysis in the hippocampal formation was performed in the CA3 and CA1 subfields of control (n = 4) and AD (n = 6) sections. The number of PTEN and phospho-Akt positive neurons was estimated using the volume fraction of tissue occupied by neuronal immunoreactivity, as determined by standard stereological point counting methods (Weibel 1979; Delatour et al. 2001). Each counting field was examined through a ×10 objective, and visualised through a camera connected to a microscope on a computer screen on which a square grid with 1225, 3-mm interspaced, points was applied. Points falling onto the immunoreactive neurons were summed for each field and divided by the total number of points to give the proportion of PTEN or phospho-Akt immunoreactivity in that field. For each area, the optimal number of fields was first determined by a pilot study to obtain a coefficient of error of <10%. Criteria published previously for the hippocampus were used to distinguish neurons from glia (Simic et al. 1997). Co-localisation of cellular immunostaining for PHF-1 and phospho-Akt, and PHF-1 and PTEN was performed in adjacent consecutive sections as outlined previously (Zhu et al. 2002). Images from adjacent consecutive sections were carefully aligned, using three landmarks in each consecutive section. For quantification of the number of PHF-1 immunoreactive neurons that were also immunoreactive to either phospho-Akt or PTEN, the number of PHF-1 and either PTEN or phospho-Akt neurons were summed in the entire CA3, and in four areas of the CA1 subfield of the AD cases. The percentage of the PHF-1 neurons which contained either phospho-Akt or PTEN immunoreactivity was determined.

Results

Increased Akt activation, loss of Akt, and altered levels and distribution of phospho-Akt in AD neurons
Studies on the mechanism of activation of Akt show that PI3-K activation, through its product phosphoinositide-3,4,5-trisphosphate (PtdIns(3,4,5)P3) promotes translocation of inactive Akt from the cytosol to the plasma membrane, with subsequent Akt activation through phosphorylation at Thr308 and Ser473 (Lawlor and Alessi 2001). In line with this, targeting of Akt to the plasma membrane promotes its constitutive activation. Akt activation is thus commonly measured as the ratio of phosphorylated-Ser473-Akt (phospho-Akt)/phospho-independent total Akt in particulate (membrane-associated), fractions. Using this approach, a significant increase in Akt activation was detected in AD particulate fractions compared with matched controls (group 1, control n = 7, AD n = 7, Fig. 1a; group 2, control n = 7, AD n = 9, Fig. 1b). This was as a result of a significant increase in phospho-Akt levels in AD particulate fractions (95%, p < 0.02 for group 1; 170%, p < 0.02 for group 2) compared with matched controls, while total Akt levels remained the same in these fractions in AD and control cases (Figs 1a and b). It is important to note that although Akt levels were identical in AD and control particulate fractions, a significant decrease in Akt levels was detected in AD cytosolic fractions compared with controls (53%, p < 0.005) (Fig. 1c, shown for group 1). This was found together with significantly decreased cytosolic phospho-Akt levels (70%, p < 0.01) (Fig. 1c). Akt activation did not differ significantly when comparing AD and control cytosolic fractions (Fig. 1c). Together, this indicates that the Akt and phospho-Akt that remains in AD cases is proportionally more recruited to particulate fractions than it is in control brain, leading to overall increased activation of Akt in AD. This was verified by western immunoblot analysis, which showed that 83 ± 16% of total phospho-Akt and 67 ± 9% of total Akt localised to particulate fractions in AD temporal cortex (Fig. 1e). Conversely, only 39 ± 4% of total Akt was detected in particulate fractions in control brain with phospho-Akt distributed equally between particulate (48 ± 5%) and cytosolic fractions (52 ± 11%) (Fig. 1d).

The alterations in phospho-Akt and Akt levels emerged at early stages of neurofibrillary pathology, and a statistically significant positive correlation was seen between phospho-Akt levels in particulate fractions and Braak staging (I–VI) for neurofibrillary changes (Fig. 2, and legend), shown for group 1 cases. This was also found for group 2 cases (r = 0.6; p < 0.02). Conversely, Akt and phospho-Akt levels in the cytosol showed a statistically significant but negative correlation with Braak staging (I–VI) for neurofibrillary changes (Fig. 2, and legend). Together, this indicates that both the loss of Akt and the increased activation of Akt link...
strongly to AD pathogenesis. Cytosolic phospho-Akt and Akt levels also correlated negatively with the number of senile plaques (phospho-Akt, \( r_s = 0.59, p < 0.05 \); Akt \( r_s = 0.58, p < 0.05 \)), suggesting a link between loss of cytosolic Akt and senile plaque accumulation.

Immunocytochemical analysis detected phospho-Akt predominantly in neurons throughout the layers of control temporal cortex. Highest levels were found in pyramidal neurons of layer III and V, which are susceptible to degeneration in AD. In agreement with the distribution detected by western immunoblot, phospho-Akt was present throughout the cell, and extended into the proximal portion of apical dendrites (Figs 3a and c). Phospho-Akt immunoreactivity was also detected in smaller cells, either interneurons or glia (Figs 3a and c). In AD, phospho-Akt immunostaining was much less dense in the cytoplasm of AD pyramidal neurons, compared with control neurons, and a significant overall loss of phospho-Akt staining was evident in AD neurons, with the phospho-Akt remaining localised in the peripheral cytoplasm towards the cell membrane (Figs 3b, d and e). Confocal microscopy was also performed to examine the localisation of phospho-Akt in AD and control temporal cortex in greater detail. These images show...
more clearly the loss of phospho-Akt from the cytosol in AD neurons (Fig. 3g) compared with control (Fig. 3f), with the phospho-Akt remaining in AD predominantly localising to the peripheral cytoplasm, towards the cell plasma membrane. Together, the western blot and immunohistochemical data of temporal cortex demonstrate that distribution and expression of Akt and phospho-Akt differs in AD neurons compared with control neurons. This is characterised by a significant loss of cytosolic Akt, and cytosolic phospho-Akt in AD, with practically all of the phospho-Akt localising to particulate fractions and to the peripheral cytoplasm, towards the cell plasma membrane in the AD cases. This differing localisation is associated with increased overall activation of Akt in AD temporal cortex.

Immunocytochemical analysis of phospho-Akt was also performed in the hippocampal formation, because of the critical involvement of this region in AD pathogenesis, where it undergoes hierarchical neurodegeneration. (Braak and Braak 1991). Relatively late stage AD (stage IV–V) cases were examined and compared with control cases (stage 0–I). Phospho-Akt was detected exclusively in hippocampal neurons of control and AD brain, with no detection in glial cells. In control brain, high levels of phospho-Akt immunoreactivity were detected in the stratum pyramidal of CA1–CA4 subfields, subicular complex, entorhinal cortex (layer II and IV) and granular layer of the dentate gyrus (Figs 4a–d). Immunostaining for phospho-Akt was less dense in the CA1 subfield compared with other hippocampal subfields (Fig. 4). Phospho-Akt was detected throughout the cell soma in neurons of the entorhinal cortex (Fig. 4a), and was present in the cytosol but absent from the nucleus of neurons of CA subfields (shown for CA1, Fig. 4b; CA4, Fig. 4c). Decreased numbers of neurons immunoreactive to phospho-Akt were detected in the entorhinal cortex (layer II) of AD brain, and remaining phospho-Akt immunoreactive neurons showed features of degeneration (Fig. 4e, compared with Fig. 4a), with loss of cytosolic phospho-Akt as was found in temporal cortex. Decreased numbers of neurons immunoreactive to phospho-Akt were detected in other hippocampal areas susceptible to neurodegeneration in AD, including the CA1 (Fig. 4f), compared with control brain CA1 (Fig. 4b), and the subiculum. Morphometric analyses of the CA1 indicated that the proportion of volume occupied by phospho-Akt immunoreactivity was reduced by 40.8% ($p < 0.05$) in AD compared with control sections. Importantly, the levels of phospho-Akt immunoreactivity were the same as those in control brain in areas of the brain not vulnerable to degeneration in AD, including CA4 (Figs 4c and g), granule cells of the dentate gyrus (Figs 4d and h), CA3 and cerebellum (results not shown). Morphometric analysis of the CA3 region indicated no significant difference in the proportion of volume occupied by phospho-Akt between AD and control groups.

Fig. 3 Paraffin-embedded sections (8 μm) of mid-temporal cortex from representative control (a, c, f) and AD (b, d, e, g) cases labelled with anti-phosphoSer473-Akt. PhosphoSer473-Akt immunoreactivity was observed in pyramidal neurons in layer III and IV of control and AD sections, seen at lower magnification (a, b) and higher magnification (c–g). In AD (b, d, e) the cytoplasm and nuclei of pyramidal neurons was not as highly immunostained for phosphoSer473-Akt [arrows in (e)] compared with pyramidal neurons in control sections (a, c). Paraffin embedded sections (8 μm) of mid-temporal cortex from representative control (f) and AD (g) cases labelled with anti-phosphoSer473-Akt, and visualised by confocal immunofluorescent microscopy. Decreased levels of phosphoSer473-Akt cytosolic immunostaining are clearly evident in the AD neuron compared with control, with phosphoSer473-Akt localising to the peripheral cytoplasm, towards the plasma membrane. Scale bar: (a–e) 50 μm; (f, g) 20 μm.
Akt substrates in AD

We found that increased activation of Akt in AD has critical downstream signalling consequences, as significant increases in the level of phosphorylation of Akt substrates were detected in particulate fractions of AD (n = 9) cases compared with controls (n = 7) (group 2, Fig. 5a). These substrates were examined initially using a phosphorylation state-specific antibody which specifically recognises Akt substrates phosphorylated on Ser or Thr in the context of the Akt consensus target motif [RXRXX(S/T)]. In control brain, prominent phosphorylation of four targets (approximate MW 33–52 kDa) and a doublet (approximate MW 160 and 165 kDa) was detected in particulate fractions, with bands of approximate MW 163 and 167 kDa represented the primary phosphorylated targets in the cytosol (Fig. 5a). In AD, highly significant increases in the phosphorylation of Akt substrates were detected in particulate fractions at stage V and VI but not in stage IV cases, with a concomitant decrease in cytosolic substrate phosphorylation (Fig. 5a).

Further examination of specific Akt substrates showed increases in the levels of phosphorylation of the Akt targets, phosphoSer9-GSK-3β (Fig. 5b), phosphoSer214-tau (Fig. 5d), and phosphoSer2448-mTOR (Fig. 5g) in AD temporal cortex compared with controls. Total GSK3β levels were decreased significantly in AD cases compared with controls, thus the ratio of phosphoSer9-GSK3β to total GSK3β was significantly...
PTEN levels controls (Figs 6a and b) shown for group 1 cases, a finding was detected in AD temporal cortex, compared with matched plaques (Fig. 5d) in AD, but not in control particulate fractions, indicating both increased, and differing Akt-induced phosphorylation of tau in AD brain. Importantly, these bands co-migrated with the AT100 (phosphoβ-tau212-Ser214-tau) PHF-immunoreactive motif (Fig. 5f), which was detected exclusively in particulate fractions of AD cases (Fig. 5e), linking Akt activation to tau pathology.

Total mTOR levels were measured in homogenates, as very low mTOR levels were detected in the cytosol. PhosphoSer2448-mTOR levels were significantly increased in AD cases compared with controls (Fig. 5g), and mTOR levels were not significantly different when comparing the same AD and control cases (Fig. 5h). Thus, the ratio of phosphoSer2448-mTOR/total mTOR was increased 2.66-fold (p < 0.02) in the AD compared with control group (Figs 5g and h), indicating Akt induced activation of mTOR in AD. Decreased (41%, p < 0.02) levels of p27kip1, which is phosphorylated and degraded as a consequence of Akt activation (Brazil et al. 2004), were detected in the AD homogenates, compared with controls (Fig. 5i). Increased levels of higher molecular weight p27kip1 immunoreactive bands were detected in AD cases compared with controls (Fig. 5i). The levels of phosphorylated protein/total protein ratio of Akt targets, and levels of phosphorylation of Akt substrates correlated with phospho-Akt levels in particulate fractions, and also with neurofibrillary disease stage (for phospho-Akt levels: phosphoSer9-GSK3β/GSK3β, r = 0.62, p < 0.02; phosphoSer2448-mTOR/mTOR, r = 0.87, p < 0.0001; phosphoSer214-tau, 68 kDa band, r = 0.59, p < 0.02; 64 kDa band, r = 0.64; p < 0.01; 60 kDa band, r = 0.52; p < 0.05; for disease stage: phosphoSer9-GSK3β/GSK3β, r = 0.7, p < 0.003; phosphoSer2448-mTOR/mTOR, r = 0.61, p < 0.02; phosphoSer214-tau, 68 kDa band, r = 0.87, p < 0.0001; 64 kDa band, r = 0.73; p < 0.004; 60 kDa band, r = 0.82; p < 0.001).

Loss and altered distribution of PTEN in AD neurons

The lipid and protein phosphatase PTEN dephosphorylates PtdIns (3, 4, 5)P3, and is a vital negative regulator of Akt activation (Leslie and Downes 2002). Our next step therefore was to investigate whether altered levels of PTEN are associated with increased Akt activation in AD. Results presented in Fig. 6 show this to be the case, as a significant decrease (p < 0.001) in PTEN (MW 57 kDa) protein levels was detected in AD temporal cortex, compared with matched controls (Figs 6a and b) shown for group 1 cases, a finding which was also replicated in the group 2 cases. PTEN levels correlated negatively with the severity of neurofibrillary pathology (r = −0.55, p < 0.04), and with the level of senile plaques (r = −0.67, p < 0.01). Immunocytochemical analysis of control hippocampal sections and temporal cortex (not shown) detected PTEN positive neurons, with no PTEN detected in glial cells (Figs 6c–e). PTEN was found in a similar regional distribution to phospho-Akt in control hippocampal formation (Figs 6c–e and 4a–c). Abundant PTEN immunoreactivity was detected throughout the neuronal cell body and within nuclei in control hippocampus and temporal cortex, with minimal staining of proximal apical and basal dendrites (Figs 6c–e).

In AD hippocampal formation, a significant loss of neurons immunoreactive to PTEN was evident in CA1 (Fig. 6f) of AD cases compared with control brain (Fig. 6c). Morphometric analysis in CA1 indicated that the proportion of volume occupied by PTEN was reduced by 42% (p < 0.05) in AD compared with control sections. In the entorhinal cortex (layer II) this loss was also apparent, and the PTEN immunopositive neurons remaining showed features of neurodegeneration (Fig. 6g) compared with control entorhinal cortex (Fig. 6d). No difference in the number of neurons immunoreactive to PTEN was apparent in CA4 (Figs 6e and h), dentate gyrus, CA3 or cerebellum (results not shown), between control and disease groups. Morphometric analysis indicated no significant difference in the proportion of volume occupied by PTEN between AD and control groups in CA3.

The distribution of PTEN differed in AD hippocampal neurons compared with control hippocampal neurons. A loss of nuclear PTEN immunoreactivity was detected in neurons of the CA1 (Fig. 6f), subiculum and entorhinal cortex (Fig. 6g) of AD cases, compared with these neurons of control brain (Figs 6c and d). In addition, significant increases in PTEN immunoreactivity were detected in apical dendrites in the CA1 and subiculum in AD cases, strikingly stretching up through the stratum radiatum towards the dentate gyrus (Fig. 6i). PTEN immunoreactivity was also detected in neurons with unusual binnuclear morphology in the CA1 and subiculum of AD cases (Fig. 6j), and in glial cells of the stratum oriens in AD brain (results not shown). Comparative analysis of PTEN immunocytochemistry was also performed in AD and control sections of temporal cortex, where a loss of PTEN immunoreactive neurons, and loss of nuclear PTEN was also detected in AD neurons compared with controls (results not shown).

Examination of the spatial relationship between phospho-Akt, PTEN and PHF-1 immunoreactivity

PHF-1 antibody is commonly used to detect NFTs in AD, and consistent with earlier reports labelled only a few immunopositive cells in control brain (Fig. 7a). In AD temporal cortex, PHF-1 primarily detects dystrophic neurites and detects intracellular NFTs less markedly (Fig. 7b), whereas PHF-1 primarily detects intracellular and extracellular tangles in the AD hippocampal formation (Figs 7e, g, i and k). In temporal cortex, 6.5 ± 1.5% of neurons immunoreactive for...
PHF-1 displayed immunoreactivity for phospho-Akt (Figs 7b and c). In CA3 pyramidal neurons, 25 ± 0.07% of PHF-1 immunoreactive neurons were immunoreactive for phospho-Akt (Figs 7d and e), and 33 ± 3.4% of PHF-1 neurons were immunoreactive to PTEN (results not shown). In CA1, 10 ± 2.5% of PHF-1 immunoreactive neurons were immunoreactive for phospho-Akt (Figs 7f and g) and 8.5 ± 2.8% of PHF-1 immunoreactive neurons were immunoreactive to PTEN (results not shown). PTEN immunopositive neurons with binuclear morphology in CA1 and subiculum, which were detected in clusters, were frequently immunoreactive to PHF-1 (Figs 7j and k). Neurons with PTEN immunopositive apical dendrites did not display immunoreactivity for PHF-1.

Regression analysis of all of the brain material (both control and AD) did not reveal any correlation between post-mortem delay interval, agonal state, age or gender and the Akt, Akt substrate or PTEN signalling parameters measured above. General increases in overall substrate phosphorylation (i.e. non-Akt substrates) were not detected in AD particulate fractions compared with control cases (results not shown). Expression of β-actin, vimentin, and cytosolic ERK, did not differ in the same AD and control fractions used for Akt, phospho-Akt and PTEN analyses (results not shown). We confirmed effective separation of particulate and cytosolic fractions in the AD and control samples by showing that particulate and cytosolic markers were detected only in the appropriate fractions.

Discussion

The present investigation supports the proposal that alterations in the coordination of Akt and PTEN signal transduction, and the impaired control of their downstream effectors, contribute to AD pathogenesis. Previous studies detected increased phospho-Thr308-Akt associated with AD neurofibrillary pathology (Pei et al. 2003), and increased phospho-Ser473 in AD temporal cortex neurons (Rickle et al. 2004), although they did not detect significant levels of phospho-Akt in control brain, possibly as a result of differing methodological approaches. We now demonstrate both a loss of neuronal cytosolic Akt in AD, and increased levels of phospho-Ser473Akt in AD particulate fractions, leading to increased Akt activity (phospho-Ser473Akt/total Akt ratio),
and increased levels of phosphorylation of critical downstream effectors of Akt in AD temporal cortex when compared with control brain; together, indicating possible constitutive activation of Akt in AD. Interestingly, studies have also found a loss of Akt in the brains of individuals with Huntington’s disease (Humbert et al. 2002) and schizophrenia (Emamian et al. 2004), suggesting the Akt pathway may be defective in other neurological disorders. We further show Akt overactivation is associated with loss and alteration in the neuronal distribution of PTEN in AD brain.

Importantly, loss of Akt, PTEN, increased activation of Akt, and increased phosphorylation of Akt downstream effectors correlate with Braak staging for neurofibrillary changes, linking them to the progression of disease pathology. These results have broad consequences for brain function in AD, as the Akt–PTEN pathway has emerged as a central focus for insulin, growth factor and cytokine signalling, modulating multiple cellular processes, including apoptosis, growth, proliferation, migration and metabolism (Brazil et al. 2004). The changes detected would also be predicted to be of central relevance to the described defects in both insulin, insulin-like growth factor (Gasparini and Xu 2003) and growth factor signalling pathways in AD.

The discovery that Akt is overactive in AD compared with normal adult human brain suggests that neurons are capable of activating this pro-survival response most probably to counteract injury in the disease, for example Ab accumulation (Martin et al. 2001; Wei et al. 2002). However, it is noteworthy that excessive and non-selective phosphorylation of total Akt substrates in AD temporal cortex is very pronounced at Braak stage V–VI of AD. This is the stage when clinical dementia is diagnosed, and deposition of NFTs and neuronal loss emerge in temporal cortex (Braak and Braak 1991). This leads to the conclusion that sustained Akt signalling, owing to its possible ineffectual inactivation by PTEN, or other inputs controlling Akt, could be deleterious to neuronal survival in AD. This may also impact on the survival of the actual Akt and PTEN neurons in AD, whose numbers were reduced in CA1 at end stages of the disease. This proposal can be difficult to reconcile with the well-known role of the PI3-kinase-Akt pathway in promoting cell survival. However, this derives predominantly from studies of mitotic cells, and it has been suggested that post-mitotic neurons may react differently to continuous activation of the Akt pathway (Marino et al. 2002; Chen et al. 2003). It has been shown that increased phosphoSer473-Akt expression, caused by conditional deletion of PTEN in mature mouse Purkinje neurons, is associated with progressive neuronal loss, characterised by vacuolation, accumulation of fibrillary inclusions and reduced p27kip1 levels (Marino et al. 2002).

The varied cellular functions of PI3-kinase-Akt are reflected in the diversity of its downstream targets, and include: GSK3; Bad; mTOR; the Forkhead family of transcription factors; p27kip1 and NF-xB. The relevance of many of these substrates in vivo awaits clarification (Lawlor and Alessi 2001). Our results show a very circumspect and specific Akt substrate phosphorylation pattern in normal control brain. We also show GSK3β, mTOR, tau, and p27kip1 to be among the Akt targets hyperphosphorylated by increased Akt activation in AD. The detection of increased phosphoSer9-GSK3β and loss of GSK3β, indicates Akt induced inactivation of GSK3β in AD. The results were unexpected, as GSK3β activity is thought to be increased in AD, causing tau hyperphosphorylation (Lovestone and...
Reynolds 1997), and strategies to inhibit GSK3β are being developed as potential AD treatments (Bhat et al. 2004). However, data supporting increased activation of GSK3β in AD brain have been equivocal, and a recent western immunoblot study also detected increased levels of phosphoSer214-GSK3β in AD pre-frontal cortex (Swatton et al. 2004). We found that levels of active GSK3β (pY216) were increased in the same AD cases (results not shown), suggesting that control of both inhibitory and stimulatory phosphorylation inputs to GSK3β may be impaired in AD. This is supported by previous immunocytochemical analyses which show both increased active GSK3β (pY216) (Yamaguchi et al. 1996; Pei et al. 1999) and inactive GSK3β (pS9) (Ferrer et al. 2002) associated with NFTs in AD.

Tau^{Ser214} is an optimal consensus sequence for Akt phosphorylation (Ksiezak-Reding et al. 2003), linking Akt and tau function directly, however, it is only recently that Akt has been considered as a potential kinase for tau phosphorylation. Previous observations indicate a protective role for phospho-tau^{Ser214}, as phosphorylation of this site is unique to the AD state and is associated with the appearance of PHF (Schneider et al. 1997), and strategies to inhibit GSK3 activity and tau overphosphorylation: effects of phospho-Akt/PTEN, AT100 and CP3 immunoreactivity which recognises phosphoSer214-tau (Jicha et al. 1999), in adjacent serial sections of the hippocampal formation. However, we had difficulty with the specificity of both AT100 and CP3 staining with immunocytochemistry. We thus employed the PHF-1 antibody, commonly used to detect NFTs in AD. Previous studies using the AT8 antibody, showed a stronger co-localisation of phospho-Akt with NFT, than was observed in the present study (Pei et al. 2003). One interpretation of the present findings is that PHF-1 accumulation in neurons may prevent detection of phospho-Akt and PTEN epitopes at late stages of AD, as was found for phospho-Akt (Thr308) and AT8 co-localisation (Pei et al. 2003). Alternatively, the phospho-Akt- and PTEN-containing neurons may resist NFT accumulation, as measured by PHF-1. Establishing the exact relationship between phospho-Akt/PTEN signalling and AD pathogenesis requires further investigation using animal and cell models, and human brain material staged for the progression of AD pathology.

In summary, this work highlights for the first time the association between the Akt/PTEN signal transduction pathway, hyperphosphorylation of critical Akt substrates, and AD neurodegeneration. The findings indicate that responses to insulin, growth factors and cytokines, all of which are activators of the PI3-kinase pathway, will differ significantly in AD compared with non-disease control brain. Importantly, treatment strategies aiming to activate of PI3-kinase-Akt to enhance neuronal survival and inhibit pathology in AD, need to seriously consider the present findings, as it is probable that the Akt signalling defects reported here would be exacerbated further by these interventions.

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References


