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A$\beta_{25–35}$ alters Akt activity, resulting in Bad translocation and mitochondrial dysfunction in cerebrovascular endothelial cells

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The amyloid-beta peptide (Aβ) induces apoptosis in cerebrovascular endothelial cells (CECs), contributing to the pathogenesis of cerebral amyloid angiopathy. We have previously shown that Aβ induces apoptosis in CECs. In the present study, we report that A$\beta_{25–35}$-induced CEC apoptosis involves the inactivation of Akt, a signaling kinase important in maintaining cell viability. Akt prevents the activation of death-signaling events by facilitating the inactivation of proapoptotic proteins such as Bad. We applied three strategies to show that A$\beta_{25–35}$ inactivation of Akt is causally related to A$\beta_{25–35}$-induced CEC death by preventing Bad activation and subsequent mitochondrial dysfunction (reflected by the release of endonuclease G and Smac, two proapoptotic intermembranous proteins of the mitochondria). Wortmannin, a PI_3-kinase inhibitor, enhanced A$\beta_{25–35}$-induced Bad activation, mitochondrial dysfunction and CEC death. Enhancement of Akt activity by a Tat–Akt fusion protein, or by viral gene transfer of a constitutively active mutant of *akt*, reduced Bad activation, mitochondrial dysfunction, and CEC death. Using a siRNA strategy to knock down the *bad* gene, we showed that Bad activation is causally related to A$\beta_{25–35}$-induced mitochondrial dysfunction and CEC death. Together, these results establish that the Akt–Bad cascade is altered by A$\beta_{25–35}$, resulting in CEC apoptosis.
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Keywords: amyloid angiopathy; apoptosis; endonuclease G; Smac; Tat–Akt

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that results from the progressive deposition of fibrillar amyloid-β peptide (Aβ) in senile plaques in the brain (Wisniewski and Wegiel, 1995; Yankner, 1996). While considerable attention has focused on parenchymal Aβ accumulation and neuronal degeneration in AD brains (Yankner *et al*, 1989; Behl *et al*, 1994), Aβ also accumulates in the cerebrovascular walls of AD patients and in nondemented elderly individuals, leading to cerebral amyloid angiopathy (Perlmutter *et al*, 1994; Wisniewski *et al*, 2000). One of the most widely recognized complications of amyloid angiopathy is hemorrhagic strokes (Walker, 1997). Amyloid-β peptide is toxic to cerebral endothelial cells (CECs) (Price *et al*, 1997; Preston *et al*, 1998; Xu *et al*, 2001; Yin *et al*, 2002*b*), and may contribute to alterations in cerebral blood flow (Iadecola *et al*, 1999). Amyloid-β peptide causes CEC death with morphological and molecular features suggestive of apoptosis (Blanc *et al*, 1997; Hase *et al*, 1997; Suo *et al*, 1997; Xu *et al*, 2001; Yin *et al*, 2002*b*). In particular, mitochondrial dysfunction has been prominently shown in CECs exposed to Aβ (Xu *et al*, 2001; Yin *et al*, 2002*b*). However, the molecular mechanism underlying Aβ-induced mitochondrial dysfunction and apoptosis in CECs remains to be fully characterized.

Akt (a.k.a. protein kinase B), a serine/threonine kinase with oncogenic properties (Burgering and Coffer, 1995; Franke *et al*, 1995), serves as an antiapoptotic regulator, controlling the balance between survival and apoptosis in growth factor-mediated cytoprotection (Burgering and Coffer, 1995; Ahmed *et al*, 1997; Dudek *et al*, 1997). Akt is activated in a PI_3-kinase-dependent manner by phosphorylation at two regulatory sites: threonine 308 (Thr 308) and serine 473 (Ser 473) (Franke *et al*,

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1995, 1997; Klippel *et al*, 1997; Sable *et al*, 1998). Fully activated Akt, in turn, functions to promote cell survival by selectively phosphorylating and inactivating several downstream proapoptotic targets including the BH3-only protein, Bad (Datta *et al*, 1997; del Peso *et al*, 1997). While a role for Akt has been established in growth factor-mediated survival, little is known regarding its potential role under conditions of pathological neurodegeneration, such as that seen in cerebral amyloid angiopathy.

Mitochondrial dysfunction has been implicated as a key event in apoptosis in a number of cell death paradigms (Green and Reed, 1998). Several cellular processes characterize mitochondrial dysfunction and activation of apoptosis in Aβ-induced CEC death. These include mitochondrial release of cytochrome *c* (Xu *et al*, 2001), Smac (Yin *et al*, 2002*b*), and endonuclease G (Endo G) (as shown in the present study). Cytochrome *c* triggers caspase activation through interaction with Apaf-1 (Liu *et al*, 1996; Li *et al*, 2000). Second mitochondria-derived activator of caspase (Smac, a.k.a. DIABLO) relieves Inhibitor of Apoptosis Proteins (IAPs) from inhibiting caspases (Du *et al*, 2000; Verhagen *et al*, 2000). However, mitochondrial release of Endo G causes nuclear chromatin condensation and fragmentation in a caspase-independent manner (Li *et al*, 2001; Cregan *et al*, 2002). The preferential release of select proapoptotic proteins from mitochondria might be subject to differential upstream regulation (Kandasamy *et al*, 2003). The Akt cascade, via its control of Bad activity, has emerged as an important regulatory mechanism upstream of mitochondria in the maintenance of cell viability. In the present study, we explore the roles of Akt and Bad and related cellular events in Aβ-induced mitochondrial dysfunction and cell death in CECs.

Materials and methods

Murine Cerebrovascular Endothelial Cell Primary Cultures

Murine CECs were prepared as described previously (Xu *et al*, 1992). Murine CECs migrating from the vessels were pooled to form a proliferating cell culture that was maintained in DMEM, with high glucose and L-glutamine supplemented with 10% FBS, 0.5 mg/ml heparin, and 75μg/ml endothelial cell growth supplements. Murine CECs of passage 4–15 that were uniformly positive for factor VIII and vimentin (>95% endothelial cell purity) and characteristic bradykinin receptors were grown to 85% to 95% confluency before use (Xu *et al*, 1992).

Treatment with Aβ and Other Chemicals

$A\beta_{1-40}$ and $A\beta_{1-42}$ are the major components of Aβ deposits in the AD brain; however, in most experimental systems, the biological effects of $A\beta_{25-35}$ are comparable (Loo *et al*, 1993; Behl *et al*, 1994). We have previously shown that $A\beta_{25-35}$ has approximately the same potency as $A\beta_{1-40}$ in inducing cell death in CECs (Xu *et al*, 2001; Yin *et al*, 2002*b*). Based on these data, we employed $A\beta_{25-35}$ in this study. Cerebrovascular endothelial cells were treated with 25 μmol/L $A\beta_{25-35}$ (Sigma, St Louis, MO, USA) in serum-free growth medium for varying times. In some experiments, the murine CECs were co-treated with wortmannin at concentrations of 0.1, 1, and 10 μmol/L.

Western Blot Analysis

Subfractionization of cellular components in CECs was performed as previously reported, with little cross-contamination of fractionated proteins (Xu *et al*, 2001; Yin *et al*, 2002*b*). Protein samples (20 to 40 μg) were electrophoresed on a 10% to 15% SDS-PAGE gel, transferred to PVDF membranes and probed with primary antibodies including rabbit anti-Endo G antibody (1:2,500, a generous gift from Dr XD Wang), goat anti-smac antibody, rabbit anti-Akt antibody, rabbit anti-phospho-Akt (Thr 308) antibody, rabbit anti-phospho-Akt (Ser 473) antibody, rabbit anti-Bad antibody, rabbit anti-phospho-Bad (Ser 136) antibody (1:1,000; Cell Signaling, Beverly, MA, USA), and mouse anti-actin antiserum (1:200; Santa Cruz, CA, USA) for 1–2 h at room temperature. The membranes were then incubated with the second antibody (1:5,000; anti-rabbit, anti-mouse or anti-goat IgG conjugated with alkaline phosphatase Promega, Madison, WI, USA) at room temperature for 1 h. The color reaction was developed using the Blot AP System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Immunofluorescent Staining

Murine CECs grown on coverslips were fixed with 4% paraformaldehyde for 30 mins and washed 3 times with 0.1 mol/L PBS (pH 7.4). The cells were then incubated with a primary rabbit anti-Endo G antibody (1:500, overnight at 4°C). The next day, the cells were incubated with fluorescein-conjugated anti-rabbit IgG (1:100; Vector Labs, Burlingame, CA, USA) for 1 h. Cerebrovascular endothelial cells were counterstained with 1 μg/mL of PI (Molecular Probes, Eugene, OR, USA) to visualize nuclear morphology. Slides were washed, wet mounted, and examined with an Olympus fluorescence microscope.

Generation and Purification of Tat–Akt Fusion Protein

The Tat–Akt fusion protein was prepared as described previously (Schwarze *et al*, 1999).

Construct design: Two mutant mouse *akt* plasmid cDNAs were kindly provided by Dr Tsichlis: pCMV-HA-K179m-AKT, a kinase-inactive mutant (Akt_m), and pCMV-HA-myr-AKT, a constitutively active myristolated mutant (Akt_{ca}) (Franke *et al*, 1995). The coding region of each plasmid was amplified by PCR with the following primers: 5′-ATGAACGACGTAGCCATTG-3′, and 5′-TCAGGCTGTG CCACTG-3′, adding *Kpn*I and *Eco*RI restriction sites at the

N and C termini, respectively. Amplimer identity was confirmed by restriction digestion, then cloned into pTAT-HA using the same restriction sites. The DNA sequence was verified by sequencing analysis.

Protein production and isolation: Recombinant plasmids were transformed into *Escherichia coli* BL21 cells and protein expression was induced using 0.1 mmol/L isopropylthiogalactoside at 37°C for 5 to 6 h. The fusion proteins were purified using a Ni-NTA superflow agarose column (Qiagen, Valencia, CA, USA), followed by a PD-10 column, pre-equilibrated with serum-free culture medium containing 10% glycerol, and then stored in 10% glycerol/PBS at −80°C until use.

Detection of protein transduction: Cerebrovascular endothelial cells were seeded on coverslips before the Tat–Akt fusion protein was added at a final concentration of 100 nmol/L, incubated for 10 to 30 mins at 37°C, and washed in PBS. Cells fixed in 4% paraformaldehyde were incubated with mouse anti-HA monoclonal antibody (1:200, Santa Cruz, CA, USA), then with fluorescein-conjugated anti-mouse IgG (1:100; Vector Labs, Burlingame, CA, USA). The coverslips were washed, wet mounted, and examined with an Olympus fluorescence microscope.

Construction of Retrovirus-Mediated Overexpression of Akt in Cerebrovascular Endothelial Cells

The mutant *akt* cDNAs (pCMV-HA-K179m-AKT, pCMV-HA-myr-AKT) (Franke *et al*, 1995) were also amplified by PCR to construct viral vectors for gene transfer. The primers used were as follows: 5′-ACAGCTAGCATGAACG ACGTAGCCATTGTG-3′ (antisense primer), 5′-ATATCTAG ATCAGGCTGTGCCACTGGCTG-3′ (sense primer). The PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and isolated by digestion with *Nhe*I–*Xba*I restriction enzymes and subcloned into the *Nhe*I–*Xba*I sites of pMX-internal ribosomal entry site (IRES)-EGFP (Kitamura *et al*, 1995). The resulting plasmid contained the *akt* gene, an IRES, and an *EGFP* gene. For production of high-titer retroviruses expressing Akt, Phoenix ecotropic packaging cells were plated at approximately 50% to 75% confluency in 15-cm-diameter tissue culture plates and pMX-AKT-IRES-EGFP vector DNAs were transfected into Phoenix retroviral producer cells by the modified calcium phosphate method. After incubation for 2 days at 32°C, viral supernatant was collected and passed through a 0.45 μm filter. Cerebrovascular endothelial cells were infected with retroviral supernatant in the presence of Polybrene at 5 μg/mL and incubated for 3 h. Retroviral supernatant was removed and replaced with normal growth medium. Cells grown for 48 to 72 h were sorted by flow cytometry. Infected populations exhibiting between 60% and 75% green fluorescent cells were used for further experimentation.

RT-PCR

Bad mRNA expression was detected by RT-PCR (Yin *et al*, 2002*a*). Total RNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). In all, 600 ng of RNA was used for the synthesis of cDNA and PCR reactions. Primers were designed based on the mouse *bad* sequences (forward: 5′-GGAAGACGCTAGTGCTACAG-3′, reverse: 5′-GAGCCTCCTTTGCCCAAGTT-3′). The relative *bad* mRNA level of was normalized to endogenous *cyclophilin* mRNA for each sample.

Co-immunoprecipitation

Co-immunoprecipitation was prepared as described previously (Xu *et al*, 2001; Yin *et al*, 2002*b*). $A\beta_{25-35}$-treated CECs were homogenized in lysis buffer and the supernatant was incubated with rabbit anti-Bcl-xL antibody. Protein G sepharose 4 fast flow (Pharmacia Biotech, Piscataway, NJ, USA) was added to the antigen–antibody mixture and incubated with gentle agitation for 1 to 2 h. The immunoprecipitate was washed with lysis buffer, resuspended in 6 × SDS loading buffer, separated in SDS-PAGE gel, transferred to PVDF membrane, and then analyzed by Western blotting with rabbit anti-Bad antibody (1:1,000) as described above.

Bad Knockdown in Cerebrovascular Endothelial Cells with RNA Interference

SiRNAs targeting the mouse *bad* gene were prepared by *in vitro* transcription using *Silencer*™ siRNA Construction Kit (Ambion, Austin, TX, USA). SiRNA was synthesized to target the coding region of mouse *bad* mRNA as follows: 5′-UUCUGCGAUCACGAUGUCUAU-3′, and scrambled: 5′-UUGUCUCAUGUCGUCUAGACA-3′. Murine CECs were transfected 24 h after plating, using 2 to 4 μL siPORT Lipid (Ambion, Austin, TX, USA) according to the manufacturer's protocol, at a final siRNA concentration of 100 nmol/L. *Bad* mRNA and Bad protein levels were examined by RT-PCR and Western blotting 48 to 72 h after transfection.

Assessment of Murine Cerebrovascular Endothelial Cell Death

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay: Cerebrovascular endothelial cells were rinsed with HBSS and replaced with DMEM containing MTT (Sigma, St Louis, MO, USA) at a final concentration of 0.5 mg/mL. The cells were then incubated for 4 h at 37°C under 5% CO_2/95% air, and for another 14 h in lysis solution (10% SDS in 0.01 N HCl) at 1:1 dilution. Absorbance of the mixture was read at 540 nm using a multiplate reader. Viability was expressed as OD_{540} of treated cells divided by OD_{540} of untreated cells (Yin *et al*, 2002*a*, *b*).

L-lactate dehydrogenase release: In all, 25 μL of medium was taken from each sample and placed into a 96-well plate; 125 μL LDH buffer (0.1 mol/L, pH 7.4, 1:2 of KH_2PO_4 and K_2HPO_4) and 100 μL NADH solution (0.03% NADH in LDH buffer) were added to each sample well. After incubation for 5 mins, 25 μL pyruvate solution

(22.7 mmol/L pyruvate in LDH buffer) was added and absorbance was determined at 340 nm. LDH release was expressed as U/mL after normalization to L-lactate dehydrogenase (LDH) standards (1000 U/mL, Sigma) (Yin *et al*, 2002*a*, *b*).

Statistical analysis: Quantitative data are expressed as mean ± s.d., based on at least three separate experiments of triplicate samples. Difference among groups was statistically analyzed by one-way analysis of variance followed by Bonferroni's *post hoc* *t*-test. Comparison between two experimental groups was based on two-tailed *t*-test. A *P*-value less than 0.05 was considered significant.

Results

Aβ_{25-35} Inactivation of Akt in Cerebrovascular Endothelial Cells

Akt plays a central role in cell survival by preventing the activation of a variety of proapoptotic pathways. Akt is activated in a PI_3-kinase-dependent manner by phosphorylation at two regulatory sites: Thr 308 and Ser 473 (Franke *et al*, 1995, 1997; Klippel *et al*, 1997; Sable *et al*, 1998). To determine whether Akt inactivation is involved in Aβ_{25-35}-induced CEC death, we first measured levels of total and phosphorylated Akt (at Thr 308 and Ser 473) by Western blot analysis. Exposure to Aβ_{25-35} (25 μmol/L) resulted in a substantial decrease in the active forms of Akt at both Thr 308 and Ser 473, as early as 1 h after treatment; but had no effect on total Akt levels until 24 h (Figures 1A and 1B). No significant changes in Akt activation were observed in control cultures between 0 and 24 h (data not shown).

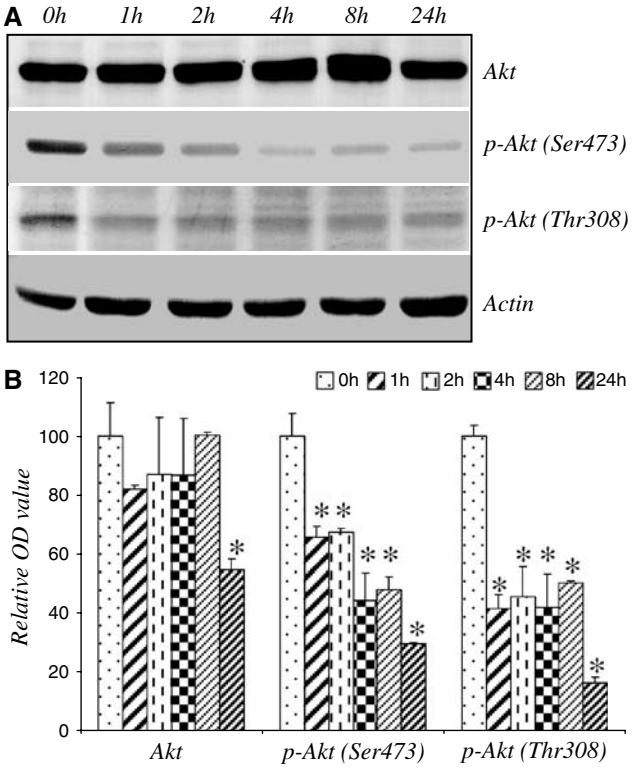


Figure 1 Aβ_{25-35} inactivation of Akt in CECs. (**A**) Cerebrovascular endothelial cells treated with 25 μmol/L Aβ_{25-35} for the indicated times were analyzed by Western blotting using anti-Akt, anti-p-Akt (at Thr 308 and Ser 473), and anti-actin antibodies. Akt inactivation reflected by a decrease in Akt phosphorylation is evident within 1 h, while little change in the total Akt level was observed until 24 h after Aβ_{25-35} exposure. (**B**) Quantitative analysis of three Western blots (normalized to actin) using the NIH Image Analysis System graphically illustrates decreased Akt phosphorylation by Aβ_{25-35}. Data shown in panel A are representative of three separate experiments with similar results. Data in panel B are expressed as mean ± s.d. *$P < 0.05$ versus 0 h exposure.

Aβ_{25-35} Dephosphorylation of Bad in Cerebrovascular Endothelial Cells

Fully activated Akt promotes cell survival by selectively phosphorylating and inactivating several downstream proapoptotic targets including the BH3-only protein, Bad (Datta *et al*, 1997; del Peso *et al*, 1997). Under normal physiological conditions, Bad, in the phosphorylated and inactive form, is known to exist almost exclusively in the cytoplasm (Cross *et al*, 1995; Datta *et al*, 1997; del Peso *et al*, 1997; Hajduch *et al*, 1998; Brunet *et al*, 1999; Kaplan and Miller, 2000). Reduced Akt activity would be expected to favor the dephosphorylation of Bad (rendering the protein active). Activated Bad, recognized as an essential initiator of the apoptotic cascade (Kelekar and Thompson, 1998; Huang and Strasser, 2000), translocates to mitochondria where it binds Bcl-x_L, an antiapoptotic member in the Bcl-2 family, resulting in mitochondrial membrane disruption, mitochondrial dysfunction, and subsequent cell death (Yang *et al*, 1995; Zha *et al*, 1996; Korsmeyer, 1999). In view of the inactivation of Akt by Aβ_{25-35}, we examined if Bad was activated after exposure to Aβ_{25-35}. Aβ_{25-35} decreased phospho-Bad (Ser 136) levels in the cytosolic fraction as early as 4 h after exposure, in parallel with an increase in the Bad content in the mitochondrial fraction (Figure 2A), indicating that dephosphorylated Bad translocated from the cytoplasm to mitochondria. We have previously reported that Aβ_{25-35} induced CEC death beginning 24 h after treatment (Xu *et al*, 2001), suggesting that Bad translocation preceded cell death. In addition, Bcl-x_L co-immunoprecipitated with Bad in Aβ-treated CECs, suggesting a physical interaction between these two Bcl-2 family members during the apoptotic process (Figure 2B). Aβ_{25-35} had little effect on *bad* mRNA levels, suggesting that Bad activation involved primarily post-transcriptional regulation (Figure 2C). No significant change in Bad phosphorylation was observed in control cultures between 0 and 48 h (data not shown).

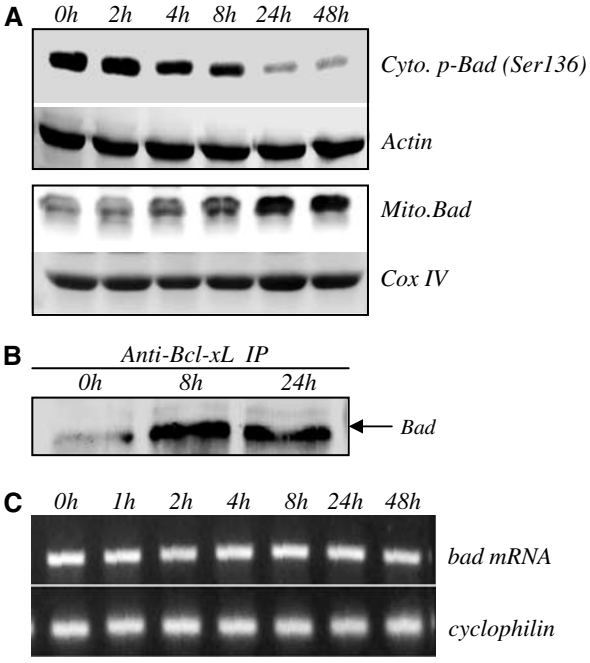


Figure 2 Aβ_{25-35} activation of Bad. Cerebrovascular endothelial cells treated with 25 μmol/L Aβ_{25-35} at the indicated times were fractionated and examined by Western blotting with anti-Bad or anti-p-Bad (Ser 136) antibodies. Cytosolic and inactive (or phosphorylated) Bad (Cyto.p-Bad) decreased 4 h after Aβ treatment, in parallel with the appearance of Bad in the mitochondrial fraction (**A**). Immunoprecipitation with anti-Bcl-x_L antibodies followed by immunoblotting with anti-Bad antibodies revealed that Bad co-immunoprecipitated with Bcl-x_L 8–24 h after Aβ_{25-35} treatment (**B**). Analysis with RT-PCR revealed that *bad* mRNA levels remained unchanged after Aβ_{25-35} exposure in CECs (**C**). Data shown are representative of three separate experiments with similar results.

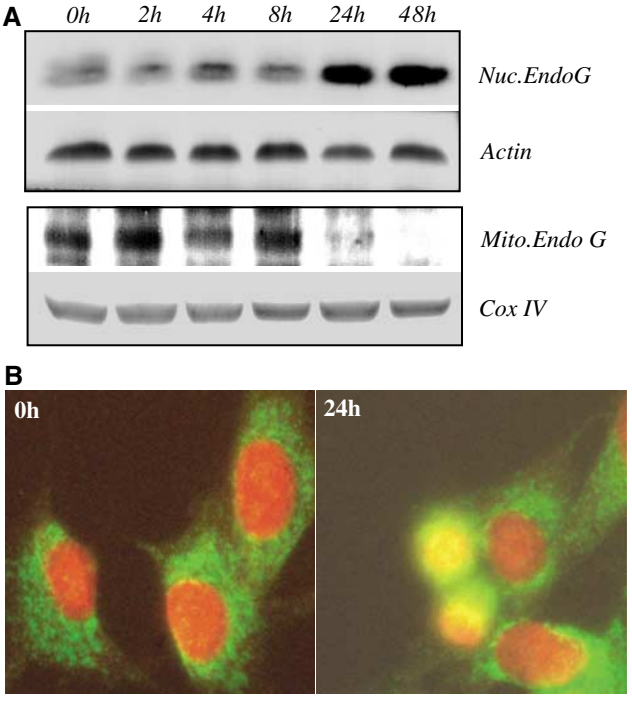


Figure 3 Aβ_{25-35}-induced translocation of Endo G from mitochondria to nucleus. Cerebrovascular endothelial cells, treated with 25 μmol/L Aβ_{25-35} and harvested at times indicated, were fractionated into mitochondrial (Mito.) and nuclear (Nuc.) extracts, separated by SDS-PAGE, and analyzed by Western blotting with anti-Endo G, anti-Actin, or anti-Cox IV antibodies (**A**). Actin and Cox IV bands reflect equal protein loading for the nuclear and mitochondrial fractions, respectively. Cerebrovascular endothelial cells, grown on coverslips and treated with 25 μmol/L Aβ_{25-35} for 24 h, were fixed and immunostained with anti-Endo G antibody (green); nuclei were counterstained with PI (red) (**B**). Note the change in Endo G immunostaining from a punctate mitochondrial to a nuclear pattern in some CECs showing condensed nuclei (yellow) after treatment with Aβ_{25-35}. Magnification: $\times$1000. Data shown are representative of three separate experiments with similar results.

Endonuclease G Translocation from Mitochondria to Nucleus After Aβ_{25-35} Exposure

Previous studies have shown that the release of mitochondrial intermembranous proteins after apoptotic stimuli might be under the control of some BH3-only family members, including Bad (Li *et al*, 2001; Wang, 2001; Madesh *et al*, 2002). These mitochondrial intermembranous proteins include cytochrome *c*, Smac and Endo G. Aβ_{25-35}-induced Bad activation and translocation to mitochondria in CECs, as shown above, would be expected to cause mitochondrial dysfunction. We have previously shown that Aβ_{25-35} induced cytochrome *c* and Smac release, leading to CEC apoptosis (Xu *et al*, 2001; Yin *et al*, 2002*b*). In the present study, we found that Aβ_{25-35} treatment caused an increase in Endo G in the nuclear fraction and a decrease in the mitochondrial fraction, indicating translocation from mitochondria to nucleus (Figure 3A). This subcellular redistribution was confirmed by immunofluorescent staining with an anti-Endo G antibody. The punctate mitochondrial staining pattern observed in control cultures changed to a dense nuclear pattern after Aβ_{25-35} treatment (Figure 3B).

Akt Regulation of Aβ_{25-35}-Induced Mitochondrial Dysfunction and Cell Death

Release of mitochondrial intermembranous proteins might be regulated by multiple upstream signaling pathways (Imaizumi *et al*, 1999; Kaltschmidt *et al*, 1999; Bozyczko-Coyne *et al*, 2001; Martin *et al*, 2001). If Bad-initiated mitochondrial dysfunction (including mitochondrial release of Smac and Endo G) and CEC death is under the regulation of Akt, alteration of Akt activity would be expected to affect cellular events downstream of Bad as well. We applied three strategies to determine if Akt was involved in Aβ_{25-35}-induced Bad activation and

subsequent release of mitochondrial intermembranous proteins. The first was to determine whether inhibition of Akt affects Aβ_{25-35}-induced mitochondrial dysfunction and cell death. Wortmannin, a PI_3/Akt inhibitor at doses between 30 nmol/L and 1 μmol/L (Dimmeler *et al*, 1998; Fujio and Walsh, 1999; Kim *et al*, 2000), enhanced Aβ_{25-35}-induced Bad translocation from the cytoplasm to mitochondria in a dose-dependent manner (Figure 4A). This effect was accompanied by enhanced Endo G translocation to the nucleus, and Smac release into cytosol, in a dose-dependent manner (Figure 4A). Wortmannin also increased Aβ_{25-35}-induced cell death (Figure 4B).

The second strategy entailed enhancement of Akt activity with cellular transfer of a constitutively active myristylated mutant Tat–Akt (Tat–Akt_{ca}) fusion protein, using a kinase-inactive mutant Tat–Akt (Tat–Akt_m) as a negative control. Successful delivery of both fusion proteins (500 nmol/L) into CECs was confirmed by Western blotting (Figure 5A). In addition, effective transduction of the Tat–Akt_{ca} and Tat–Akt_m protein was further confirmed by immunostaining with anti-HA antibody in CECs (Figure 5B). As expected, Tat–Akt_{ca}, but not Tat–Akt_m, increased phospho-Akt (Thr 308 and Ser 473) (Figure 5A). Treatment with Tat–Akt_{ca}, but not Tat–Akt_m, effectively inhibited Aβ_{25-35}-induced Bad translocation to mitochondria and subsequent mitochondrial release of Smac and Endo G to cytosol and nucleus, respectively (Figure 5C). Tat–Akt_{ca} protected CECs from Aβ_{25-35}-induced cell death (Figure 5D), while Tat–Aktm had no effect on Aβ_{25-35}-induced mitochondrial protein release, and subsequent cell death (Figures 5C and 5D).

The third strategy involved the upregulation of Akt activity in Aβ_{25-35}-treated CECs using retroviral gene transfer of akt_{ca} (pMX/Akt_{ca}), or akt_m (pMX/Akt_m), which served as a negative control. Infection with both *akt*-carrying viruses increased total Akt protein levels, while only pMX/Akt_{ca} increased phospho-Akt levels in CECs (Figure 6A). Effective gene transfer was also confirmed by immunofluorescence, which revealed abundant cytoplasmic staining of the virally expressed reporter, GFP (Figure 6B). Akt_{ca} gene transfer attenuated Aβ_{25-35}-induced Bad translocation to the mitochondria and the subsequent release of Endo G (Figure 6C) and Smac (data not shown), and cell death (Figure 6D), while gene transfer of the kinase inactive mutant akt_m had none of these effects. Results of these experiments further implicate the causal role of Akt in regulating Aβ_{25-35}-induced Bad activation and mitochondrial dysfunction, leading to CEC apoptosis.

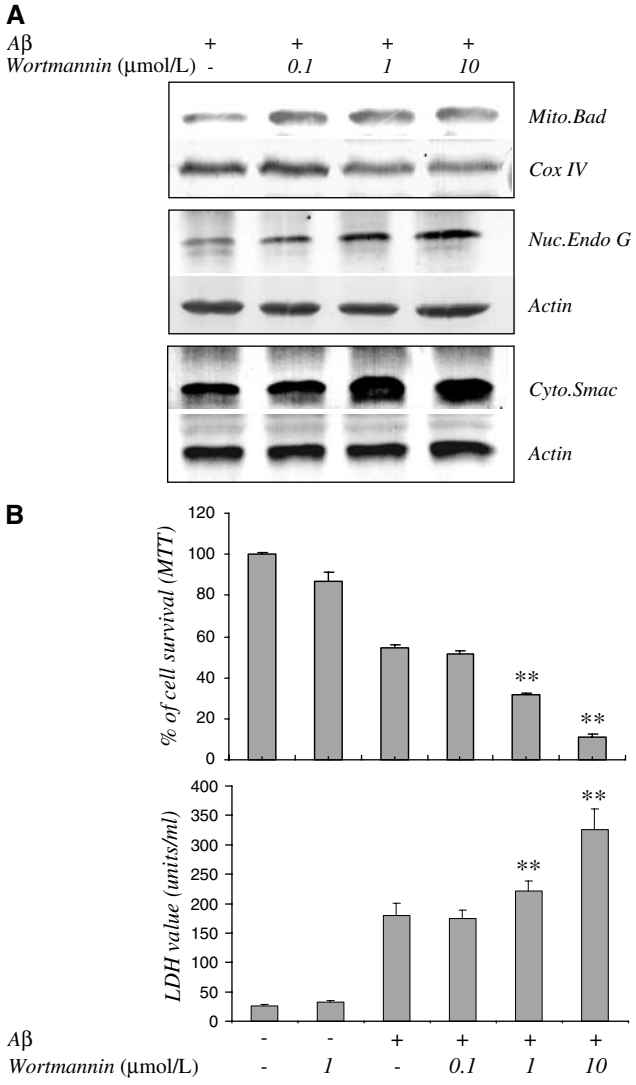


Figure 4 Effects of Akt inhibition on Bad translocation, mitochondrial intermembranous protein release, and CEC death induced by Aβ_{25-35}. (**A**) Cerebrovascular endothelial cells, treated with 25 μmol/L Aβ_{25-35} in the presence or absence of the Akt inhibitor, wortmannin, were fractionated and analyzed by Western blotting with antibodies indicated. Wortmannin significantly increased Aβ_{25-35}-induced Bad translocation to mitochondria (Mito. Bad) after Aβ_{25-35} exposure for 4 h, Endo G translocation to nucleus (Nuc.Endo G) and Smac release into cytoplasm (Cyto.Smac) after Aβ_{25-35} exposure for 24 h in a dose-dependent manner. Western blots are representative of three separate experiments with similar results. (**B**) Wortmannin increased CEC death (determined by MTT assay and LDH assay) after Aβ_{25-35} exposure for 24 h. Data shown are expressed as mean $\pm$ s.d. from three separate experiments in quadruplicate. $^{**}P<0.05$ versus the control groups (CECs treated with Aβ_{25-35} only).

Effects of *bad* Knockdown with siRNA on Mitochondrial Dysfunction and Cell Death

Since mitochondrial release of intermembranous proteins such as Smac and Endo G is under the control of BH3-only family members including Bad (Li *et al*, 2001; Wang, 2001; Madesh *et al*, 2002), we examined if Bad activation was causally related to Aβ_{25-35}-induced mitochondrial dysfunction and CEC death. Cerebrovascular endothelial

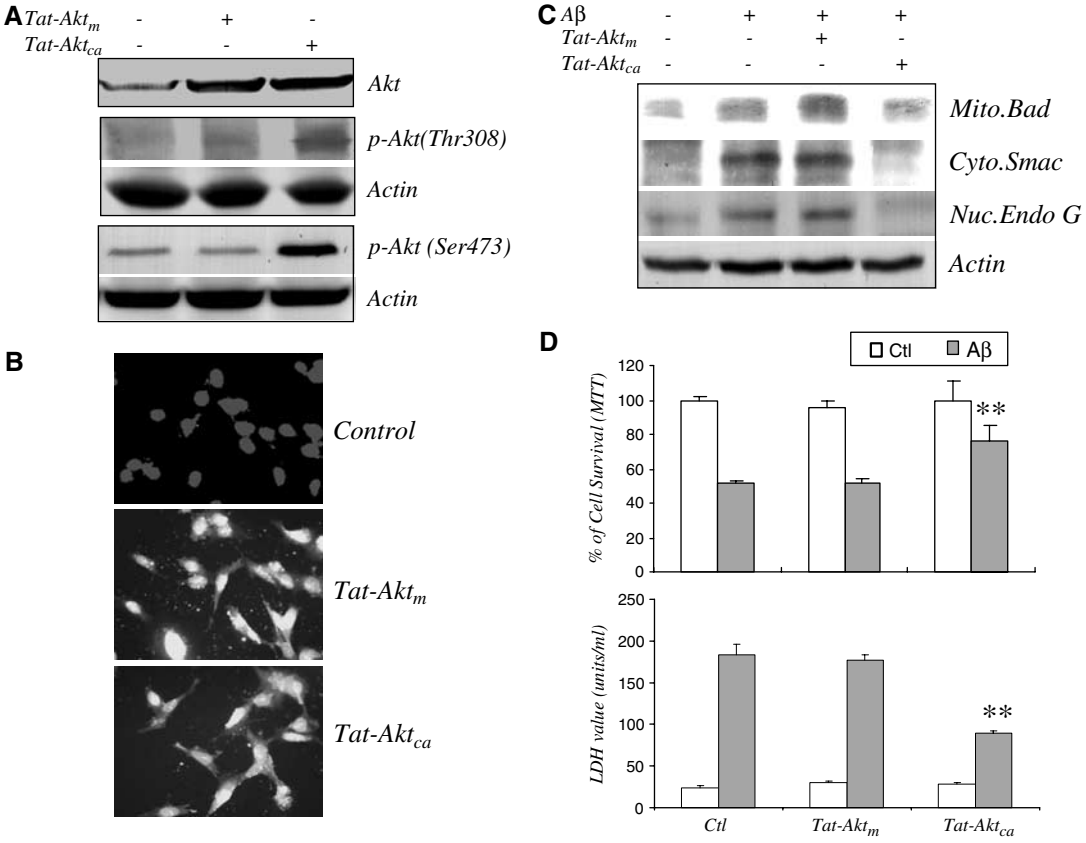


Figure 5 Effects of Tat–Akt fusion protein delivery on $A\beta_{25-35}$-induced Bad translocation, Endo G and Smac release, and CEC death. (**A**) Cerebrovascular endothelial cells, treated with 25 μmol/L $A\beta_{25-35}$ in the presence or absence of 500 nmol/L constitutively active myristylated mutant Tat–Akt fusion protein (Tat–Akt_{ca}) or a kinase-inactive mutant Tat–Akt fusion protein (Tat–Akt_m), were fractionated and examined by Western blotting. An increase in cytosolic p-Akt (Thr 308 and Ser 473) was only observed with Tat–Akt_{ca} treatment. (**B**) Tat–Akt_{ca} or Tat–Akt_m fusion protein delivery was confirmed by immunostaining using an anti-HA monoclonal antibody. Magnification: $\times$ 200. (**C**) Western blotting showed that Tat–Akt_{ca}, but not Tat–Akt_m, delivery significantly reduced $A\beta_{25-35}$-induced Bad translocation and subsequent mitochondrial Endo G and Smac release (Mito.Bad, mitochondrial Bad; Nuc.Endo G, nuclear Endo G; Cyto.Smac, cytosolic Smac). (**D**) Tat–Akt_{ca}, but not Tat–Akt_m, treatment decreased CEC death after $A\beta_{25-35}$ exposure for 24 h. Data shown in panels A, B, and C are representative of three separate experiments with similar results. Data shown in panel D are expressed as mean $\pm$ s.d. from three separate experiments in quadruplicate. $^{**}P<0.05$ versus the control groups (CECs treated with $A\beta_{25-35}$ or $A\beta_{25-35}$ plus Tat–Akt_m delivery).

cells were treated with siRNA specific for *bad* without $A\beta_{25-35}$ exposure. *Bad* mRNA levels were significantly repressed by treatment with 100 nmol/L *bad* siRNA (Figure 7A). SiRNA treatment also decreased $A\beta_{25-35}$-induced Bad translocation to mitochondria (Mito Bad, Figure 7B), and reduced Endo G translocation from mitochondria to nucleus (Nuc Endo G, Figure 7B). Smac translocation from mitochondria to cytoplasm was also decreased (Cyto Smac, Figure 7B). Finally, *bad* knockdown with siRNA also attenuated $A\beta_{25-35}$-induced CEC death (Figure 7C). The specificity of this *bad* siRNA strategy was supported by the finding that scrambled *bad* siRNA was without effect on $A\beta_{25-35}$-induced Bad activation, mitochondrial release of Smac and Endo G, and the extent of CEC death. Furthermore, *bad* siRNA did not alter *cyclophilin* mRNA levels.

Discussion

We have shown that $A\beta_{25-35}$-induced CEC death was accompanied by Akt inactivation by means of dephosphorylation, occurring within 1 h after exposure to $A\beta_{25-35}$. That Akt inactivation was causally related to $A\beta_{25-35}$-induced mitochondrial dysfunction and subsequent CEC apoptosis was supported by two lines of evidence. First, Akt inhibition by wortmannin exacerbated $A\beta_{25-35}$-induced Bad activation, mitochondrial dysfunction, and CEC death. Second, enhancing Akt activity using a Tat–Akt_{ca} fusion protein or by viral gene transfer of a consitutively active mutant akt_{ca} attenuated $A\beta_{25-35}$-induced Bad activation, mitochondrial dysfunction, and CEC death. These results suggest that $A\beta_{25-35}$-induced Akt dephosphorylation activates Bad, releasing mitochondrial Endo G and Smac, and

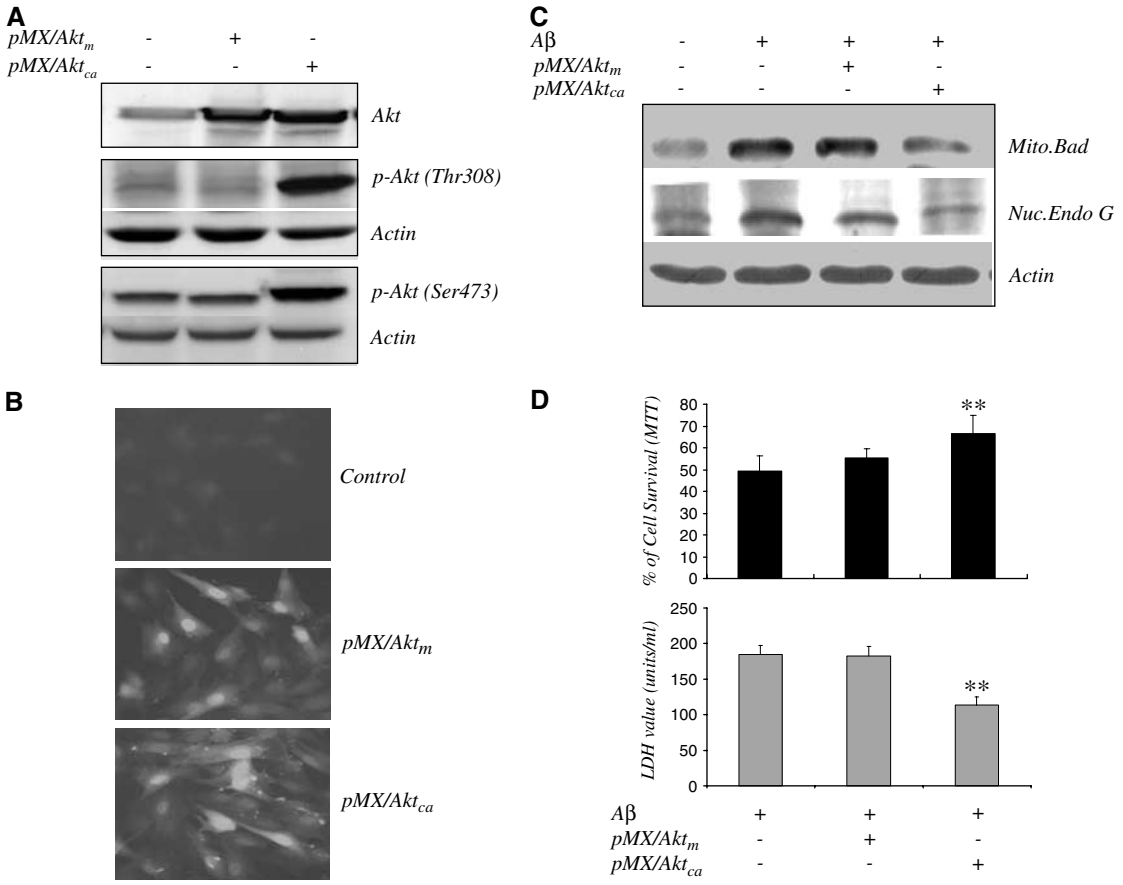


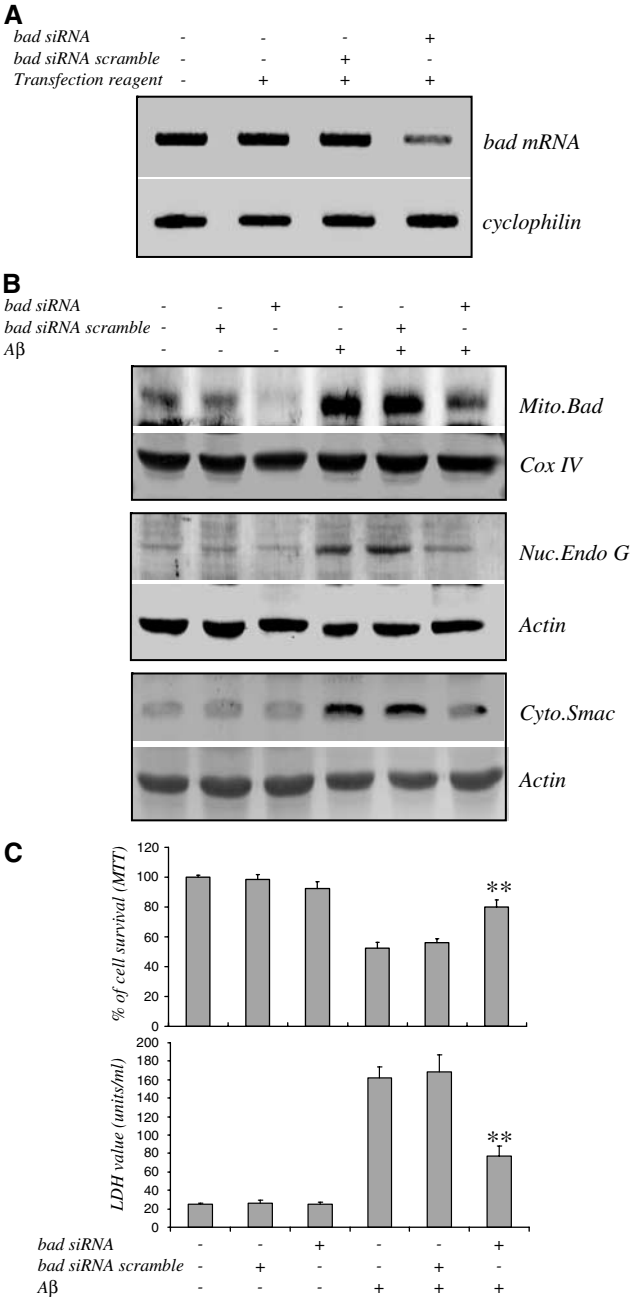
Figure 6 Effects of Akt upregulation by retroviral *akt* gene transfer on Aβ_{25-35}-induced Bad translocation, Endo G and CEC death. (**A**) Western blotting shows total Akt and phospho-Akt levels after infection with retroviral vectors carrying kinase-inactive mutant *akt* (pMX/Akt$_m$) or constitutively active myristylated *akt* (pMX/Akt$_{ca}$). (**B**) Effective gene transfer to CECs was confirmed by GFP fluorescence. Magnification: $\times$ 200. (**C**) Constitutively active *akt*, but not kinase-inactive *akt*, gene transfer significantly reduced Bad translocation and subsequent mitochondrial Endo G release after Aβ_{25-35} treatment for 24 h (Mito.Bad, mitochondrial Bad; Nuc.Endo G, nuclear Endo G). (**D**) Transfer of the constitutively active *akt*, but not kinase inactive *akt*, gene decreased Aβ_{25-35}-induced CEC death. Data shown in panels A, B, and C are representative of three separate experiments with similar results. Data shown in panel D are expressed as mean $\pm$ s.d. from three separate experiments in quadruplicate. $^{**}P<0.05$ versus the control groups (CECs treated with Aβ_{25-35} or Aβ_{25-35} plus pMX/Akt$_m$ gene transfer).

contributes to Aβ_{25-35}-induced CEC death. The survival-promoting activity of several growth factors, including neurotrophins, is mediated by enhancement of Akt activity (Kaplan and Miller, 2000). Conversely, there is growing evidence that a number of death-promoting stimuli cause Akt inactivation. For example, *N*-methyl-D-aspartate excitotoxicity is accompanied by Akt inactivation and Akt upregulation using viral gene transfer of a constitutively active *akt* gene prevents excitotoxic neuronal cell death (Luo *et al*, 2003).

Although the PI3K/Akt pathway protects cells from apoptosis caused by diverse stress stimuli, its protective role in the setting of Aβ exposure is unclear, and several reports are contradictory. For example, a recent study by Wei *et al* (2002) shows that Aβ_{25-35} induced a weak activation of Akt together with JNK and ERK but not p38 kinase in

Figure 7 Effects of *bad1* knockdown on Endo G and Smac translocation and CEC death. (**A**) Total RNA from CECs transfected with 100 nmol/L *bad* siRNA or scrambled *bad* siRNA was analyzed by RT-PCR 48 h after transfection. *Bad*, but not scrambled, siRNA reduced *bad* mRNA levels. *Bad* siRNA did not alter *cyclophilin* mRNA levels, suggesting the specificity of this knockdown strategy. (**B**) Cerebrovascular endothelial cells treated with *bad* siRNA for 48 h before Aβ_{25-35} treatment were fractionated and analyzed by Western blotting with the antibodies indicated. *Bad* siRNA effectively reduced mitochondrial Bad translocation (Mito.Bad), nuclear Endo G (Nuc. Endo G) and cytosolic Smac (Cyto.Smac) 24 h after Aβ_{25-35} treatment. (**C**) *Bad* knockdown also reduced CEC death (determined by MTT assay and LDH assay). RT-PCR (**A**) and Western blots (**B**) are representative of three separate experiments with similar results. Data shown in panel C are expressed as mean $\pm$ s.d. from three separate experiments in quadruplicate. $^{**}P<0.05$ versus control (CECs treated with Aβ_{25-35} in the absence of siRNA or in the presence of a scrambled siRNA).

SH-5Y cells. Martin *et al* (2001) have also shown that Akt activity was activated shortly after incubation with Aβ_{25-35} and Aβ_{1-40} with kinetics different from that of nerve growth factor in PC12 cells. However, consistent with the results of the current study, several other groups have observed down-regulation of Akt after Aβ exposure. For example, Suhara *et al* (2003) recently showed that virally encoded Aβ_{1-42} was proapoptotic and inhibitory to Akt phosphorylation in human umbilical vein endothelial cells, which was characterized by mitochondrial dysfunction, DNA condensation, and activation of caspase-3. Kubo *et al* (2002) also found that Aβ_{1-40} suppressed the PI-3K-dependent Akt phosphorylation but not mitogen-activated protein kinase phosphorylation in HeLa cells. These conflicting data might be due to differences in cell types and experimental conditions, and underscore the complexity of intrinsic apoptosis-regulatory genes in cell systems.



Akt phosphorylates a number of proteins which are known to regulate apoptosis (Cross *et al*, 1995; Datta *et al*, 1997; del Peso *et al*, 1997; Hajduch *et al*, 1998; Brunet *et al*, 1999; Kaplan and Miller, 2000); prominent among these proteins is the BH3-only protein, Bad. In this study, subsequent to Aβ_{25-35}-induced Akt inactivation, we observed Bad translocation from cytoplasm to mitochondria. On translocation, we found that Bad physically interacted with Bcl-x_L; Bcl-x_L, in the absence of Bad, interacts with Bax to neutralize its proapoptotic activity (Zamzami *et al*, 1998). That Bad activation was indeed causally related to Aβ_{25-35}-induced mitochondrial dysfunction including Endo G and Smac release was confirmed by the observations that specific *bad* knockdown with a siRNA strategy resulted in a reduction in Aβ_{25-35}-induced Endo G and Smac release and CEC death. Endonuclease G is an effector enzyme in the apoptotic cascade, cleaving chromatin on translocation from mitochondria to nucleus (Susin *et al*, 1999; Li *et al*, 2001). Smac, released to the cytoplasm, relieves IAP protein inhibition of caspases, resulting in the propagation of the apoptotic cascade (Du *et al*, 2000).

Together, these results establish an Aβ_{25-35}-activated death signaling cascade in CECs involving Akt inactivation and resulting in a series of cellular events. The downstream pathway involves Bad activation and translocation from the cytoplasm to mitochondria, and binding to Bcl-x_L, resulting in disruption of the mitochondrial membrane and release of cytochrome *c*, Smac, and Endo G. These events triggered by mitochondrial dysfunction are likely the underlying mechanism of Aβ_{25-35}-induced CEC apoptosis. Understanding the death signaling processes activated by Aβ will contribute to future development of strategies to prevent vascular degeneration in cerebral amyloid angiopathy.

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