Journal of Neuroscience Research 79:798–808 (2005)

Inactivation of Bad by Site-Specific Phosphorylation: The Checkpoint for Ischemic Astrocytes to Initiate or Resist Apoptosis

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Bcl-2-associated death protein (Bad), a member of the Bcl family, directs astrocytes in primary cultures to enter or resist apoptosis during ischemia in vitro. Under ischemia, Bad was the only Bcl family member whose expression was upregulated significantly during the early stages of an ischemic insult. Increased endogenous Bad was translocated from the cytoplasm to mitochondria to induce apoptosis in astrocytes. Concurrently, ischemia also induced Bad phosphorylation specifically on Ser112 to promote survival. This site-specific phosphorylation of Bad was mediated by an early activation of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) intracellular signaling pathway. This study demonstrates that ischemia-induced Bad plays a dual role in determining whether astrocytes enter or resist apoptosis after an ischemic insult.

Key words: ischemia; astrocyte; Bad; apoptosis; LY294002; U0126

Bad, Bcl-2-associated death protein, is a member of the Bcl-2 protein family that plays a critical role in apoptosis. A prevailing hypothesis is that the balance of pro-apoptotic (e.g., Bad and Bcl-associated X (Bax)) and antiapoptotic (e.g. Bcl-2 and Bcl-X_L) members in mitochondria may determine the control of apoptosis (Desagher and Martinou, 2000; Gross, 2001). Originally identified as a binding partner of Bcl-2 (Yang et al., 1995), Bad promotes apoptosis by binding with Bcl-2 or Bcl-X_L located in the mitochondrial membrane (Yang et al., 1995; Zha et al., 1996). In the FL5.12 cell line, however, overexpressing *Bad* does not result in cell death after interleukin-3 (IL-3) withdrawal (Yang et al., 1995), whereas under certain conditions, overexpression of Bad even promotes growth as in chicken embryo fibroblasts (Maslyar et al., 2001). Bad may thus play a dual role, one in cell apoptosis and the other in cell survival.

The function of Bad depends on site-specific phosphorylation on Ser112, 136, and 155 by factors such as nerve growth factor (NGF), stem cell factor, granulocyte/macrophage colony-stimulating factor, IL-3, and insulin (Zha et al., 1996, Hinton and Welham, 1999; Harada et al., 1999; Datta et al., 2000). There are limited studies on Bad expression and phosphorylation under ischemia and previous results have been contradictory (Friguls et al., 2001; Simakajornboon et al., 2001). *Bad* has been reported to be uninvolved in the protective role of estradiol after ischemia (Dubal et al., 1999), whereas Zhu et al. (2002) observed that transforming growth factor β-1 prevented ischemic damage by increasing phosphorylated (p)-Bad 112. The activation of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and phosphatidylinositol-3-kinase/protein kinase B (PI-3/Akt) pathways after ischemia also varies among different

Abbreviations: Bad, Bcl-associated death protein; DMEM, Dulbecco's modified Eagle medium; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; GFP, green fluorescent protein; LF2000, LipofectAMINE 2000; MAPK, mitogen-activated protein kinase; p-AKT, phosphorylated AKT; PBS, phosphate-buffered saline; p-ERK, phosphorylated ERK; PI-3/Akt, phosphatidylinositol-3-kinase/protein kinase B; RT, room temperature; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

Contract grant sponsor: Hong Kong Research Grants Council; Contract grant number: HKUST6177/97M; Contract grant sponsor: North American Medical Association Foundation (Hong Kong); Contract grant number: NAMA 94/95.SC01; Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 30270426; Contract grant sponsor: Beijing Natural Science Foundation; Contract grant number: 7032026.

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Received 25 October 2004; Accepted 15 November 2004

Published online 25 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20396

models (Wu et al., 2000; Friguls et al., 2001; Shibata et al., 2002; Zhu et al., 2002). Although many studies support the protective role of activated MAPK/ERK and PI-3/Akt pathways, the damaging effect of such activation has also been reported (Hinton and Welham, 1999; Datta et al., 2000; Aki et al., 2001; Namura et al., 2001; Zhu et al., 2002). Some reports indicate increased levels of p-Bad 136 after ischemia whereas others show that levels of p-Bad are not affected by PI-3/Akt pathway activation (Friguls et al., 2001; Simakajornboon et al., 2001).

Elucidating possible roles of MAPK/ERK and PI-3/Akt pathways under ischemia would reveal the importance of site-specific Bad phosphorylation, and the mechanisms involved in ischemia-induced cell death of neural cells. Our laboratory used an in vitro ischemia model in which primary cultures of cerebral cortical astrocytes were incubated in an anaerobic chamber to investigate various aspects of ischemia-induced cell death in the nervous system (Ho et al., 2001; Yu et al., 2001, 2002; Chen and Yu, 2002; Jiang et al., 2002, 2003; Chen et al., 2003). Using this model, we demonstrated that ischemia induced apoptosis in astrocytes (Yu et al., 2001). We have also studied the responses of the PI-3/Akt and MAPK/ERK pathways in astrocytes under ischemia (Jiang et al., 2002, 2003).

In this report, we demonstrate that Bad is activated and site-specifically phosphorylated during ischemia, indicating that Bad plays a critical role in ischemia-induced activation of survival signal pathways as well as in ischemia-induced apoptosis in astrocytes. Moreover, we identified a discrepancy between the responses of the MAPK/ERK and PI-3/Akt pathways in astrocytes and neurons during ischemia, which might be one of the reasons why astrocytes seem more resistant than neurons to ischemic insult (Nawashiro et al., 2000; Xu et al., 2001).

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) and Opti-MEM were from Gibco-BRL, Life Technologies, Inc., fetal calf serum (FCS) was from HyClone (Logan, UT), and Hoechst 33342 and MitoTracker Red CMXRos were from Molecular Probes (Eugene, OR). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) reagents were from Boehringer Mannheim (Germany); p-EBG-mBad, antibodies to Bad, p-Bad 112, p-Bad 136, p-Bad 155, LY294002, and U0126 were from Cell Signaling Technology (Beverly, MA). Antibodies to ERK, phosphorylated ERK at Tyr204 (p-ERK), Akt, phosphorylated Akt at Ser473 (p-Akt), Bax, Bcl-2, Bcl-X_L, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). LipofectAMINE 2000 (LF2000) was from Invitrogen Life Technology (Carlsbad, CA), and p-EGFP-N1 and pDsRed1-N1 vectors were from Clontech (Palo Alto, CA).

Primary Cultures of Cerebral Cortical Astrocytes

Astrocyte cultures were prepared from cerebral cortices of newborn ICR mice, as reported previously (Ho et al., 2001; Yu et al., 2001; Chen and Yu, 2002; Jiang et al., 2003), and 10 ml of cell suspension was plated on each 100-mm Falcon tissue culture dish (Becton-Dickinson, Franklin Lakes, NJ). All cultures were incubated in a Napco CO_2 incubator (Precision Scientific Inc.) at 37°C with 95% air/5% CO_2 (vol/vol) and 95% humidity. The culture medium was changed after 2 days and subsequently twice per week with DMEM containing 10% (vol/vol) FCS for the first 2 weeks and 7% (vol/vol) FCS afterwards. Cultures of at least 4 weeks old were used for experiments.

Primary Cultures of Cerebral Cortical Neurons

Neuron cultures were prepared from cerebral cortices of ICR mice of embryonic Day 16 as reported previously (Yu et al., 1984, 1986; Li et al., 2001, 2002). All cultures were incubated in a Napco CO_2 incubator at 37°C with 95% air/5% CO_2 (vol/vol) and 95% humidity. Cultures 7 days old were used for experiments.

Anaerobic Chamber-Induced Ischemia and Inhibitor Treatment

The in vitro ischemic model mimics an in vivo ischemic insult by incubating the cultures in a limited amount of anoxic glucose-free medium inside an anaerobic chamber (Yu and Lau, 2000; Ho et al., 2001; Yu et al., 2001; Chen and Yu, 2002; Jiang et al., 2003). Briefly, ischemia media (free of glucose and serum) was degassed for 30 min with N_2 and regassed with N_2:CO_2:H_2 (85:5:10) for 20 min before use. The ischemia media and cultures were transferred to an anaerobic chamber (Forma Scientific Inc.) saturated with N_2:CO_2:H_2 (85:5:10). The oxygen concentration in the ischemia media and the anaerobic chamber was 0.1 parts per million (ppm) as measured by a dissolved oxygen meter (HI9142; Hanna Instruments, Italy) and 0.1% (vol/vol) as detected by gas meter (MSA; Passport Personal Alarm, USA). All culture dishes were wrapped with Parafilm to prevent evaporation during incubation. The cultures were washed three times with ischemia media and then cells were covered with 6.4 ml or 0.78 ml ischemia media for 100-mm or 35-mm culture dishes, respectively. LY294002 at a final concentration of 20 μM, or U0126 at a final concentration of 10 μM, was added to the ischemia media in the chamber just before ischemia was carried out. Dimethylsulfoxide (DMSO) was added to some cultures as the vehicle control for inhibitors.

Immunostaining and TUNEL Staining

All procedures for immunostaining were carried out at room temperature (RT) (Chen and Yu, 2002; Chen et al., 2003). Before staining, cultures of astrocytes were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. After washing twice with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 min and blocked with 3% bovine serum albumin (BSA) for 2 hr. After incubation with primary antibody for 2 hr and washing three times with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 hr. After washing five times with PBS, nuclei were stained using Hoechst 33342 (2 μg/ml) for 5 min before the coverslip was mounted. TUNEL staining was carried out as described previously (Yu et al., 2001) before Hoechst 33342 staining.

Western Blot Analysis

Cell extracts were prepared by lysing cells in ice-cold lysis buffer (150 mM NaCl, 0.1% [wt/vol] Triton, 20 mM Tris, pH 7.6, 0.1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin, and 0.5 μg/ml pepstatin) (Chen and Yu, 2002; Chen et al., 2003), and then 10 μg of total protein were resolved on 12% reducing SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) nonfat dried milk in TBST buffer (0.1 M Tris-HCl, pH 8.0, 0.9% [wt/vol] NaCl, and 0.1% [vol/vol] Tween-20) and probed with rabbit polyclonal antibody to p-Bad 112, p-Bad 136, p-Bad 155, p-ERK, total ERK, p-Akt 473 and total Akt (using dilution folds recommended by the manufacturers) in TBST for 2 hr at RT. After incubation with goat anti-rabbit IgG with horseradish peroxidase (HRP) conjugate (1:4,000; Amersham Biosciences, France) for 1 hr at RT, detection of antigens was accomplished using a chemiluminescence kit (Amersham Biosciences). Blots were stripped in a denaturing buffer (0.5 M Tris-HC, pH 6.8, 10% SDS, and β-mercaptoethanol) and reprobed with antibody to total Bad (Santa Cruz Biotechnology) or actin. Results were quantified by normalizing to controls (0-hr ischemia).

Coimmunoprecipitation

Coimmunoprecipitation was carried out on a rocking platform in a cold room (Chen and Yu, 2002; Chen et al., 2003). Total protein (0.4 ml, 1 μg/μl) from each sample was precleaned with 10 μl of protein A/G-Agarose slurry (Boehringer Mannheim, Germany) for 1 hr. After centrifugation (5 min × 2,000) at 4°C, the supernatant was transferred to a new tube and incubated with 1 μg of Bad protein for 2 hr. Protein A/G-Agarose (20 μl) was added and incubated for 1 hr. After centrifugation, the immunoprecipitate was washed twice with lysis buffer and PBS, respectively. Finally, 20 μl of 1.5× sample loading buffer was added to the immunoprecipitate and boiled for 5 min before Western blot analysis. Normal rabbit IgG was used as negative controls.

Lipofection of Primary Cultures of Astrocytes

Mouse *Bad* DNA fragment was inserted into p-EGFP-N1 and pDsRed1-N1 vectors. The coding region of mouse *14-3-3γ* DNA was PCR amplified from primary cultures of astrocytes and then sequenced (Chen et al., 2003). The sequence was identical to that registered in GenBank (GenBank Accession number BC008129; data not shown). Plasmid DNA for transfection was extracted with Wizard Plus Midipreps DNA purification kit or Wizard Plus Maxipreps DNA purification kit (Promega, Madison, WI). For transient transfection of primary cultures of astrocytes, 3 μg of total DNA and 6 μl of LF2000 were used. DNA and LF2000 reagent were diluted in 0.15 ml Opti-MEM for 5 min separately, then combined and incubated for 30 min. All procedures were carried out at RT. The DNA/LF2000 mixture was added into the cultures and incubated for 6 hr at 37°C with 95% air/5% CO_2 (vol/vol) and 95% humidity. After LF2000 treatment, cultures were washed twice with DMEM and fresh culture media added. Cultures were used for experiments 24 or 48 hr after transfection.

Statistical Analysis

All data are presented as means ± standard error of the mean (SEM) of at least three independent experiments. Statistical analysis was carried out using unpaired Student's *t*-tests at a confidence level of 95% (i.e., $P \leq 0.05$ was considered statistically significant).

RESULTS

Ischemic Astrocytes Exhibit Condensed Nuclei and Morphologic Changes

Changes in astrocyte morphology during ischemia were examined by phase contrast microscopy whereas nuclear changes were visualized by Hoechst 33342 immunostaining (data not shown). Morphologic changes and cell death, typical of apoptosis, increased gradually after 1–8 hr exposure to ischemia. Condensed nuclei were shown to result from apoptosis by coimmunostaining the culture with Hoechst and TUNEL reagents. After 6-hr ischemia, about 90% of the extensively condensed Hoechst-positive nuclei were also TUNEL positive. As both Hoechst and TUNEL staining could reveal apoptotic cells with similar efficiency, Hoechst staining was subsequently used to distinguish apoptotic astrocytes in all experiments.

Cytochrome c Release From Mitochondria in Ischemic Astrocytes

The release of cytochrome c from mitochondria into the cytoplasm, a critical step for a cell to commit to apoptosis with the activation of caspase-3 (Sims and Anderson 2002), has never been reported in ischemic astrocytes. Here, the release of cytochrome c from mitochondria of ischemic astrocytes is demonstrated clearly (Fig. 1). Cytochrome c, appearing filamentous in control astrocytes under confocal microscopy, was stained with a specific monoclonal antibody. Mitochondria stained with a specific mitochondrion-selective dye revealed similar filamentous structures, indicating that cytochrome c is confined within the mitochondria in astrocytes under normal conditions. After 4-hr ischemia, cytochrome c had diffused to the cytoplasm in astrocytes exhibiting condensed nuclei, a hallmark of apoptosis in these cells. Furthermore, mitochondria in these astrocytes seemed swollen and perforated and were distributed throughout the cytoplasm. Ischemia thus induces the release of cytochrome c from mitochondria into the cytoplasm.

Translocation of Activated Bad Into Mitochondria to Induce Apoptosis in Ischemic Astrocytes

Endogenous Bad protein expression and distribution in surviving and apoptotic astrocytes under ischemia was examined by immunostaining. Bad was almost undetectable in normal astrocytes (Fig. 2A). After 4-hr ischemia, Bad staining was observed as perforated aggregates only in astrocytes that seemed to be undergoing apoptosis (Fig. 2A, arrows). Costaining with anti-Bad antibodies and a specific mitochondrion-selective dye revealed the subcellular localization of Bad in these apoptotic astrocytes. The colocalization of Bad aggregates and mitochondria

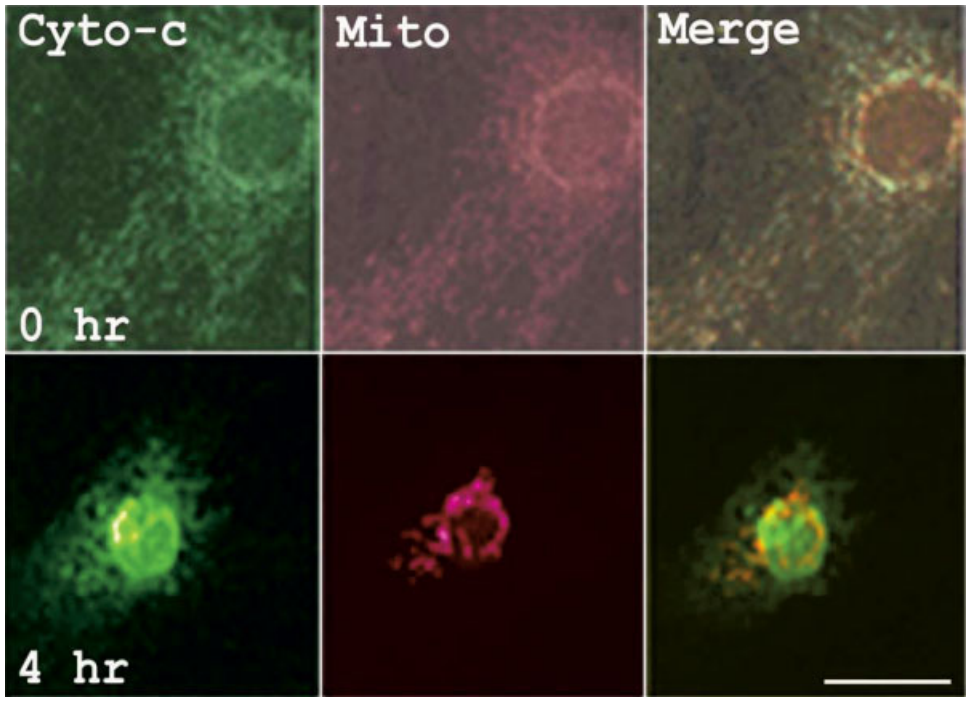

Fig. 1. Mitochondrial cytochrome c release in astrocytes under ischemia. Cultured astrocytes at 0 (top) and 4 hr (bottom) of ischemia were stained simultaneously with cytochrome c (left) and MitoTracker (middle). Micrographs show distribution of cytochrome c (cyto-c) and mitochondria (mito) and the merged image of the same fields (right). Scale bar = 20 μm. Figure can be viewed in color online via www.interscience.wiley.com.

(Fig. 2B, merge arrows) indicate the accumulation of Bad in the mitochondria of apoptotic astrocytes under ischemia.

The study of Bad was facilitated by cotransfecting cultured astrocytes with wild type *Bad* (75%) and green fluorescent protein (*GFP*; 25%) (Fig. 3). Using fluorescent microscopy, GFP-positive astrocytes showed a higher intensity of Bad staining than did the untransfected controls, indicating that the transfected astrocytes overexpressed Bad. Transfected astrocytes underwent apoptosis (Fig. 3A, arrow), in contrast to neighboring untransfected astrocytes. Apoptosis due to Bad overexpression was assessed by the GFP/actin ratio in the transfected cultures. The GFP/actin ratio represents the relative viability of the transfected cells. As 14-3-3 proteins are considered antiapoptotic, *14-3-3*γ was used as a control gene for cotransfection in this experiment (Zha et al., 1996; Masters et al., 2001). Cotransfecting *14-3-3*γ and *GFP* genes did not cause any observable cell death in astrocytes (data not shown). Under similar conditions, cotransfection of *Bad* and *GFP* resulted in significant decreases in GFP expression in the culture, indicating that many *Bad/GFP* cotransfected cells died (Fig. 3B). The level of GFP/actin in cultures cotransfected with *Bad* and *GFP* was only 20% of that cotransfected with *14-3-3*γ and *GFP*, demonstrating that overexpression of *Bad* leads to extensive cell death in astrocytes.

Bad Is Selectively Upregulated During Ischemia

Western blot analysis showed that among the Bcl-2 family, only Bad increased significantly after ischemia (Fig. 4A). In astrocytes, levels of Bad doubled after 1-hr ischemia and increased by fivefold after 2-hr ischemia. Bad expression peaked after 4-hr ischemia. At 6-hr ischemia, levels of Bad declined but remained significantly higher than that at 0-hr ischemia (Fig. 4A). In contrast, levels of Bax, Bcl-2, and Bcl-X_L did not change significantly in astrocytes after 1- and 2-hr ischemia (Fig. 4B). Bcl-2 levels decreased after 4- and 6-hr ischemia whereas Bax and Bcl-X_L levels did not change significantly throughout the entire ischemic treatment.

Association of Site-Specific Phosphorylation of Bad With Cell Survival

Bad phosphorylation on Ser112 and 136 under ischemia has been observed in a few studies, although discrepancies exist (Dubal et al., 1999; Friguls et al., 2001; Simakajornboon et al., 2001; Zhu et al., 2002), but phosphorylation on Ser155 has not been reported previously. The dynamics of Bad phosphorylation on Ser112, 136, and 155 under ischemia therefore were studied using Western blot analysis. Low levels of endogenous p-Bad 112, p-Bad 136, and p-Bad 155 in astrocytes at 0-hr ischemia were detected (Fig. 5A), although only p-Bad 112 levels increased significantly under ischemia (Fig. 5A). In fact, p-Bad 112 levels doubled after 1-hr ischemia (Fig. 5B), and increased more than fivefold at 2- and 4-hr ischemia. Levels of p-Bad 112 diminished after 6-hr ischemia. The amounts of p-Bad 136 and p-Bad 155 did not change significantly after 1, 2, 4, and 6 hr of ischemia (Fig. 5B). These results demonstrate that ischemia induces site-specific phosphorylation of Bad on Ser112 in cultured astrocytes.

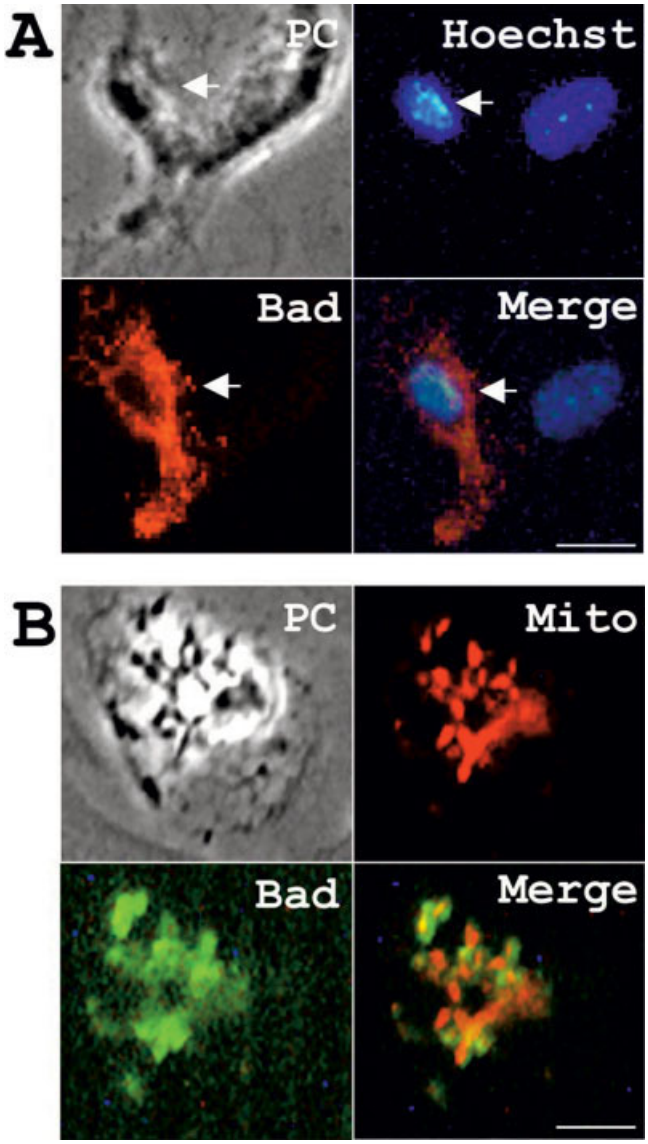

Fig. 2. Activation of endogenous Bad in apoptotic astrocytes under ischemia. **A:** Aggregation of Bad in apoptotic astrocytes. Cultured astrocytes after 4-hr ischemia were costained with Bad antibodies and Hoechst 33342. Micrographs show phase contrast (PC), Hoechst staining, Bad staining, and the merged image of the same field. Arrows indicate astrocytes with Bad aggregates undergoing apoptosis. **B:** Translocation of Bad in apoptotic astrocytes. Micrographs show PC, MitoTracker staining (Mito), Bad staining, and the merged image of the same field. Scale bars = 20 μm. Figure can be viewed in color online via www.interscience.wiley.com.

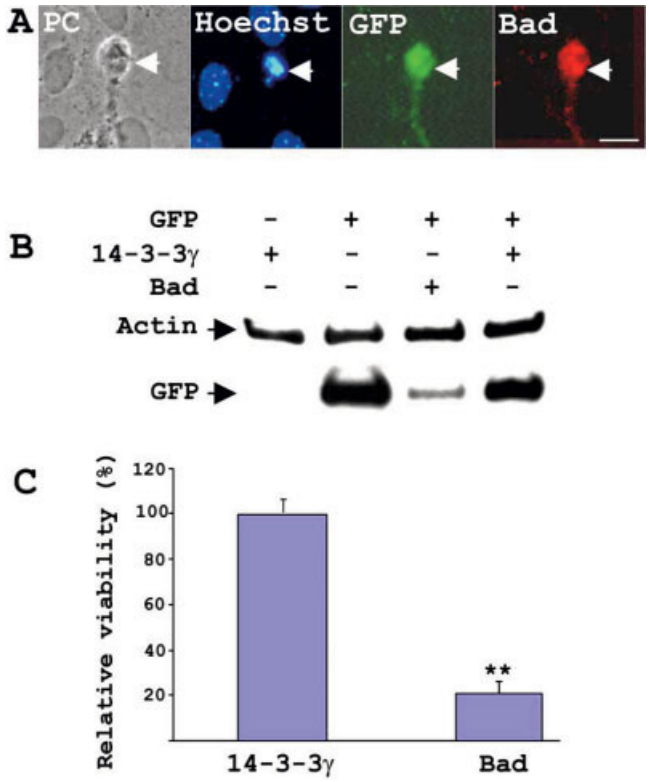

Fig. 3. Apoptotic effect of Bad in astrocytes. **A:** Immunostaining. Primary cultures of astrocytes were cotransfected with *Bad* (75%) and *GFP* (25%) genes. At 2 days after lipofection, cells were stained simultaneously for Bad and with Hoechst 33342. Micrographs show phase contrast (PC), Hoechst staining, GFP expression, and Bad staining of the same field. Arrows indicate transfected astrocytes. Scale bar = 20 μm. **B:** Viability of transfected cells. Cultured astrocytes were transfected with *14-3-3γ*, *GFP*, *Bad* (75%) + *GFP* (25%), and *14-3-3γ* (75%) + *GFP* (25%) genes, respectively. At 2 days after transfection, total protein was extracted from the culture and 10 μg soluble protein used for Western blotting. The same membrane was blotted with GFP and actin antibodies. **C:** Relative viability of transfected astrocytes calculated by the GFP/actin ratio. Data represent the mean ± SEM of results from three independent experiments. $^{**}P < 0.01$ vs. control. Figure can be viewed in color online via www.interscience.wiley.com.

The site-specific phosphorylation of Bad in astrocytes during ischemia was confirmed further by transfecting *Bad* into cultured astrocytes. At 24 hr after *Bad* transfection, the cultures were subjected to 4-hr ischemia. Bad phosphorylation on Ser112, 136, and 155 was revealed by Western blot analysis. Ischemia increased p-Bad 112 levels only and did not alter the levels of p-Bad 136 and p-Bad 155 (Fig. 5C). To confirm that phosphorylation of Bad on Ser112 is essential for cell survival, the cellular distribution of Bad and p-Bad 112 in *Bad*-transfected astrocytes was compared. One day after transfection, the culture was treated simultaneously with Hoechst and antibodies specific to total Bad and p-Bad 112 (Fig. 5D). In untransfected astrocytes, the intensity of Bad staining was too low to be observed. Astrocytes with increased intensity of Bad staining indicated *Bad*-transfected cells. As demonstrated above, overexpression of Bad in astrocytes led to extensive cell death in astrocytes. In surviving astrocytes that overexpressed Bad, however, both Bad and p-Bad 112 were distributed throughout the cytoplasm (Fig. 5D, arrows). The detection of p-Bad 112 in surviving astrocytes therefore strongly suggests that Bad site-specific phosphoryla-

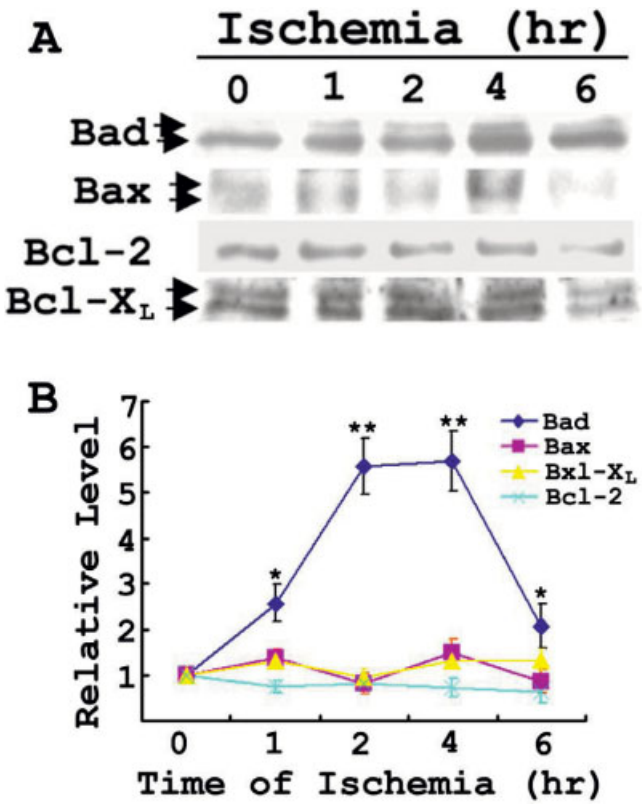

Fig. 4. Differential expression of endogenous Bad, Bax, Bcl-2 and Bcl-X_L in astrocytes under ischemia. **A:** Western blot analysis of Bad, Bax, Bcl-2, and Bcl-X_L proteins. Total proteins were extracted from cultured astrocytes after 1, 2, 4, and 6 hr of ischemia. Equal amounts of soluble proteins were subjected to electrophoresis. The membranes were probed with specific antibodies to Bad, Bax, Bcl-2, and Bcl-X_L. **B:** Statistical analysis of quantified Western blot results. Data represent the mean ± SEM from three independent results. $\star P < 0.05$ and $\star\star P < 0.01$ vs. controls. Figure can be viewed in color online via www.interscience.wiley.com.

tion on Ser112 in ischemic astrocytes is required for cell survival.

Involvement of MAPK/ERK and PI-3/Akt Pathways in Site-Specific Phosphorylation of Bad

The involvement of MAPK/ERK and PI-3/Akt pathways in Bad phosphorylation was investigated using phosphorylation inhibitors, LY294002 and U0126, of ERK and Akt, respectively. Basal amounts of p-Bad 112, p-Bad 136, and p-Bad 155 were detected in untreated astrocytes (Fig. 6A). Only p-Bad 112 levels increased significantly after 4-hr ischemia in the DMSO vehicle control, which were reduced significantly in the presence of LY294002 and U0126; p-Bad 136 and p-Bad 155 levels remained unaffected (Fig. 6A). This suggests that phosphorylation of Bad on Ser112 in ischemic astrocytes may be mediated by the MAPK/ERK and PI-3/Akt pathways.

Coimmunoprecipitation revealed that apoptosis in astrocytes was mediated by the binding of Bad to Bcl-X_L but not Bcl-2 under normal or ischemic conditions (Fig. 6B). In normoxic astrocytes, binding between Bad and Bcl-X_L indicated that a small amount of Bad might be localized in mitochondria. After 4-hr ischemia, Bcl-X_L binding with Bad increased slightly, but LY294002 and U0126 treatment increased the binding of Bcl-X_L to Bad significantly under ischemia (Fig. 6B).

The temporal expression profiles of ERK, Akt, and their phosphorylation in cultured astrocytes were determined by Western blot analysis under ischemic conditions. Levels of p-ERK increased significantly after 1, 2, 4 and 6 hr of ischemia whereas that of total ERK did not change significantly throughout the entire ischemic treatment (Fig. 7A). Statistical analysis demonstrated that ERK phosphorylation increased more than tenfold after 1-hr ischemia and was maintained at high levels after 2, 4, and 6 hr of ischemia (Fig. 7B). The increase of p-ERK/ERK levels paralleled the increase of p-Bad 112 levels at 1, 2 and 4 hr of ischemia (Fig. 5A). Levels of p-Akt/Akt remained unchanged until 4-hr ischemia (Fig. 7A), and a two- to threefold increase was observed at 6-hr ischemia (Fig. 7B). To clarify whether the activation of these signaling pathways was specific to astrocytes, the expression profiles of ERK and Akt were also measured in neurons under similar ischemic conditions. After 2-hr ischemia, however, levels of p-ERK in neurons under ischemia decreased, whereas p-Akt levels decreased from 0-hr to undetectable levels at 2- and 4-hr ischemia (Fig. 7A).

DISCUSSION

Although it is well known that both necrosis and apoptosis occur in neurons and astrocytes after cerebral ischemia (Li et al., 1995; Nawashiro et al., 2000; Yu et al., 2001), the cellular mechanisms underlying ischemic death are unclear. Increased interest in understanding the apoptotic mechanisms underlying ischemia may lead to the possibility of delaying or even preventing apoptosis. This may enable astrocytes to survive, and perhaps influence the recovery of neuronal functions during and after ischemia (Rosenberg, 1991; Han et al., 2001). In this investigation, we demonstrate a possible role for Bad in apoptotic signaling in cultured cerebral cortical astrocytes under ischemia.

We have shown that cytochrome c is released from mitochondria in intact astrocytes dying from ischemia (Fig. 1). As the release of cytochrome c is a critical event in mitochondria-mediated apoptosis, this observation provides solid evidence for ischemia-induced apoptosis in astrocytes via a mitochondrial cytochrome c-dependent pathway. This observation helps explain the increase of cytochrome c in the cytosol after cerebral ischemia, as detected by Western blot analysis (Yenari et al., 2002). We have used previously a similar in vitro injury model using Annexin V staining, TUNEL staining, electron micrography, and DNA laddering to demonstrate apoptosis in astrocytes (Yu et al., 2001). The release of cytochrome c that occurred within 4 hr of ischemia is consistent with the changes in plasma membrane seen in early stages of apoptosis, as detected by Annexin V staining (Yu et al., 2001).

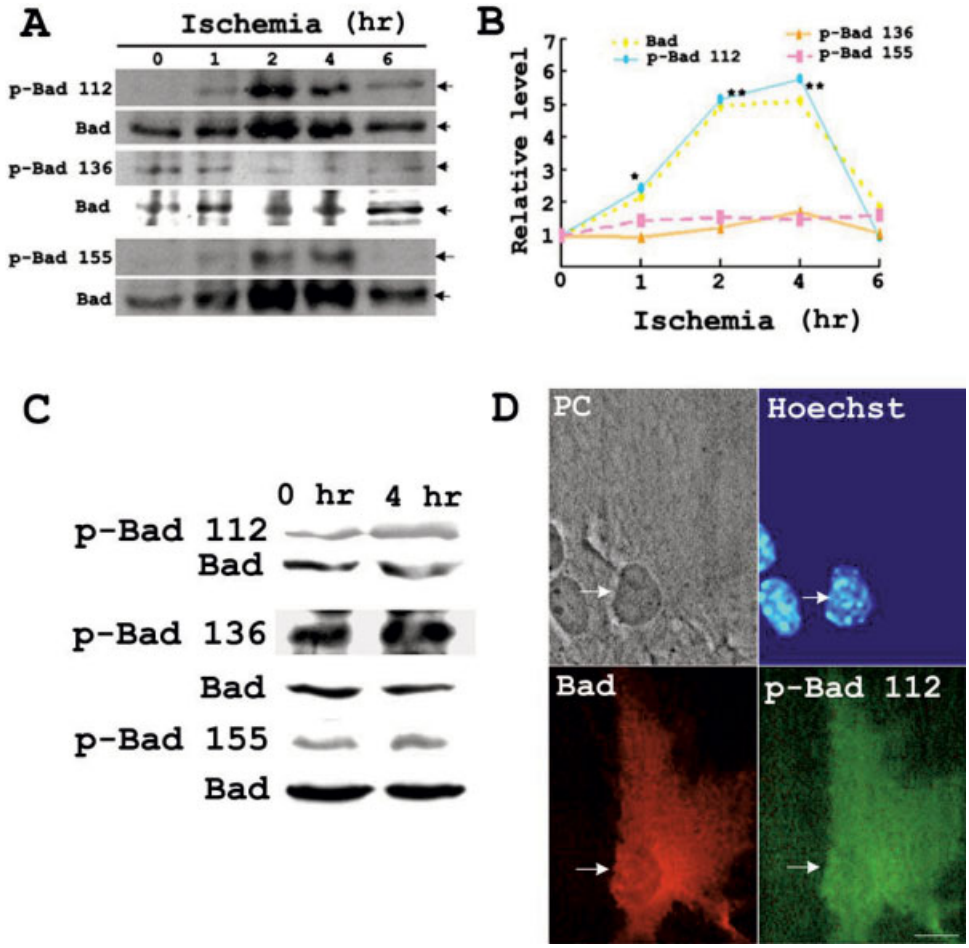

Fig. 5. Site-specific phosphorylation of Bad in astrocytes is associated with cell survival under ischemia. **A:** Western blot analysis of p-Bad 112, p-Bad 136, and p-Bad 155. Total protein (0.2 mg) from astrocytes at 0, 1, 2, 4 and 6 hr of ischemia was used for Western blot analysis with specific antibodies to p-Bad 112, p-Bad 136, or p-Bad 155. After reacting with anti-phosphorylated Bad antibodies, the same membrane was stripped and probed with anti-Bad antibodies. It is shown clearly that p-Bad 112 levels increased after 1, 2, and 4 hr of ischemia, whereas those of p-Bad 136 and p-Bad 155 did not change significantly. **B:** Statistical analysis of quantified Western blot results. Data represent the means ± SEM of three independent results. $\star P < 0.05$; $\star\star P < 0.01$ vs. controls. **C:** Ischemia-induced site-specific phosphorylation of Bad in astrocytes with *Bad* transfection. *Bad* gene was transfected into primary cultures of astrocytes and 1 day after transfection, cultures were subjected to 4-hr ischemia. Western blot analysis was carried out as in A. Results show specific elevation of p-Bad 112 levels after 4-hr ischemia when compared to levels of p-Bad 136 and p-Bad 155. **D:** Phosphorylation of Bad on Ser112 in surviving astrocytes. At 1 day after *Bad* transfection, the culture was stained simultaneously with Hoechst, Bad, and p-Bad antibodies. Micrographs show phase contrast (PC), Hoechst, Bad, and p-Bad staining of the same field. Arrows indicate surviving astrocytes with *Bad* overexpression. Scale bar = 20 μm. Figure can be viewed in color online via www.interscience.wiley.com.

In our system, 4 hr of ischemia is a critical point for many cultured astrocytes to switch to apoptotic pathways. Other critical changes, such as decreases in ADP/ATP levels, coincide with this point as well (Yu et al., 2002).

The balance between proapoptotic (e.g., Bad and Bax) and antiapoptotic (e.g., Bcl-2 and Bcl-X_L) members of the Bcl-2 family is critical to control mitochondria-induced apoptosis. Although ischemia-induced changes in Bcl-2, Bax, or Bad have been reported in the central nervous system (Gillardon et al., 1996; Chen et al., 1997; Schwarz et al., 2002), the temporal expression profiles of these proteins under ischemia have not been compared in a single study. We found that only Bad increased significantly when the expression of Bad, Bax, Bcl-2, and Bcl-X_L proteins were compared simultaneously in ischemic astrocytes (Fig. 4). It is intriguing to observe that the elevation of proapoptotic Bad occurs significantly earlier than mitochondrial cytochrome c release and turnover of membrane phosphatidylserine (Annexin V staining). Bad expression during early stages of ischemic insult, therefore, plays an important role in determining the survival of astrocytes under ischemia. In contrast, Bcl-2 and Bax are not likely to be key initiators of apoptosis in response to ischemia in astrocytes, as their expression profiles re-

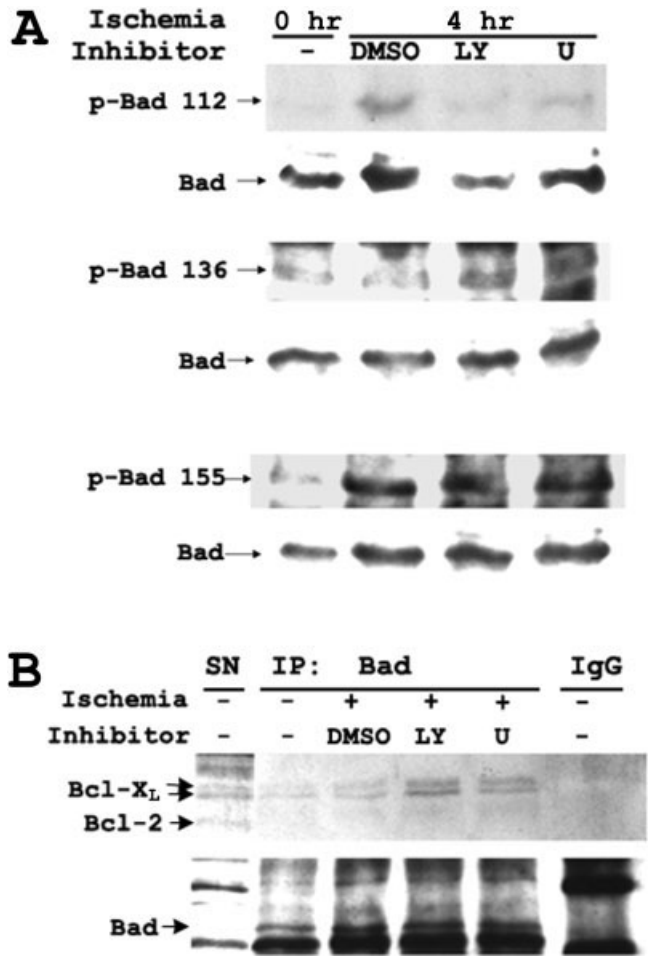

Fig. 6. Role of MAPK/ERK and PI-3/Akt pathways in Bad phosphorylation and its binding with Bcl-X_L in astrocytes under ischemia. **A:** LY294002 and U0126 blocked Bad phosphorylation on Ser112. Cultured astrocytes were subjected to 4-hr ischemia in the presence of inhibitors of PI-3 kinase (LY294002 [LY], 20 μM) and MAPK (U0126 [U], 10 μM). Equal volumes of DMSO were used as vehicle control for the inhibitors. Total protein (0.2 mg) from astrocytes after 0 and 4 hr of ischemia in the presence of DMSO, LY, and U was used for Western blot analysis. After probing with antibodies to p-Bad 112, 136, and 155, the same membranes were reprobed with antibodies to total Bad. Results show that LY294002 and U0126 blocked Bad phosphorylation specifically at Ser112 but not at Ser136 or p-Bad 155. **B:** LY294002 and U0126 promote the binding of Bad and Bcl-X_L. Cultured astrocytes were subjected to 4-hr ischemia in the presence of LY294002 (LY, 20 μM), U0126 (U, 10 μM) or DMSO. Total protein (0.4 mg) was used for immunoprecipitation (IP). Total protein (10 μg) from the supernatant (SN) of normal astrocytes was used as control in Western blot analysis. The same membrane was probed with antibodies to Bad, Bcl-X_L, and Bcl-2, respectively. Results show that Bad bound to Bcl-X_L but not Bcl-2. The binding of Bad and Bcl-X_L increased in the presence of LY294002 and U0126.

mained unaffected by the insult (Fig. 7C). The binding of Bad to Bcl-X_L but not Bax or Bcl-2 implies that Bad is also the key factor determining the balance of pro- and antiapoptotic Bcl-2 members in astrocyte mitochondria. The early increase of Bad during 1–2 hr of ischemia upsets the balance between pro- and antiapoptotic members of the Bcl-2 family and influences the sensitivity of astrocytes to ischemic insult, thus committing these cells to apoptosis should the ischemic insult extend to 4 hr and beyond (Fig. 7D).

Bad plays diverse and complex roles in cell death. Overexpression of *Bad* in many cell lines induces apoptosis (Zha et al., 1996; Harada et al., 1999; Hinton and Welham, 1999; Masters et al., 2001). Overexpression of *Bad* in astrocytes caused extensive cell death (Fig. 3), indicating that Bad protein in ischemic astrocytes is apoptotic. Bad also aggregated in the mitochondria of dying astrocytes (Fig. 2), demonstrating its proapoptotic effect in ischemic astrocytes. It has been shown that Bad does not induce apoptosis unless it is dephosphorylated and translocated into mitochondria (Yang et al., 1995; Harada et al., 1999). The translocation of Bad into mitochondria during the later stages of ischemia is thus critical in shifting the balance between pro- and antiapoptotic Bcl-2 family members in the mitochondria to cause apoptosis. Bad binds with Bcl-2 and Bcl-X_L and dissociates Bax from Bcl-2/X_L to exert its apoptotic effect (Yang et al., 1995). Bad also induces apoptosis by binding to other members of the Bcl-2 family (Bae et al., 2001). It should be noted that aggregation of Bad in mitochondria itself might cause mitochondrial dysfunctions and the release of mitochondrial cytochrome c. Interestingly, overexpression of *Bad* is not always associated with cell death in some systems (Maslyar et al., 2001).

In astrocytes, endogenous Bad binds to Bcl-X_L but not Bcl-2, indicating that Bcl-X_L may play a major antiapoptotic role in astrocytes. In addition, the activity of the MAPK/ERK and PI-3/Akt intracellular signaling pathways influence the binding of Bad to Bcl-X_L (Fig. 6). These results suggest that Bad induces apoptosis by aborting the antiapoptotic effect of Bcl-X_L by binding to Bcl-X_L in the later stages of ischemia. As Bad also binds to Bcl-X_L in normal astrocytes (Fig. 6), however, the binding of Bad and Bcl-X_L is not simply apoptotic as proposed previously (Yang et al., 1995; Zha et al., 1996). The balance of endogenous Bad and Bcl-X_L within the mitochondria may be more important in allowing apoptosis to occur.

In astrocytes, ischemia induces significant phosphorylation of endogenous Bad, though only on Ser112 (Fig. 5B), and Bad phosphorylation is associated with cell survival (Fig. 5D). Some growth factors and cytokines also phosphorylate Bad. Phosphorylation of Bad on Ser112, 136, or 155 is associated with its binding to 14-3-3 proteins and disassociation from Bcl-2/X_L (Yang et al., 1995; Zha et al., 1996; Datta et al., 2000). This study is the first to compare the phosphorylation of endogenous Bad on Ser112, 136, and 155 in a single experiment. Bad is phosphorylated on all three serine sites in astrocytes under normal conditions. Only phosphorylation of endogenous Bad on Ser112 is significantly affected by ischemia in astrocytes (Fig. 5B). The importance of site-specific phos-

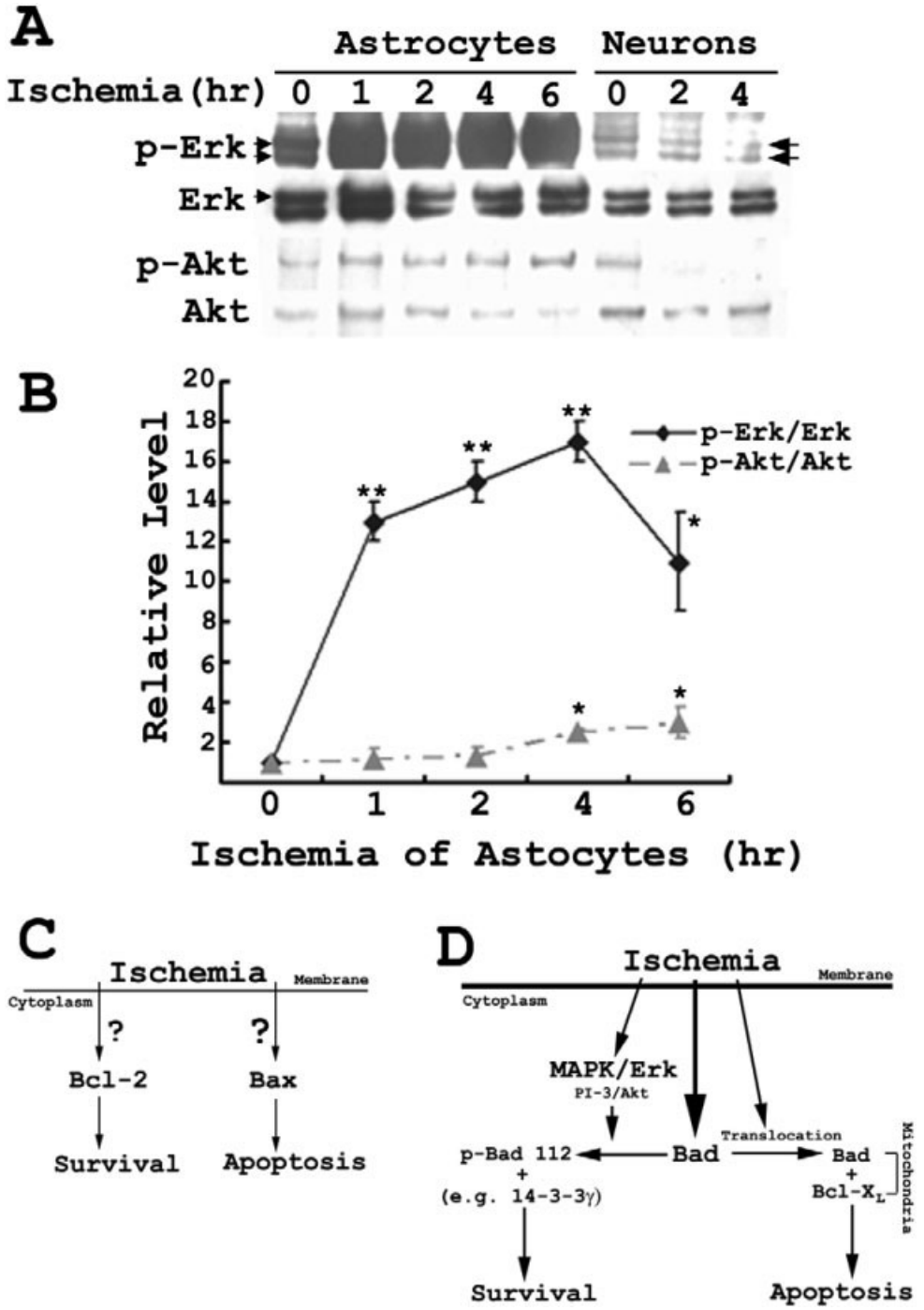

Fig. 7. Differential activation of MAPK/ERK and PI-3/Akt pathways in astrocytes and neurons and the proposed role of Bad in astrocytes during ischemia. **A:** Western blot analysis of p-ERK and p-Akt in ischemic astrocytes and neurons. Total proteins were extracted from primary cultures of cerebral cortical astrocytes and neurons after various periods of ischemia. Soluble protein (10 μg) was used for Western blot analysis with specific antibodies to p-ERK and p-Akt. The same membrane was probed with antibodies to total ERK and Akt after stripping. Results show that p-ERK and p-Akt increased in astrocytes under ischemia but decreased in neurons. **B:** Statistical analysis of Western blot results. Data represent the mean ± SEM of results from three independent experiments. $*P < 0.05$; $**P < 0.01$ vs. controls. **C:** Role of Bad under ischemia based on previous results. Data suggest that ischemia elevates Bcl-2 or Bax to protect neural cells or induce apoptosis. **D:** Role of Bad under ischemia based on this study. Ischemia induces expression of Bad protein and promotes its activation. Activated Bad translocates to mitochondria, where it binds to Bcl-X_L and induces apoptosis. Ischemia also induces a site-specific phosphorylation of Bad at Ser112, mediated by the MAPK/ERK and PI-3/Akt pathways. Phosphorylated Bad binds to 14-3-3γ and promotes survival. Arrow indicates induction or activation.

phorylation of Bad on Ser112 for cell survival is confirmed further by overexpressing Bad in astrocytes, and the detection of elevated p-Bad 112 in surviving astrocytes by immunostaining (Fig. 5D). These results complement previous reports of enhanced cell death in cells with mutated Bad phosphorylation sites (Zha et al., 1996; Datta et al., 2000; Masters et al., 2001). Although a variety of evidence supports a survival role for Bad phosphorylation (Zha et al., 1996; Harada et al., 1999; Datta et al., 2000; Maslyar et al., 2001; Masters et al., 2001), this is the first study to report the phosphorylation of Bad in surviving cells (Fig. 5D). We speculate, however, that different site-specific phosphorylation of Bad may lead to distinct functions under various physiologic conditions besides ischemic insult. Phosphorylation of Bad on Ser112 may be sufficient to inhibit its proapoptotic activity in astrocytes through its binding to other proteins, causing p-Bad to remain in the cytoplasm instead of entering mitochondria to mediate apoptosis.

We investigated further whether phosphorylation of Bad on Ser112, 136, and 155 involved the PI-3/Akt and MAPK/ERK signaling pathways. The MAPK/ERK and PI-3/Akt pathways are the two major cell survival signal pathways in a variety of systems (Cantley, 2002; Irving and Bamford, 2002). MAPK/ERK and PI3/Akt pathways promote survival by phosphorylating Bad in the presence of growth factors or cytokines (Zha et al., 1996; Harada et al., 1999; Datta et al., 2000). LY294002 and U0126, specific inhibitors of the MAPK/ERK and PI3/Akt pathways, respectively, blocked the elevation of p-Bad 112 but not p-Bad 136 and p-Bad 155 in astrocytes under ischemia (Fig. 6), indicating the involvement of both pathways in phosphorylating Bad on Ser112, specifically during ischemia. In ischemic astrocytes, however, MAPK/ERK pathway activation occurred early after ischemia, whereas PI-3/Akt pathway activation occurred after 4 hr. The PI-3/Akt pathway may therefore not be involved directly in Bad phosphorylation during early stages of ischemia, although it may play a role during the later stages. In astrocytes, p-ERK levels increased more than tenfold after 1-hr ischemia; however, p-Akt levels increased about two- to threefold only after 4-hr ischemia (Fig. 7B). In addition to promoting Bad phosphorylation, the MAPK/ERK pathway also promotes survival by inducing Bcl-2 protein in astrocytes after 4-hr ischemia (Jiang et al., 2002). Moreover, inhibition of the MAPK/ERK pathway during ischemia is damaging whereas inhibition of the PI-3/Akt pathway is protective in astrocytes (Jiang et al., 2002). Taken together, these studies suggest that rather than the PI-3/Akt pathway, MAPK/ERK is the major survival signal pathway in astrocytes under ischemia.

Whether activation of the MAPK/ERK and PI-3/Akt pathways is a common survival response of neural cells under ischemia is worth clarifying. We compared the amounts of p-ERK and p-Akt in primary cultures of astrocytes and neurons under similar ischemic conditions. Interestingly, the levels of neither p-ERK nor p-Akt were elevated in neurons after various periods of ischemic treatment (Fig. 7A). Ischemia-induced Bad is phosphorylated on Ser112 by the early activation of the MAPK/ERK pathway. Phosphorylated Bad 112 binds to protective proteins, thus preventing the entry of Bad into mitochondria; therefore, early and prominent activation of the MAPK/ERK pathway is protective. This may be one factor contributing to the relative resistance to ischemia of astrocytes compared to that of neurons. Another factor that may be important in protecting astrocytes from ischemia-induced apoptosis is the significant increase in 14-3-3γ proteins after 1-hr ischemia (Chen et al., 2003), which specifically binds p-Bad 112 over other forms of p-Bad (Chen et al., in press).

In conclusion, ischemia induces apoptosis by elevating Bad protein and promoting its translocation to mitochondria, where it binds Bcl-X_L (Fig. 7D). In astrocytes, ischemia induces an elevation of Bad and an early activation of the MAPK/ERK pathway, resulting in the phosphorylation of Bad specifically at Ser112. Bad phosphorylation promotes its interaction with protective proteins, such as 14-3-3γ preventing its translocation to mitochondria. Neurons do not show a comparable early activation of the MAPK/ERK and PI-3/Akt pathways, which may explain the higher tolerance of astrocytes to ischemic insult than neurons.

ACKNOWLEDGEMENT

This work was supported by research grants 30270426 and 30470543 (Natural Science Foundation of China) and 7032026 and 7051004 (Beijing National Science Foundation).

REFERENCES

Aki T, Mizukami Y, Oka Y, Yamaguchi K, Uemura K, Fujimiya T, Yoshida K. 2001. Phosphoinositide 3-kinase accelerates necrotic cell death during hypoxia. Biochem J 358:481–487.

Bae J, Hsu SY, Leo CP, Zell K, Hsueh AJ. 2001. Underphosphorylated BAD interacts with diverse antiapoptotic Bcl-2 family proteins to regulate apoptosis. Apoptosis 6:319–330.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. Science 296: 1655–1657.

Chen J, Graham SH, Nakayama M, Zhu RL, Jin K, Stetler RA, Simon RP. 1997. Apoptosis repressor genes Bcl-2 and Bcl-x-long are expressed in the rat brain following global ischemia. J Cereb Blood Flow Metab 17:2–10.

Chen XQ, Chen JG, Zhang Y, Hsiao WW, Yu AC. 2003. 14-3-3gamma is upregulated by in vitro ischemia and binds to protein kinase Raf in primary cultures of astrocytes. Glia 42:315–324.

Chen XQ, Fung YW, Yu AC. 2005. Association of 14-3-3gamma and phosphorylated Bad protects astrocytes from ischemic injury. J Cereb Blood Flow Metab (in press).

Chen XQ, Yu AC. 2002. The association of 14-3-3 gamma and actin plays a role in cell division and apoptosis in astrocytes. Biochem Biophys Res Commun 296:657–663.

Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME. 2000. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell 6:41–51.

Desagher S, Martinou JC. 2000. Mitochondria as the central control point of apoptosis. Trends Cell Biol 10:369–377.

Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM. 1999. Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors. J Neurosci 19:6385–6393.

Friguls B, Justicia C, Pallas M, Planas AM. 2001. Focal cerebral ischemia causes two temporal waves of Akt activation. Neuroreport 12:3381–3384.

Gillardon F, Lenz C, Waschke KF, Krajewski S, Reed JC, Zimmermann M, Kuschinsky W. 1996. Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. Brain Res Mol Brain Res 40:254–260.

Gross A. 2001. BCL-2 proteins: regulators of the mitochondrial apoptotic program. IUBMB Life 52:231–236.

Han BH, DeMattos RB, Dugan LL, Kim-Han JS, Brendza RP, Fryer JD, Kierson M, Cirrito J, Quick K, Harmony JA, Aronow BJ, Holtzman DM. 2001. Clusterin contributes to caspase-3-independent brain injury following neonatal hypoxia-ischemia. Nat Med 7:338–343.

Harada H, Becknell B, Wilm M, Mann M, Huang LJ, Taylor SS, Scott JD, Korsmeyer SJ. 1999. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 3:413–422.

Hinton HJ, Welham MJ. 1999. Cytokine-induced protein kinase B activation and Bad phosphorylation do not correlate with cell survival of hemopoietic cells. J Immunol 162:7002–7009.

Ho MC, Lo AC, Kurihara H, Yu AC, Chung SS, Chung SK. 2001. Endothelin-1 protects astrocytes from hypoxic/ischemic injury. FASEB J 15:618–626.

Irving EA, Bamford M. 2002. Role of mitogen- and stress-activated kinases in ischemic injury. J Cereb Blood Flow Metab 22:631–647.

Jiang Z, Zhang Y, Chen X, Lam PY, Yang H, Xu Q, Yu AC. 2002. Activation of Erk1/2 and Akt in astrocytes under ischemia. Biochem Biophys Res Commun 294:726–733.

Jiang Z, Zhang Y, Chen XQ, Lam PY, Yang H, Xu QY, Yu AC. 2003. Apoptosis and activation of Erkl/2 and Akt in astrocytes postischemia. Neurochem Res 28:831–837.

Li Y, Chopp M, Jiang N, Yao F, Zaloga C. 1995. Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 15:389–397.

Li Z, Li Q, Sun CX, Hertz L, Yu AC. 2001. Cloning and identification of differentially expressed transcripts in primary culture of GABAergic neurons. Neurochem Res 26:1101–1105.

Li Q, Li Z, Sun CX, Yu AC. 2002. Identification of transcripts expressed under functional differentiation in primary culture of cerebral cortical neurons. Neurochem Res 27:147–154.

Maslyar DJ, Aoki M, Vogt PK. 2001. The growth-promoting activity of the Bad protein in chicken embryo fibroblasts requires binding to protein 14-3-3. Oncogene 20:5087–5092.

Masters SC, Yang H, Datta SR, Greenberg ME, Fu H. 2001. 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. Mol Pharmacol 60:1325–1331.

Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JV, Alessandrini A. 2001. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. Proc Natl Acad Sci USA 98:11569–11574.

Nawashiro H, Brenner M, Fukui S, Shima K, Hallenbeck JM. 2000. High susceptibility to cerebral ischemia in GFAP-null mice. J Cereb Blood Flow Metab 20:1040–1044.

Rosenberg PA. 1991. Accumulation of extracellular glutamate and neuronal death in astrocyte-poor cortical cultures exposed to glutamine. Glia 4:91–100.

Schwarz DA, Barry G, Mackay KB, Manu F, Naeve GS, Vana AM, Verge G, Conlon PJ, Foster AC, Maki RA. 2002. Identification of differentially expressed genes induced by transient ischemic stroke. Brain Res Mol Brain Res 101:12–22.

Shibata M, Yamawaki T, Sasaki T, Hattori H, Hamada J, Fukuuchi Y, Okano H, Miura M. 2002. Upregulation of Akt phosphorylation at the early stage of middle cerebral artery occlusion in mice. Brain Res 942:1–10.

Simakajornboon N, Szerlip NJ, Gozal E, Anonetapipat JW, Gozal D. 2001. In vivo PDGF beta receptor activation in the dorsocaudal brainstem of the rat prevents hypoxia-induced apoptosis via activation of Akt and BAD. Brain Res 895:111–118.

Sims NR, Anderson MF. 2002. Mitochondrial contributions to tissue damage in stroke. Neurochem Int 40:511–526.

Wu DC, Ye W, Che XM, Yang GY. 2000. Activation of mitogen-activated protein kinases after permanent cerebral artery occlusion in mouse brain. J Cereb Blood Flow Metab 20:1320–1330.

Xu L, Sapolsky RM, Giffard RG. 2001. Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. Exp Neurol 169:416–424.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80:285–291.

Yenari MA, Iwayama S, Cheng D, Sun GH, Fujimura M, Morita-Fujimura Y, Chan PH, Steinberg GK. 2002. Mild hypothermia attenuates cytochrome c release but does not alter Bcl-2 expression or caspase activation after experimental stroke. J Cereb Blood Flow Metab 22:29–38.

Yu AC, Chan PH, Fishman RA. 1986. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. J Neurochem 47:1181–1189.

Yu AC, Fisher TE, Hertz E, Tildon JT, Schousboe A, Hertz L. 1984. Metabolic fate of [14C]-glutamine in mouse cerebral neurons in primary cultures. J Neurosci Res 11:351–357.

Yu AC, Lau LT. 2000. Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury. Neurochem Int 36:369–377.

Yu AC, Lau AM, Fu AW, Lau LT, Lam PY, Chen XQ, Xu ZY. 2002. Changes of ATP and ADP in cultured astrocytes under and after in vitro ischemia. Neurochem Res 27:1663–1668.

Yu AC, Wong HK, Yung HW, Lau LT. 2001. Ischemia-induced apoptosis in primary cultures of astrocytes. Glia 35:121–130.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X (L). Cell 87:619–628.

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, Klumpp S, Krieglstein J. 2002. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci 22: 3898–3909.