A role for c-Jun N-terminal kinase 1 (JNK1), but not JNK2, in the β -amyloid-mediated stabilization of protein p53 and induction of the apoptotic cascade in cultured cortical neurons

Marie P. FOGARTY, Eric J. DOWNER and Veronica CAMPBELL¹

Department of Physiology, Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland

β -Amyloid ($A\beta$) peptide has been shown to induce neuronal apoptosis; however, the mechanisms underlying $A\beta$ -induced neuronal cell death remain to be fully elucidated. The stress-activated protein kinase, c-Jun N-terminal kinase (JNK), is activated in response to cellular stress and has been identified as a proximal mediator of cell death. In the present study, expression of active JNK was increased in the nucleus and cytoplasm of $A\beta$ -treated cells. Evaluation of the nature of the JNK isoforms activated by $A\beta$ revealed a transient increase in JNK1 activity that reached its peak at 1 h and a later activation (at 24 h) of JNK2. The tumour suppressor protein, p53, is a substrate for JNK and can serve as a signalling molecule in apoptosis. In cultured cortical neurons, we found that $A\beta$ increased p53 protein expression and phosphorylation of p53 at Ser¹⁵. Thus it appears that $A\beta$ increases p53 expression via phosphorylation-mediated stabilization of the protein. Given the lack of availability of a JNK inhibitor that

can distinguish between JNK1- and JNK2-mediated effects, we employed antisense technology to deplete cells of JNK1 or JNK2 selectively. Using this strategy, the respective roles of JNK1 and JNK2 on the $A\beta$ -mediated activation of the apoptotic cascade (i.e. p53 stabilization, caspase 3 activation and DNA fragmentation) were examined. The results obtained demonstrate a role for JNK1 in the $A\beta$ -induced stabilization of p53, activation of caspase 3 and DNA fragmentation. In contrast, depletion of JNK2 had no effect on the proclivity of $A\beta$ to activate caspase 3 or induce DNA fragmentation. These results demonstrate a significant role for JNK1 in $A\beta$ -mediated induction of the apoptotic cascade in cultured cortical neurons.

Key words: antisense, apoptosis, β -amyloid, c-Jun N-terminal kinase, p53, caspase-3.

INTRODUCTION

Deposition of β -amyloid ($A\beta$) protein around neurons is a feature of Alzheimer's disease (AD) [1]. Although the 1–42 fibrillar form of $A\beta$ is the most abundant in the AD senile plaque, the 1–40 peptide also has the proclivity to form fibrils and is present in the senile plaque, but to a lesser extent than the 1–42 species [2]. In AD brains, neuropathological hallmarks of apoptosis such as DNA fragmentation have been detected [3] and $A\beta$ is assumed to contribute directly to this pathology by inducing neuronal apoptosis [4]. In support of the hypothesis that $A\beta$ deposition drives the neurodegenerative process, both $A\beta_{1-40}$ and $A\beta_{1-42}$ have been shown to induce neuronal apoptosis *in vitro* [5,6]. There has been a significant interest in identifying the pathways that underlie neuronal responses to $A\beta$ in an attempt to develop novel neuroprotective therapeutic strategies. To date, many signalling pathways have been proposed as mediators of $A\beta$ -induced neuronal apoptosis and these include reactive oxygen species [7], Ca²⁺-sensitive proteases [8] and cysteine proteases [9]. The stress-activated protein kinase, c-Jun N-terminal kinase (JNK), has been proposed as a mediator of cell death in response to a variety of stimuli such as growth factor deprivation [10], excitotoxicity [11] and oxidative stress [12]. Recent studies [13–15] have also suggested that JNK plays a role in $A\beta$ -mediated effects. In sympathetic neurons and PC12 cells, the 1–42 fragment of $A\beta$ activates JNK and the synthetic JNK inhibitor, CEP-1347, blocks $A\beta$ -mediated neurotoxicity [14]. Three JNK isoforms have been identified, JNK1, JNK2 and JNK3, and these are encoded

by independent genes. Since each of these isoforms is expressed in the brain [16], $A\beta$ has the potential to couple with JNK1, JNK2 or JNK3. Thus although the study of Troy et al. [14] provides compelling evidence of a role for JNK in $A\beta$ -mediated neurodegeneration, the synthetic JNK inhibitor used in their study is unable to distinguish between the different JNK isoforms. In the present study, we used antisense technology to determine the respective roles of the JNK isoforms in $A\beta_{1-40}$ -mediated neurodegeneration of cultured cortical neurons.

The multifunctional tumour suppressor protein, p53, has been suggested to play a role in the cell death pathway as well as having a role in the regulation of the cell cycle and DNA repair [17]. An increase in p53 expression has been reported in the brain of patients with AD [18] and in transgenic mice that overexpress $A\beta$ [19]. cDNA expression of a dominant-negative p53 mutant [20] and application of a chemical inhibitor of p53 [21] offer protection against $A\beta$ -mediated toxicity. Taken together, these studies provide compelling evidence of a role for p53 in $A\beta$ -mediated neurodegeneration. However, the mechanisms that underlie $A\beta$ -mediated activation of p53 remain to be elucidated. p53 has the proclivity to regulate a variety of functions and this diversity in function arises from the presence of several phosphorylation sites on p53 [22]. Thus phosphorylation on Ser¹⁵ is associated with the apoptotic effect of p53 [23], whereas phosphorylation at Ser³⁹² promotes formation of the p53 tetramer that activates transcription from several cell-cycle-regulating genes in response to DNA damage [24]. The stress-activated protein kinase, p38, has been shown to phosphorylate p53 at Ser¹⁵ during hypoxic cell

Abbreviations used: $A\beta$, β -amyloid; AD, Alzheimer's disease; JNK, c-Jun N-terminal kinase; TUNEL, terminal transferase deoxytidyl uridine end-labelling.

¹ To whom correspondence should be addressed (e-mail vacmpbll@tcd.ie).

death [25] and chemical stress-induced apoptosis [26]. p53 is also a substrate for JNK [27] and there is evidence for a role for JNK signalling in the regulation of p53 stability and apoptotic capacity [28]. Given that A β can couple with the JNK pathway [13,15], the aim of the present study was to evaluate the role of the JNK isoforms, JNK1 and JNK2, in the A β -mediated activation of p53 and induction of the apoptotic cascade.

The results from the present study demonstrate a differential time frame of JNK1 and JNK2 activation by A β in this system with JNK1 being activated rapidly by A β (within minutes), whereas JNK2 activation was delayed by 24 h. A β caused an increase in p53 protein expression and phosphorylation of p53 at Ser¹⁵. Use of JNK antisense demonstrated that the regulation of p53 by A β_{1-40} was mediated via JNK1 as was the A β -mediated activation of caspase 3 and DNA fragmentation. These results demonstrate that A β has a differential pattern of signalling via the JNK pathway and provide evidence for an interaction between JNK1 and p53 in this system.

EXPERIMENTAL

Culture of cortical neurons

Primary cortical neurons were established from postnatal 1-day-old Wistar rats and maintained in neurobasal medium (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.). Rats were decapitated and cerebral cortices removed. The dissected cortices were incubated in PBS containing trypsin (0.25 %) for 20 min at 37 °C. The tissue was then triturated (5 $\times$) in PBS containing soyabean trypsin inhibitor (0.1 %) and DNase (0.2 mg/ml) and gently filtered through a sterile mesh filter. After centrifugation (2000 g for 3 min at 20 °C), the pellet was resuspended in neurobasal medium, supplemented with heat-inactivated horse serum (10 %, v/v), penicillin (100 units/ml), streptomycin (100 units/ml) and glutamax (2 mM). Suspended cells were plated out at a density of 0.25×10^6 cells on circular 10 mm diameter coverslips, coated with 60 μ g/ml of poly(L-lysine) and incubated in a humidified atmosphere containing 5 % CO₂/95 % air at 37 °C. After 48 h, 5 ng/ml of cytosine arabinofuranoside was included in the culture medium to prevent proliferation of non-neuronal cells. Culture media were exchanged every 3 days and cells were grown in culture for 12 days before A β treatment.

Drug treatment

A β_{1-40} (BioSource International, U.K.) was made up as a 1 mM stock solution in PBS and allowed to aggregate for 48 h at 30 °C. For treatment of cortical neurons, A β was diluted to a final concentration of 2 μ M in prewarmed neurobasal medium. This concentration has been previously used to induce activation of the apoptotic cascade in these cells [8].

Antisense-mediated depletion of JNK1 and JNK2

20-mer phosphorothioate oligonucleotides were purchased from Biognostik (Göttingen, Germany), and the sequences used were complementary with the mRNA encoding rat JNK1 and JNK2 proteins [26]. The sequences used were: JNK1 antisense oligonucleotide, 5'-CTCATGATGGCAAGCAATTA-3'; JNK1 scrambled oligonucleotide, 5'-ACTACTACACTAGACTAC-3'; JNK2 antisense oligonucleotide, 5'-GCTCAGTGGACATGGATGAG-3'; and JNK2 scrambled oligonucleotide, 5'-GGACTACTACACTAGACTAC-3'. Neurons were incubated with 1.2 μ M oligonucleotide in serum-free prewarmed neurobasal medium

containing 5 μ g/ml of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium/dioleoyl phosphatidylethanolamine (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.). After 4 h, the medium was replaced with supplemented prewarmed neurobasal medium containing 1.2 μ M oligonucleotide. The cells were incubated for a further 48 h before treatment with A β .

Western-immunoblot analysis of phospho-JNK and phospho-p53 expression

After incubation with A β , cortical neurons were harvested in lysis buffer (20 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 5 μ g/ml pepstatin A, 2 μ g/ml leupeptin and 2 μ g/ml aprotinin; pH 7.4) and left on ice for 20 min. The cells were centrifuged (15 000 g for 20 min at 4 °C) and the supernatant was diluted to 50 μ g of protein/ml with sample buffer [150 mM Tris/HCl (pH 6.8), 10 % (v/v) glycerol, 4 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 0.002 % (w/v) Bromophenol Blue]. Samples were then heated to 100 °C for 3 min. Proteins (1 μ g/lane) were separated by electrophoresis on a 10 % (v/v) polyacrylamide minigel, transferred on to a nitrocellulose membrane and immunoblotted with an anti-active JNK monoclonal antibody (1 : 1000; Santa Cruz Biotechnology, CA, U.S.A.) purified from mouse serum, which recognizes the active forms of JNK1 (p46), JNK2 (p54) and JNK3 (p57) after phosphorylation on Thr¹⁸³ and Tyr¹⁸⁵. To monitor expression of non-phosphorylated JNK, the blots were stripped and re-probed with an anti-JNK polyclonal antibody purified from rabbit serum (1 : 1000; Santa Cruz Biotechnology), which recognizes JNK1, JNK2 and JNK3. To monitor p53 phosphorylation, the anti-[phosphoSer¹⁵]p53 (1 : 1000; Cell Signaling Technology, Beverly, MA, U.S.A.) and anti-[phosphoSer³⁹²]p53 (Biosource International, Nivelles, Belgium) polyclonal antibodies purified from rabbit serum, which recognize p53 phosphorylated on Ser¹⁵ and Ser³⁹² respectively, were used. Expression of the non-phosphorylated form of p53 was established using an anti-p53 polyclonal antibody purified from rabbit serum (Santa Cruz Biotechnology). Immunoreactive bands were detected using the horseradish peroxidase-conjugated anti-mouse IgG (for active JNK) or anti-rabbit IgG (for non-phosphorylated JNK, active p53 and non-phosphorylated p53) and enhanced chemiluminescence. Bandwidths were quantified by densitometry (D-Scan PC software).

p53 mRNA expression

Total RNA was extracted from cortical neurons using TRI Reagent (Sigma-Aldrich). cDNA synthesis was performed on 1 μ g of total RNA using oligo(dT) primer according to the manufacturer's instructions (Life Technologies). The RNA was treated with RNase-free DNase I (Life Technologies) at 1 unit/ μ g of RNA for 15 min at 37 °C. Equal amounts of cDNA were used for PCR amplification for a total of 28 cycles. The cycling conditions were 95 °C for 5 min, followed by cycles of 95 °C for 1 min 15 s, 52 °C for 1 min 15 s and 72 °C for 1 min 30 s. A final extension step was performed at 70 °C for 10 min. Primers were pretested through an increasing number of amplification cycles to obtain reverse transcriptase-PCR products in the exponential range. The PCR products were analysed by electrophoresis on 1.5 % (w/v) agarose gels, visualized by ethidium bromide staining, photographed and quantified using densitometry. Primers used were as follows: rat p53 sense, 5'-GGCCATCTACAAGAAGTCAC-3' and antisense, 5'-CCAGAAGATTCCCACTGGAG-3'; rat β -actin

sense, 5'-GAAATCGTGCGTGACATTAAAGAGAAGCT-3' and antisense, 5'-TCAGGAGGAGCAATGATCTTGA-3' [30]. The primers generated a p53 PCR product of 317 bp and a β -actin PCR product of 360 bp. To control for any residual genomic DNA contamination, β -actin primers were chosen to span an intron. This meant that any amplification of genomic, rather than cDNA, would result in larger products than those expected. This semi-quantitative technique facilitated estimation of p53 mRNA expression using β -actin as a reference gene. No observable change in β -actin mRNA was observed in any of the treatment conditions.

Terminal transferase deoxythymine end-labelling (TUNEL)

Apoptotic cell death was assessed using the DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI, U.S.A.). Cells were treated with A β for 72 h to evoke maximal DNA fragmentation [8]. Cells were then fixed with paraformaldehyde (4%, w/v), permeabilized with Triton X-100 (0.1%) and biotinylated nucleotide was incorporated at 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase. Horseradish peroxidase-labelled streptavidin then bound to the biotinylated nucleotide and this was detected using the peroxidase substrate H₂O₂ and the chromogen diaminobenzidine. Cells were then viewed under light microscopy at $\times 40$ magnification, where the nuclei of TUNEL-positive cells stained brown. Apoptotic cells (TUNEL-positive) were counted and expressed as a percentage of the total number of cells examined. To exclude the possibility that the number of living cells present on the coverslip had an effect on the TUNEL-positive ratio, the same number of cells (approx. 400) was counted for each treatment.

Measurement of caspase 3 activity

Caspase 3 activity was assessed by immunocytochemistry using an anti-active caspase 3 antibody (Promega). Briefly, cells were treated with A β for 24 h, fixed with 4% paraformaldehyde, blocked with 5% (v/v) goat serum in PBS, incubated with anti-active caspase 3 antibody (1:200 dilution, overnight incubation at 4 °C). Immunoreactivity was detected using a biotinylated anti-rabbit IgG antibody and streptavidin conjugated to horseradish peroxidase. Immunoreactive cells were visualized with the chromogen diaminobenzidine, and the number of immunoreactive cells was counted and expressed as a percentage of the total number of cells examined (400–500 cells/coverslip).

Fluorescence immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and non-reactive sites were blocked with 5% goat serum in PBS. To determine the intracellular distribution of active JNK, the cells were incubated overnight with an anti-active JNK antibody (Santa Cruz Biotechnology) purified from mouse serum. To evaluate the efficacy of the antisense oligonucleotides in down-regulating JNK expression, immunolocalization of non-phosphorylated JNK1 and JNK2 was assessed using anti-JNK1 or anti-JNK2 monoclonal antibodies purified from mouse serum antibody (Santa Cruz Biotechnology). Cells were then incubated with anti-mouse IgG conjugated to FITC (Sigma-Aldrich) and viewed by fluorescence microscopy at an excitation of 510 nm.

Statistics

Statistical analysis was performed using ANOVA followed by the *post hoc* Student–Newman–Keuls test and the significance (at the 0.05 level) was indicated. When comparisons were being made between two treatments, a paired Student's *t* test was performed and $P < 0.05$ or $P < 0.01$ were considered significant.

RESULTS

A β activates JNK in cortical neurons

Cortical neurons were exposed to A β (2 μ M) for 1 and 24 h and JNK activity was examined by fluorescence immunocytochemistry using an antibody that recognizes phosphorylated JNK (Figure 1A). In control conditions phospho-JNK immunoreactivity was detected in some cells, demonstrating a basal level of JNK activation. However, in cells treated with A β , a higher intensity of phospho-JNK immunoreactivity was observed in the cytoplasm and nucleus, a distribution which indicates that activated JNK has both cytosolic and nuclear substrates. Potential nuclear substrates of JNK include the transcription factor, c-Jun [16], which has been shown to be responsible for regulating gene expression in response to cellular insults such as A β accumulation [15]. The finding that activated JNK also localized in the cytosol may reflect the association of phospho-JNK with cytosolic substrates such as p53 [28].

Time course of A β -mediated activation of JNK1 and JNK2

Western-immunoblot analysis was employed to characterize the time course of A β -mediated JNK activation and to determine the nature of the JNK isoforms activated by A β in lysates prepared from cortical neurons. A β evoked a rapid increase in JNK1 activation, as assessed by expression of phosphorylated JNK1 (Figure 1B). Thus at 5 min, the expression of active JNK1 in control cells was 449 ± 36 (mean bandwidth $\pm$ S.E.M.; arbitrary units) and this was significantly increased to 541 ± 45 in A β -treated cells ($P < 0.05$, Student's paired *t* test, $n = 6$). Similarly, a significant increase in JNK1 activity was observed after A β treatment for 1 and 6 h. At the 1 h time point, JNK1 activity in control cells was 491 ± 12 and this was significantly increased to 593 ± 45 by A β ($P < 0.01$, Student's paired *t* test, $n = 6$). At the 6 h time point, JNK1 activity in control cells was 450 ± 15 and this was significantly increased to 522 ± 20 by A β ($P < 0.05$, Student's paired *t* test, $n = 6$). In contrast, cells treated with A β for 18, 24 and 48 h had no observable increase in JNK1 activation. This result demonstrates the temporal nature of the A β -mediated activation of JNK1. Although JNK1 activation in response to A β occurs rapidly and is sustained for several hours, the activation is decreased by 18 h. A sample immunoblot illustrating the time-dependent activation of JNK1 by A β is shown in Figure 1(D). Also shown is an immunoblot demonstrating that total JNK1 protein expression is unaffected by A β .

In contrast, a different pattern of JNK2 activation by A β was observed. A β had no effect on JNK2 activity at the earlier time points of 5 min–18 h; however, JNK2 activity was significantly increased by A β at the later time points of 24 and 48 h ($P < 0.05$, Student's *t* test, $n = 6$; Figure 1C). A sample immunoblot illustrating the time-dependent activation of JNK2 by A β is shown in Figure 1(D). Also shown is an immunoblot demonstrating that total JNK2 protein expression is unaffected by A β . It is to be noted that significant basal activity of JNK2 is observed in untreated cells; this is consistent with our immunocytochemical results,

Figure 1 Aβ activates JNK1 and JNK2 within different time frames

(A) Cortical neurons were exposed to Aβ (2 μM) for 1 and 24 h and expression of phospho-JNK was monitored by immunofluorescence. In untreated cells (i, iii) active JNK immunoreactivity is apparent in a subpopulation of cells. In cells exposed to Aβ for 1 h (ii) or 24 h (iv) more intense phospho-JNK immunostaining is observed in the nucleus and in discrete areas within the cytosol. Arrows indicate cells displaying phospho-JNK immunoreactivity. Scale bar = 20 μm. (B) Cells were exposed to Aβ over a range of time points (5 min to 48 h) and phospho-JNK1 expression was monitored by Western-immunoblot analysis. Aβ caused a significant increase in phospho-JNK1 expression at 5 min, 1 and 6 h, but not at subsequent time points. Results are means ± S.E.M. from six independent observations. **P* < 0.05, ***P* < 0.01. Statistical analysis was performed using Student's paired *t* test. (C) Cells were incubated with Aβ over a range of time points (5 min to 48 h) and phospho-JNK2 expression was monitored by Western-immunoblot analysis. Aβ caused a significant increase in phospho-JNK2 expression at 24 and 48 h, but not at earlier time points. Results are means ± S.E.M. from six independent observations. **P* < 0.05. Student's paired *t* test was used for statistical analysis. (D) Sample immunoblot demonstrating the Aβ-mediated increase in phospho-JNK1 expression with no concomitant change in expression of phospho-JNK2 or non-phosphorylated forms of JNK1 or JNK2 at (a) 5 min, 1 h, (b) 6 h and (c) 24 and 48 h.

where phospho-JNK immunoreactivity was detected in some cells in the absence of Aβ. Basal JNK activity also has been observed in other studies [31] and may reflect an endogenous role for JNK2 in regular cell physiology.

Effect of Aβ on p53 mRNA and protein expression

Since p53 is a target for modulation by JNK [27], the effect of Aβ on p53 expression and phosphorylation was examined

Figure 2 Aβ increases p53 expression and phosphorylation at Ser¹⁵

(A) Cells were exposed to Aβ (2 μM) for 1 h and p53 expression was assessed by Western-immunoblot analysis. Aβ evoked a significant increase in p53 expression. A sample immunoblot demonstrating the Aβ-mediated increase in p53 expression is shown as an inset. Results are means ± S.E.M. from six independent observations. **P* < 0.05. Statistical analysis was performed using Student's paired *t* test. (B) Cells were treated with Aβ for a range of time points (5 min to 18 h), total RNA was extracted and expression of p53 mRNA was examined by PCR. A representative image of the ethidium bromide-stained gel is shown as an inset. No change in basal (lane 1) p53 mRNA expression was observed after treatment with Aβ for 5 min (lane 2), 30 min (lane 3), 1 h (lane 4) or 18 h (lane 5). Results are means ± S.E.M. from six independent observations. (C) After incubation of the cells with Aβ, the expression of p53 phosphorylated on Ser¹⁵ was examined by Western-immunoblot analysis. Aβ caused a significant increase in phosphorylation of p53 at Ser¹⁵ at 5 min and 1 h. A sample immunoblot demonstrating the Aβ-mediated increase in [phosphoSer¹⁵]p53 is shown as an inset. At 5 min, basal expression of [phosphoSer¹⁵]p53 (lane 1) is increased by Aβ (lane 2). Similarly, at 1 h, basal expression of [phosphoSer¹⁵]p53 (lane 3) is increased by Aβ (lane 4). Results are means ± S.E.M. from eight independent observations. **P* < 0.05, ***P* < 0.01. Statistical analysis was performed using Student's paired *t* test. (D) The effect of Aβ on phosphorylation of p53 at Ser³⁹² was assessed by Western-immunoblot analysis. Aβ had no effect on the expression of p53 phosphorylated at Ser³⁹² at 5 min or 1 h. A sample immunoblot demonstrating the lack of effect of Aβ on phosphorylation of p53 at Ser³⁹² is shown as an inset. At 5 min, basal expression of [phosphoSer³⁹²]p53 ('phospho-p53^{ser392}') (lane 1) is unaffected by Aβ (lane 2). Similarly, at 1 h basal expression of [phosphoSer¹⁵]p53 ('phospho-p53^{ser15}') (lane 3) is unaffected by Aβ (lane 4). Results are means ± S.E.M. from six independent observations.

in the present study (Figure 2). Figure 2(A) demonstrates that Aβ (2 μM, 1 h) evoked a significant 30 % increase in p53 protein expression from 905 ± 78 (means ± S.E.M.; arbitrary units) to 1141 ± 88 (*P* < 0.01, Student's paired *t* test, *n* = 6). This increase in p53 expression was not attributable to a transcription event, since Aβ had no modulatory effect on p53 mRNA expression at any of the time points examined (5 and 30 min, 1 and 18 h; Figure 2B). There was no time-dependent

effect on basal levels of p53 mRNA expression and hence the control data were pooled on the graph for clarity. Since p53 phosphorylation at Ser¹⁵ can stabilize p53 by preventing ubiquitin-mediated degradation [33], we examined the effect of A β on p53 phosphorylation using an antibody that recognizes phospho-Ser¹⁵ on p53 (Figure 2C). A β significantly increased expression of [phosphoSer¹⁵]p53 from 587 ± 55 to 1030 ± 116 at 5 min ($P < 0.01$, Student's paired *t* test, $n = 8$) and from 837 ± 19 to 1265 ± 118 at 1 h ($P < 0.05$, Student's paired *t* test, $n = 6$). In contrast, A β failed to induce phosphorylation of p53 at Ser³⁹² at these time points. This result suggests that A β increases p53 protein expression by stabilizing the p53 protein via phosphorylation at Ser¹⁵.

Depletion of JNK1 and JNK2 by antisense oligonucleotides

To examine the respective roles of JNK1 and JNK2 in the A β -mediated modulation of p53 stabilization, antisense technology was employed. We first evaluated the effectiveness of the selected antisense oligonucleotide sequences in down-regulating expression of the JNK1 or JNK2 proteins using immunocytochemistry and Western-immunoblot analysis (Figure 3). Immunocytochemical analysis of JNK1 (Figure 3A, i and ii) and JNK2 (Figure 3A, iii and iv) expression revealed intra-cellular JNK1 and JNK2 immunoreactivity in control cells that was markedly down-regulated in cells transfected with the respective JNK1 and JNK2 antisense sequences. To quantify the antisense-mediated depletion of the respective JNK isoforms, Western-immunoblot analysis was employed. Figure 3(B) demonstrates that there was no difference in JNK1 expression in untreated cells when compared with cells treated with the scrambled JNK1 control oligonucleotide. However, in cells treated with the antisense JNK1 oligonucleotide for 48 h, JNK1 expression was significantly decreased by 80 % ($P < 0.01$, Student's paired *t* test; compared with cells treated with JNK1 scrambled oligonucleotide $n = 4$). Figure 3(C) demonstrates an identical result for JNK2. However, in cells treated with the antisense JNK2 oligonucleotide for 48 h, JNK2 expression was also significantly attenuated. Sample immunoblots demonstrating the efficacy of the JNK1 and JNK2 antisense oligonucleotides in down-regulating expression of JNK1 and JNK2 proteins respectively are shown as insets in Figures 3(B) and 3(C). We also verified that the JNK1 antisense had no effect on JNK2 expression (Figure 3D) and that the JNK2 antisense too had no effect on expression of JNK1 (Figure 3E). These results demonstrate that the JNK1 and JNK2 antisense sequences evoke a selective down-regulation in expression of their respective target proteins.

We confirmed that this extent of JNK depletion was sufficient to abrogate A β -mediated JNK activation (Figures 3F and 3G). Since the maximal activation of JNK1 and JNK2 occurred after 1 and 24 h treatment with A β respectively, the effect of JNK depletion on JNK1 and JNK2 activation was examined at these time points. For JNK1 (Figure 3F) in scrambled oligonucleotide-treated cells, A β ($2 \mu\text{M}$, 1 h) evoked a significant increase in expression of phospho-JNK1 ($P < 0.05$, ANOVA; compared with cells treated with JNK1 scrambled oligonucleotide alone, $n = 6$). However, in JNK1 antisense oligonucleotide-treated cells, the basal expression of activated phospho-JNK1 was 613 ± 39 , and 503 ± 57 after incubation with A β ($2 \mu\text{M}$, 1 h, $n = 6$), demonstrating that the JNK1 antisense was effective in reducing the A β -mediated stimulation of JNK1 phosphorylation. Figure 3(G) demonstrates that in cells treated with the JNK2 scrambled oligonucleotide, expression of activated phospho-JNK2 was significantly increased by A β ($2 \mu\text{M}$, 24 h; $P < 0.05$, ANOVA; compared with cells

Figure 3 Determination of the efficacy of the JNK antisense sequences in down-regulating JNK1/JNK2 expression

(A) Cells were treated with either JNK1 or JNK2 antisense ($2 \mu\text{M}$) for 48 h and the consequence on JNK1 and JNK2 expression was examined by fluorescence immunocytochemistry using monoclonal JNK1 or JNK2 antibodies. (i) In untreated cells, JNK1 expression was localized throughout the cytoplasm with a high level of immunoreactivity at the inner plasma membrane; (ii) JNK1 immunostaining was significantly decreased in JNK1 antisense-treated cells; (iii) in untreated cells, JNK2 expression was abundant in the cytosol and inner plasma membrane; and (iv) JNK2 immunostaining was significantly decreased in JNK2 antisense-treated cells. Cells that have been successfully depleted of JNK are indicated by the arrows. Scale bar = $5 \mu\text{m}$. (B) Antisense-mediated depletion of JNK1 was quantified by Western-immunoblot analysis. The JNK1 scrambled sequence had no effect on JNK1 expression; however, the JNK1 antisense significantly decreased JNK1 expression by approx. 80 %. A sample immunoblot demonstrating the JNK1 antisense-mediated depletion of JNK1 is shown as an inset. JNK1 is abundant in JNK1 scrambled-treated cells (lane 1) and JNK1 expression is down-regulated in JNK1 antisense-treated cells (lane 2). Results are expressed as means $\pm$ S.E.M. from four observations. $**P < 0.01$. Student's paired *t* test was used for statistical analysis. (C) Antisense-mediated depletion of JNK2. The procedure is same as in (B). (D) Neither JNK2 scrambled nor JNK2 antisense oligonucleotides had any effect on JNK1 expression. Results are expressed as means (arbitrary units) $\pm$ S.E.M. from four observations. (E) Same as in (D), but with JNK2. (F) In JNK1 scrambled oligonucleotide-treated cells, A β ($2 \mu\text{M}$, 1 h) retained the ability to stimulate JNK1 activity, as assessed by phospho-JNK1 expression. However, JNK1 antisense abrogated the A β -mediated increase in JNK1 activity. Results are expressed as means $\pm$ S.E.M. from six observations. $**P < 0.05$. Statistical analysis was performed using ANOVA. (G) In JNK2 scrambled oligonucleotide-treated cells, A β ($2 \mu\text{M}$, 24 h) retained the ability to stimulate JNK2 activity, as assessed by phospho-JNK2 expression. However, JNK2 antisense abrogated the A β -mediated increase in JNK2 activity. Results are expressed as means $\pm$ S.E.M. from six observations, $**P < 0.05$. Statistical analysis was performed using ANOVA.

Figure 4 Depletion of JNK1 prevents the A β -mediated stimulation of p53 phosphorylation

The consequence of JNK1 depletion on the ability of A β to impact on p53 was examined by Western-immunoblot analysis using an antibody that recognizes p53 phosphorylated at Ser¹⁵. In control scrambled oligonucleotide-treated cells, A β (2 μ M, 1 h) significantly increased [phosphoSer³⁹²]p53 expression. In contrast, JNK1 depletion prevented the A β -mediated increase in [phosphoSer¹⁵]p53 expression. Results are the means $\pm$ S.E.M. from six independent observations. ** P < 0.05. Statistical analysis was performed using ANOVA.

treated with JNK2 scrambled oligonucleotide alone, n = 6). However, in JNK2 antisense oligonucleotide-treated cells the expression of activated phospho-JNK2 was unaffected by A β (2 μ M, 24 h, n = 6) and this result demonstrates that the JNK2 antisense had the capacity to reduce the A β -mediated stimulation of phospho-JNK2.

Having established the efficacy of the JNK1 and JNK2 antisense sequences in depleting expression of their respective target proteins and abrogating the stimulatory effect of A β on JNK1 and JNK2 phosphorylation, we used the antisense approach to determine the role of the JNK isoforms in A β -mediated activation of p53 and induction of the apoptotic cascade.

JNK1 is involved in A β -mediated phosphorylation of p53

Since JNK2 activation by A β was only observed at the later time point of 24 h, it seemed unlikely that JNK2 was involved in p53 phosphorylation, since the impact of A β on p53 occurred within 60 min. In contrast, A β -mediated activation of JNK1 was an earlier event that could potentially be upstream of p53 phosphorylation. Therefore the antisense approach was used to examine the role of JNK1 in the A β -mediated phosphorylation of p53 at Ser¹⁵ (Figure 4). After treatment with A β (2 μ M, 1 h), phospho-p53 expression is significantly increased from 84 ± 1 (means $\pm$ S.E.M.; arbitrary units) to 109 ± 8 (P < 0.05, ANOVA; compared with control cells, n = 8). Although the JNK1 antisense oligonucleotide alone had no effect on p53 phosphorylation, it abolished the stimulatory effect of A β on p53 phosphorylation at Ser¹⁵. A sample immunoblot demonstrating that JNK1 antisense abrogates the A β -mediated increase in phospho-p53 expression in shown as an inset in Figure 4.

Thus this result provides evidence of a role for JNK1 in the A β -mediated phosphorylation of p53 at Ser¹⁵.

Role of JNK1 and JNK2 in A β -mediated activation of apoptotic cascade

A downstream consequence of JNK activation and p53 phosphorylation at Ser¹⁵ is a commitment to the cell death pathway

Figure 5 Consequences of JNK1 and JNK2 depletion on A β -induced activation on caspase 3 activation

(A) The percentage of cells displaying anti-active caspase 3 immunoreactivity was assessed by immunocytochemistry. In cells that had not been treated with oligonucleotide (untreated), A β (2 μ M, 24 h) significantly increased the percentage of cells displaying active caspase 3 immunostaining by 40%. In JNK1 scrambled oligonucleotide-treated cells, A β also evoked a significant increase in active caspase 3 immunoreactivity. However, this effect was abrogated in JNK1 antisense-treated cells. In contrast, neither the JNK2 scrambled nor the JNK2 antisense oligonucleotide had any effect on the proclivity of A β to evoke the increase in active caspase 3 immunoreactivity. Results are the means $\pm$ S.E.M. from six independent observations. ** P < 0.05; + P < 0.05. (B) The effect of JNK1 and JNK2 depletion on the A β -mediated activation of caspase 3 at 48 h was assessed. Other details are the same as in (A). ** P < 0.05; ++ P < 0.05. All statistical analyses were performed using ANOVA.

[17,32]. Since A β activates both JNK1 and JNK2 in cortical neurons, the respective roles of JNK1 and JNK2 in coupling A β with activation of the apoptotic cascade were examined using the JNK1 and JNK2 antisense oligonucleotides. The components of the apoptotic cascade that were examined in the present study were activation of the caspase 3 and DNA fragmentation.

Caspase 3 activity was examined by immunocytochemistry using an anti-active caspase 3 antibody. Figure 5(A) demonstrates that in untreated cells, the percentage of cells displaying active caspase 3 immunoreactivity was $18 \pm 2\%$ (means $\pm$ S.E.M.) and this was significantly increased to $45 \pm 4\%$ after treatment with A β for 24 h (P < 0.05, ANOVA; compared with untreated cells, n = 6 coverslips). After transfection with the JNK1 scrambled oligonucleotide, the A β -mediated stimulation of caspase 3 was retained (P < 0.05, ANOVA, n = 6 coverslips). In contrast, the JNK1 antisense oligonucleotide significantly decreased the A β -mediated stimulation of caspase 3 activity (P < 0.05, ANOVA; compared with cells treated with A β + JNK1 scrambled oligonucleotide, n = 6 coverslips). Thus in cells transfected with JNK1 antisense alone, the percentage of cells with active caspase 3 immunostaining was $21 \pm 3\%$ and, in cells treated with A β in the presence of JNK1 antisense

oligonucleotide, $24 \pm 4\%$ of cells displayed active caspase 3 immunoreactivity ($n=6$ coverslips). This result demonstrates that JNK1 is pertinent in the $A\beta$ -mediated activation of caspase 3 in cultured cortical neurons. In contrast, depletion of JNK2 had no effect on $A\beta$ -mediated activation of caspase 3 at 24 h. Thus in cells treated with JNK2 antisense alone, $25 \pm 3\%$ of cells displayed active caspase 3 immunoreactivity and this was significantly increased to $38 \pm 4\%$ in JNK2-depleted cells treated with $A\beta$ for 24 h ($P < 0.05$, ANOVA; compared with cells treated with JNK2 antisense alone, $n=6$). Since the $A\beta$ -mediated stimulation of JNK2 was retained after depletion of JNK2, we conclude that JNK2 is not involved in the $A\beta$ -mediated activation of caspase 3 that is found to occur at the 24 h time point.

Since cells had to be incubated with $A\beta$ for 24 h before JNK2 activation was detected, our finding that JNK2 was not involved in the $A\beta$ -mediated activation of caspase 3 at 24 h is not surprising. To determine whether JNK2 was involved in caspase 3 activation at later time points, we repeated the experiment by monitoring the effect of JNK1 and JNK2 antisense on $A\beta$ -mediated activation of caspase 3 at 48 h, a time point that would be downstream of JNK2 activation. Figure 5(B) demonstrates that in untreated cells, the percentage of cells displaying active caspase 3 immunoreactivity was $13 \pm 3\%$ (means $\pm$ S.E.M.) and this was significantly increased to $45 \pm 8\%$ after treatment with $A\beta$ for 48 h ($P < 0.05$, ANOVA; compared with untreated cells, $n=6$ coverslips). After transfection with the JNK1 scrambled oligonucleotide, the $A\beta$ -mediated stimulation of caspase 3 was retained ($P < 0.05$, ANOVA; $n=6$ coverslips). In contrast, the JNK1 antisense oligonucleotide significantly decreased the $A\beta$ -mediated stimulation of caspase 3 activity ($P < 0.05$, ANOVA; compared with cells treated with $A\beta$ + JNK1 scrambled oligonucleotide, $n=6$ coverslips). Thus in cells transfected with JNK1 antisense alone, the percentage of cells with active caspase 3 immunostaining was $19 \pm 4\%$ and, in cells treated with $A\beta$ in the presence of JNK1 antisense oligonucleotide, $25 \pm 4\%$ of cells displayed active caspase 3 immunoreactivity ($n=6$ coverslips). This result demonstrates that JNK1 is pertinent in the $A\beta$ -mediated activation of caspase 3 in cultured cortical neurons at 48 h. In contrast, depletion of JNK2 had no effect on $A\beta$ -mediated activation of caspase 3 at 48 h. Thus in cells treated with JNK2 antisense alone, $13 \pm 6\%$ of cells displayed active caspase 3 immunoreactivity and this was significantly increased to $46 \pm 6\%$ in JNK2-depleted cells treated with $A\beta$ for 48 h ($P < 0.05$, ANOVA; compared with cells treated with JNK2 antisense, $n=6$). These results demonstrate that JNK1, and not JNK2, is involved in the coupling of $A\beta$ with caspase 3 activation.

Role of JNK1 and JNK2 in $A\beta$ -mediated DNA fragmentation

One of the later stages in apoptosis is the fragmentation of nuclear DNA. We have previously shown that $A\beta_{1-40}$ has no effect on DNA fragmentation before 48 h, but a maximal 35% increase in DNA fragmentation is observed after exposure of cortical neurons to $A\beta_{1-40}$ for 72 h [8]; we therefore chose to use that time point in the present study. The effect of $A\beta$ on DNA fragmentation was analysed by TUNEL and the roles of JNK1 and JNK2 in $A\beta$ -mediated DNA fragmentation were assessed after antisense-mediated depletion of the JNK1 and JNK2 isoforms (Figure 6). In untreated cortical neurons, the percentage of cells with fragmented DNA (TUNEL-positive cells) was $21 \pm 2\%$ (means $\pm$ S.E.M.) and this was maximally increased to $52 \pm 8\%$ by $A\beta$ (72 h, $2 \mu\text{M}$) ($P < 0.05$, ANOVA, $n=6$ coverslips)

Figure 6 Effect of JNK1 and JNK2 depletion on $A\beta$ -induced DNA fragmentation

(A) The effect of JNK1 and JNK2 depletion on the ability of $A\beta$ ($A\beta$) to induce DNA fragmentation (TUNEL-positive) was assessed. In untreated cells, $A\beta$ significantly increased the percentage of TUNEL-positive cells by 40%. For JNK1 scrambled oligonucleotide, $A\beta$ also evoked a significant increase in TUNEL-positive cells; however, this effect was significantly decreased in JNK1 antisense-treated cells. Neither the JNK2 scrambled nor the JNK2 antisense oligonucleotides had any effect on the proclivity of $A\beta$ to evoke an increase in active DNA fragmentation. Results are the means $\pm$ S.E.M. from six independent observations. ** $P < 0.05$; ++ $P < 0.05$. Statistical analysis was performed using ANOVA. con, control. (B) Representative TUNEL staining for cells treated with (i) JNK1 scrambled oligonucleotide, (ii) $A\beta$ + JNK1 scrambled oligonucleotide, (iii) $A\beta$ + JNK1 antisense oligonucleotide, (iv) JNK2 scrambled oligonucleotide, (v) $A\beta$ + JNK2 scrambled oligonucleotide and (vi) $A\beta$ + JNK2 antisense oligonucleotide. Scale bar = $20 \mu\text{m}$.

as has been previously reported [5]. After transfection with the JNK1 scrambled control oligonucleotide, the $A\beta$ -induced DNA fragmentation was retained ($P < 0.05$, ANOVA, $n=6$ coverslips). In contrast, the JNK1 antisense oligonucleotide abolished the $A\beta$ -mediated stimulation in DNA fragmentation. Thus in cells transfected with JNK1 antisense oligonucleotide, the percentage of cells with DNA fragmentation was $20 \pm 2\%$ in control cells and $28 \pm 3\%$ in $A\beta$ -treated cells. This result demonstrates that JNK1 is pertinent in the induction of DNA fragmentation by $A\beta$. Since JNK1 was also found to have a role in the $A\beta$ -mediated activation of caspase 3, a key executioner of apoptosis, the JNK1-dependent DNA fragmentation probably involves caspase 3 as an upstream component of this pathway. To determine the role of JNK2 in $A\beta$ -mediated DNA fragmentation, the cells were transfected with the JNK2 antisense oligonucleotide. Figure 6 demonstrates that after depletion of JNK2, $A\beta$ retained the ability to evoke a significant increase in DNA fragmentation ($P < 0.05$, ANOVA, $n=6$). Thus JNK1 is the principal isoform involved in

coupling A β with activation of caspase 3 and DNA fragmentation in cortical neurons.

DISCUSSION

The aim of the present study was to examine the roles of JNK1 and JNK2 in A β -mediated induction of the apoptotic cascade in cultured cortical neurons. In the absence of a suitable pharmacological inhibitor to distinguish between JNK1- and JNK2-mediated effects, antisense technology was employed to cause a selective down-regulation in expression of JNK1 and JNK2 to examine their respective roles in the apoptotic pathway. A β increased phospho-JNK expression in isolated cortical neurons with activated JNK immunoreactivity being localized to discrete regions within the nucleus and cytosol. This pattern of distribution is consistent with the distribution of phospho-JNK in response to other stress stimuli, where nuclear staining may reflect the ability of JNK to regulate transcription of stress-induced genes [33]. The punctate distribution of phospho-JNK that we detect in the cytosol after exposure to A β may represent mitochondrial targeting of JNK [34] or association of JNK with cytosolic substrates such as p53 [28]. The results obtained from Western-immunoblot analysis of cell lysates demonstrate a differential temporal activation of JNK1 and JNK2 in cultured cortical neurons. JNK1 was activated rapidly (within 5 min) by A β and sustained for several hours. By 18 h, the A β -mediated activation of JNK1 had returned to basal levels, demonstrating the temporal effectiveness of this signalling cascade. In contrast, A β failed to induce an early activation of JNK2 but JNK2 activity was increased by A β at the later time points of 24 and 48 h. The finding that A β activates JNK1 and JNK2 within dissimilar time frames suggests that JNK1 and JNK2 may have distinct roles in cellular stress responses to A β . Expression of p53 and phosphorylation of p53 at Ser¹⁵ were increased by A β . Phosphorylation of p53 at Ser¹⁵ has been reported as a proximal event in cellular apoptosis [23] and the ability of A β to phosphorylate p53 was abolished in JNK1-depleted cells. The effect of A β on downstream apoptotic events, namely caspase 3 activation and DNA fragmentation, was also significantly attenuated after JNK1 antisense treatment. In contrast, depletion of JNK2 had no effect on the ability of A β to activate caspase 3 or induce DNA fragmentation.

Our finding that A β_{1-40} increases phospho-JNK expression in cortical neurons agrees closely with the effects of other A β fragments in neuronal systems. A β_{1-42} and A β_{25-35} have been reported to increase JNK activity in cortical neurons [13] and A β_{1-42} increases JNK phosphorylation in sympathetic neurons and PC12 cells [14]. Although there are similarities in the effects of different A β peptides on the JNK pathway, it is to be noted that the time frame for JNK activation varies considerably between different A β peptide species. In the present study, we have demonstrated that JNK1 is activated within 5 min of exposure to A β_{1-40} , whereas the A β -mediated activation of JNK2 is not apparent until 24 h. In contrast, A β_{25-35} increases JNK1 activity at 4 h, but not at earlier time points [13], and A β_{1-42} causes peak activation of both JNK1 and JNK2 within 2–6 h in PC12 cells [14]. It is therefore probable that temporal variations in JNK activation may depend on the nature of the A β fragment and cell type.

There has been much interest in the signalling events that underlie A β -mediated induction of the apoptotic cascade. The present study and the work of other groups [13,15] provide evidence for A β impinging on the JNK signalling pathway. Induction of the apoptotic cascade has been attributed to activation of JNK in several systems [32]. In support of a role for JNK in A β -mediated cell death, synthetic inhibitors of JNK protect against

A β -mediated cell [13], although the exact nature of the JNK isoforms that mediate the A β neurodegeneration remains to be established. In the present study, we have successfully employed antisense technology to cause a selective down-regulation in expression of JNK1 and JNK2 proteins. After antisense-mediated down-regulation of JNK1 and JNK2, the ability of A β to induce phosphorylation of JNK1 and JNK2 was selectively abolished. However, it was surprising that the antisense-mediated down-regulation in JNK1/2 protein expression was not accompanied by a reduction in basal phospho-JNK expression. This may reflect endogenous activity of the residual JNK that remained after antisense treatment or may be due to an enhanced stability of the phosphorylated form of JNK. Since the selected antisense oligonucleotides blocked A β -mediated activation of each of the JNK isoforms, this approach was used to elucidate the respective roles of JNK1 and JNK2 in the A β -mediated induction of apoptotic events such as phosphorylation of p53, caspase 3 activation and DNA fragmentation.

Our results demonstrate that A β_{1-40} increases p53 expression and phosphorylation of p53 at Ser¹⁵ in cortical neurons. A β had no effect on p53 mRNA expression, although other A β fragments (i.e. A β_{31-35}) have been found to increase p53 mRNA expression within 1–3 h of exposure to A β in murine neurons [35]. Thus in the rat cortex, the increase in p53 expression occurs in a transcription-independent manner. p53 has a short half-life, which is prolonged in response to cellular stress [22]. Stress-mediated phosphorylation of p53 at residue 15 disrupts its association with the oncoprotein, Mdm-2, preventing ubiquitin-mediated degradation of p53 and thus stabilizing the p53 protein [36]. The finding that A β rapidly phosphorylates p53 at Ser¹⁵ provides a mechanism for the A β -mediated increase in p53 expression. In contrast with the ability of A β to phosphorylate p53 at Ser¹⁵ within 5–60 min, no phosphorylation of the Ser³⁹² epitope was observed at this time point. Phosphorylation at Ser³⁹² occurs in response to DNA damage [37], but since A β does not induce DNA damage until 48–96 h [8], phosphorylation at Ser³⁹² would be unlikely to occur within the time frame (5–60 min) used here. Thus phosphorylation of p53 at Ser¹⁵ is an early event in A β signalling that may serve to act as an upstream event in the degenerative pathway. The evidence from the present study and by others [16,21], supports an interaction between A β and p53. However, the mechanism of A β -induced stabilization of p53 remains to be elucidated. Several mechanisms for p53 stabilization have been proposed and these include caesin kinase [38], DNA-activated kinase [39] and JNK [27,40]. Since we have demonstrated that JNK1 is activated rapidly by A β in cortical neurons, we used selective antisense oligonucleotides to down-regulate JNK1 expression to elucidate the role of JNK1 in A β -mediated activation of p53. Depletion of JNK1 prevented the A β -mediated phosphorylation of p53 at Ser¹⁵ and this result demonstrates that JNK1 is upstream of p53. In non-neuronal cells, JNK1 has also been shown to bind to and phosphorylate p53 [41], although there is also evidence for activation of p53 in response to DNA damage being upstream of JNK activation [42]. Thus the interrelationship between JNK and p53 signalling probably depends on the type of stress stimulus and status of the cell. JNK2 and JNK3 can also serve as p53 N-terminal Ser³⁴ kinases in response to cellular stress [40]. However, since the JNK1 antisense completely abrogated the A β -mediated phosphorylation of p53 and A β -mediated activation of JNK2 was only detected at the later time points of 24–48 h, which was much later than the time required to stabilize p53, we conclude that JNK2 was not pertinent in the A β -mediated activation of p53.

After phosphorylation at Ser¹⁵, p53 can contribute to apoptosis by inducing transcription of proapoptotic genes such as *bax*,

leading to release of mitochondrial cytochrome *c* and activation of caspase 3, a key executioner of apoptosis [23]. Alternatively, p53 may target directly to the mitochondria to induce a transcription-independent apoptotic signal [43]. Thus potential downstream consequences of JNK1-mediated activation of p53 include induction of caspase 3 and the appearance of apoptotic hallmarks such as DNA fragmentation. After depletion of JNK1, the proclivity of $A\beta$ to activate caspase 3 and induce DNA fragmentation was significantly attenuated. JNK2 depletion had no effect on the $A\beta$ -mediated activation of caspase 3 or increase in DNA fragmentation and this suggests that JNK1 is the principal JNK isoform involved in $A\beta$ -mediated activation of the apoptotic cascade. Although $A\beta$ induced caspase 3 cleavage in this system, it is important to note that the role of caspase 3 in $A\beta$ -mediated neurodegeneration remains a matter of debate. In cortical neurons, a caspase 3 inhibitor has been found to protect neurons against some aspects of $A\beta$ -mediated cell death [44], whereas caspase 3 has been found to have no role in $A\beta$ -mediated hippocampal cell death [5]. The role of caspase 3 in $A\beta$ neurodegeneration has therefore been proposed to be brain-region-specific [45]. Our findings that JNK1 and JNK2 have distinct activation profiles and different roles in the $A\beta$ -mediated induction of the apoptotic cascade highlights the complexity of JNK signalling. In ischaemic death, differential roles of JNK1 and JNK2 have also been reported [29] and there is evidence that the profile of JNK activation is dependent on neuronal cell type and the applied stress paradigm [46]. Furthermore, the use of *jnk3* knockout mice has identified a role for JNK3 in $A\beta$ -mediated neurotoxicity [15], whereas in the present study, we found no evidence of JNK3 activation by $A\beta_{1-40}$ (results not shown) and our result suggests a prominent role for JNK1 in $A\beta$ -mediated DNA fragmentation in this system. This lends further support for the idea that the patterns of JNK signalling utilized by $A\beta$ are diverse and may be subject to developmental factors, cell type and animal species. The present study provides evidence for the 1–40 fragment of $A\beta$ coupling with JNK1 upstream of cell death in the cortex and this is relevant since $A\beta_{1-40}$ is detected in the mature senile plaque [2]. However, the nature of the JNK isoform responsible for evoking cell death by $A\beta_{1-42}$, the principal component of the plaque, remains to be resolved.

In conclusion, $A\beta$ induces phosphorylation of JNK1 and JNK2 in cortical neurons with a distinct temporal pattern. Although JNK1 is pertinent in the $A\beta$ -mediated stabilization of p53, activation of caspase 3 and induction of DNA fragmentation, JNK2 is not involved in coupling $A\beta$ with these apoptotic events. An increase in phospho-JNK expression in the AD brain has been reported [47] and our study indicates that JNK1 may contribute to the pathology of the disease by activating p53 and inducing the apoptotic cascade.

This work was supported by the Health Research Board, Ireland.

REFERENCES

- Miller, D. L., Papayannopoulos, I. A., Styles, J., Bobin, S. A., Lin, Y. Y., Bieman, K. and Iqbal, K. (1993) Peptide composition of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Arch. Biochem. Biophys.* **301**, 41–52
- Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N. and Ihara, Y. (1994) Visualization of $A\beta_{42}$ and $A\beta_{40}$ in senile plaques with end-specific β monoclonals: evidence that an initially deposited species is $A\beta_{42}$. *Neuron* **13**, 45–53
- Adamec, E., Vonsattel, J. P. and Nixon, R. A. (1999) DNA strand breaks in Alzheimer's disease. *Brain Res.* **849**, 67–77
- Li, Y. P., Bushnell, A. F., Lee, C. M., Perlmutter, L. S. and Wong, S. K. (1996) β -Amyloid induces apoptosis in human-derived neurotypic SH-SY5Y cells. *Brain Res.* **738**, 196–204
- Troy, C. M., Rabacchi, S. A., Friedman, W. J., Frappier, T. F., Brown, K. and Shelanski, M. L. (2000) Caspase-2 mediates neuronal cell death induced by β -amyloid. *J. Neurosci.* **20**, 1386–1392
- White, A. R., Guirguis, R., Brazier, M. W., Jobling, M. F., Hill, A. F., Beyreuther, K., Barrow, C. J., Master, C. L., Collins, S. J. and Cappai, R. (2001) Sublethal concentration of prion peptide PrP106–126 or the amyloid peptide protein of Alzheimer's disease activates expression of proapoptotic markers in primary cortical neurons. *Neurobiol. Dis.* **8**, 299–316
- Suo, Z., Fang, C., Crawford, F. and Mullan, M. (1997) Superoxide free radical and intracellular calcium mediate $A\beta_{1-42}$ -induced endothelial toxicity. *Brain Res.* **762**, 144–152
- Boland, B. and Campbell, V. (2003) β -Amyloid $_{1-40}$ -induced apoptosis of cultured cortical neurons involves calpain-mediated cleavage of poly-ADP ribose polymerase. *Neurobiol. Aging* **24**, 179–186
- Jordan, J., Galindo, M. F. and Miller, R. J. (1997) Role of calpain and interleukin-1 β converting enzyme-like proteases in the β -amyloid-induced death of rat hippocampal neurons in culture. *J. Neurochem.* **68**, 1612–1621
- Eilers, A., Whitfield, J., Shah, B., Spadoni, C., Desmond, H. and Ham, J. (2001) Direct inhibition of c-jun N-terminal kinase in sympathetic neurons prevents c-jun promoter activation and NGK-withdrawal-induced death. *J. Neurochem.* **76**, 439–454
- Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P. and Flavell, R. A. (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the *jnk3* gene. *Nature (London)* **389**, 865–870
- Yoshizumi, M., Kogame, T., Suzuki, Y., Fujita, Y., Kyaw, M., Kirima, K., Ishizawa, K., Tsuchiya, K., Kagami, S. and Tamaki, T. (2002) Ebselen attenuates oxidative stress-induced apoptosis via the inhibition of the c-jun N-terminal kinase and activator protein-1 signalling pathway in PC12 cells. *Br. J. Pharmacol.* **136**, 1023–1032
- Bozczyko-Coyne, D., O'Kane, T. M., Wu, Z.-L., Dobrzanski, P., Murthy, S., Vaught, J. L. and Scott, R. W. (2001) CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with $A\beta$ -induced cortical neuron apoptosis. *J. Neurochem.* **77**, 849–863
- Troy, C. M., Rabacchi, S. A., Xu, Z., Maroney, A. C., Connors, T. J., Shelanski, M. L. and Greene, L. A. (2001) β -Amyloid-induced neuronal apoptosis requires c-jun N-terminal kinase activation. *J. Neurochem.* **77**, 157–164
- Morishima, Y., Gotoh, Y., Zieg, J., Barrett, T., Takano, H., Flavell, R., Davis, R. J., Shirasaki, Y. and Greenberg, M. E. (2001) β -Amyloid induces neuronal apoptosis via a mechanism that involves the c-jun N-terminal kinase pathway and the induction of the Fas ligand. *J. Neurosci.* **21**, 7551–7560
- Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J.* **15**, 2760–2770
- Oren, M. (1994) Relationship of p53 in the control of apoptotic cell death. *Semin. Cancer Biol.* **5**, 221–227
- Kitamura, Y., Shimohama, S., Kamoshima, W., Matsuoka, Y., Nomura, Y. and Taniguchi, T. (1997) Changes in p53 in the brains of patients with Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **232**, 418–421
- LaFerla, F. M., Hall, C. K., Ngo, L. and Jay, G. (1996) Extracellular deposition of β -amyloid upon p53-dependent cell death in transgenic mice. *J. Clin. Invest.* **98**, 1626–1632
- Zhang, Y., McLaughlin, R., Goodyer, C. and LeBlanc, A. (2002) Selective cytotoxicity of intracellular β -amyloid peptide 1–42 through p53 and bax in cultured primary human neurons. *J. Cell Biol.* **156**, 519–529
- Culmsee, C., Zhu, X., Yu, Q.-S., Chan, S. L., Camandol, S., Guo, Z., Greig, N. H. and Mattson, M. P. (2001) A synthetic inhibitor of p53 protects neurons against death induced by ischemic and excitotoxic insults, and amyloid β -peptide. *J. Neurochem.* **77**, 220–228
- Miller, F. D., Pozniak, C. D. and Walsh, G. S. (2000) Neuronal life and death: an essential role for the p53 family. *Cell Death Differ.* **7**, 880–888
- Unger, T., Sionov, R. V., Moallem, E., Yee, C. L., Howley, P. M., Oren, M. and Haupt, Y. (1999) Mutation in serines 15 and 20 of human p53 impair its apoptotic activity. *Oncogene* **18**, 3205–3212
- Sakaguchi, K., Sakamoto, H., Lewis, M. S., Anderson, C. W., Erickson, J. W., Appella, E. and Xie, D. (1997) Phosphorylation of serine 392 stabilizes the tetramer formation of tumor-suppressor protein p53. *Biochemistry* **36**, 10117–10124
- Zhu, Y., Mao, X. O., Sun, Y., Xia, Z. and Greenberg, D. A. (2002) p38 mitogen-activated protein kinase-mediated hypoxic regulation of Mdm2 and p53 in neurons. *J. Biol. Chem.* **277**, 22909–22914
- Kwon, Y. W., Ueda, S., Ueno, M., Yodoi, J. and Masutani, H. (2002) Mechanism of p53-dependent apoptosis induced by 3-methylcholanthrene: involvement of p53 phosphorylation and p38 MAPK. *J. Biol. Chem.* **277**, 1837–1844
- Adler, V., Pincus, M. R., Minamoto, T., Fuchs, S. Y., Bluth, M. J., Brandt-Rauf, P. W., Friedman, F. K., Robinson, R. C., Chen, J. M., Wang, X. W. et al. (1997) Conformation-dependent phosphorylation of p53. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 1686–1691

- 28 Fuchs, S. Y., Adler, V., Pincus, M. R. and Ronai, Z. (1998) MEKK1/JNK signaling stabilizes and activates p53. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10541–10546
- 29 Hreniuk, D., Garay, M., Gaarde, W., Monia, B. P., McKay, R. A. and Cioffi, C. L. (2001) Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes. *Mol. Pharmacol.* **59**, 867–874
- 30 Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) Molecular structure of the human cytoplasmic β -actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6133–6137
- 31 Lee, J. K., Park, J., Lee, S. H. and Han, P. L. (1999) Distinct localization of SAPK isoforms in neurons of adult mouse brain implies multiple signaling modes of SAPK pathway. *Brain Res. Mol. Brain Res.* **70**, 116–124
- 32 Mielke, K. and Herdegen, T. (2000) JNK and p38 stress kinases – degenerative effectors of signal transduction cascades in the nervous system. *Prog. Neurobiol.* **61**, 45–60
- 33 Pena, E., Berciano, M. T., Fernandez, R., Crespo, P. and Lafarga, M. (2000) Stress-induced activation of c-jun N-terminal kinase in sensory ganglion neurons: accumulation in nuclear domains enriched in splicing factors and distribution in perichromatin fibrils. *Exp. Cell Res.* **256**, 179–191
- 34 Ito, Y., Mishra, N. C., Yoshida, K., Kharbanda, S., Saxena, S. and Kufe, D. (2001) Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells. *Cell Death Differ.* **8**, 794–800
- 35 Yan, X.-Z., Xia, R., Dou, Y., Wang, S.-D., Qiao, Z.-D. and Qiao, J.-T. (2000) Carbachol blocks β -amyloid fragment 31–35-induced apoptosis in cultured cortical neurons. *Brain Res. Bull.* **51**, 465–470
- 36 Shieh, S. Y., Ikeda, M., Taya, Y. and Prives, C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by Mdm2. *Cell (Cambridge, Mass.)* **91**, 325–334
- 37 Lu, H., Taya, Y., Ikeda, M. and Levine, A. J. (1998) Ultraviolet radiation, but not γ radiation or etoposide-induced DNA damage, results in phosphorylation of the murine p53 protein at serine 389. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6399–6402
- 38 Wang, Y. and Eckhart, W. (1992) Phosphorylation sites in the amino-terminal region of mouse p53. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4231–4235
- 39 Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E. and Anderson, C. W. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* **12**, 5041–5049
- 40 Hu, M. C., Qiu, W. R. and Wang, Y. P. (1997) JNK1, JNK2, and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene* **15**, 2277–2287
- 41 Wang, J. and Friedman, E. (2000) Downregulation of p53 by sustained JNK activation during apoptosis. *Mol. Carcinog.* **29**, 179–188
- 42 Inamaura, N., Enokido, Y. and Hatanaka, H. (2001) Involvement of c-jun N-terminal kinase and caspase-3-like protease in DNA damage-induced, p53-mediated apoptosis of cultured mouse cerebellar granule neurons. *Brain Res.* **904**, 270–278
- 43 Marchenko, N. D., Zaika, A. and Moll, U. M. (2000) Death signal-induced localization of p53 protein to mitochondria. *J. Biol. Chem.* **275**, 16202–16212
- 44 Harada, J. and Sugimoto, M. (1999) Activation of caspase-3 in β -amyloid-induced apoptosis of cultured rat cortical neurons. *Brain Res.* **842**, 311–323
- 45 Selznick, L. A., Holtzman, D. M., Han, B. H., Gokden, M., Srinivasan, A. N., Johnson, E. M. and Roth, K. A. (1999) *In situ* immunodetection of neuronal caspase-3 activation in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* **58**, 1020–1026
- 46 Mielke, K., Damm, A., Yang, D. D. and Herdegen, T. (2000) Selective expression of JNK isoforms and stress-specific JNK activity in different neuronal cell lines. *Mol. Brain Res.* **75**, 128–137
- 47 Zhu, X., Raina, A. K., Rottkamp, C. A., Aliiev, G., Perry, G., Bux, H. and Smith, M. A. (2001) Activation and redistribution of c-jun N-terminal kinase/stress-activated protein kinase in degenerating neurons in Alzheimer's disease. *J. Neurochem.* **76**, 435–441

Received 23 October 2002/16 December 2002; accepted 21 January 2003

Published as BJ Immediate Publication 21 January 2003, DOI 10.1042/BJ20021660