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Apoptosis and Activation of Erk1/2 and Akt in Astrocytes Postischemia

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We have shown previously that in vitro ischemia could induce apoptosis in primary culture of astrocytes. In this paper we demonstrate that astrocytes in culture could undergo apoptosis during in vitro incubation postischemia. We also measured the changes of phosphorylated Erk1/2 (p-Erk1/2) and phosphorylated Akt (p-Akt) in order to determine whether these two pathways play a role in apoptosis. After 4 h in vitro ischemic incubation of cultured astrocytes, a limited amount of nuclear condensation was demonstrated by Hoechst 33342 staining. When ischemic incubation was halted and the cultures transferred to standard normoxic incubation (postischemia) conditions, DNA fragmentation and apoptosis were demonstrated by TUNEL and DNA laddering analysis. TUNEL-positive astrocytes began to appear at 6 h postischemia and increased in number from 12 h postischemia. Western blot analysis showed that both p-Erk1/2 and p-Akt were elevated in astrocytes subjected to 4 h of ischemia. Elevated p-Erk1/2 levels were sustained during the post-ischemia incubation for 12 h and decreased significantly afterward, but did not return to the levels in the control cultures that did not experience ischemic insult. In contrast, the p-Akt level continued to increase at 6 and 12 h postischemia before declining significantly. The changes in p-Erk1/2 and p-Akt correlated well with the appearance of apoptotic astrocytes in the culture.

KEY WORDS: Astrocyte; apoptosis; Erk; Akt; ischemia.

INTRODUCTION

Astrocytes are the most abundant cell type in the central nervous system (CNS). They have physiological and pathological roles in the CNS and outnumber neurons by at least 10-fold. They provide a structural and nutritive support to the neuronal cells and maintain CNS homeostasis (1). Their end feet surround the capillaries in the CNS, and thus they are the first neural cells to suffer ischemic injury (2). The dysfunction of astrocytes resulting from ischemia insult influences the responses of other neural cells to injury. Plum (3) and Largo et al. (4) suggested that the viability of astrocytes was important to the degree of infarction in ischemia. Evidence that blocking gap junctions between astrocytes and neurons increased glutamate cytotoxicity (5) further indicates the important roles of astrocytes in brain injury.

It has been reported in many in vivo systems that apoptosis could be induced under ischemia and during postischemia (6–9). We have shown previously that in vitro ischemia could induce apoptosis in primary culture of astrocytes (10). However, the responses of intracellular signaling pathways in astrocytes after ischemia

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treatment and to apoptosis have not been fully defined. We have previously reported changes to Erk1/2 (extracellular signal-regulated kinases 1 and 2) and Akt (protein kinase B) in astrocytes under an in vitro ischemia model (11). In the present study we used an anaerobic chamber to induce in vitro ischemia in astrocytes (10,11) and then incubated these cultures in normal medium (postischemia incubation) and observed changes in cellular morphology and levels of Erk1/2 and Akt.

EXPERIMENTAL PROCEDURE

Culture Preparation. Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice (Animal Care Facility, HKUST) (10). In brief, meninges-free cortices diced into cubes of ~1 mm^3 and suspended in modified Dulbecco's Minimum Essential Medium (DMEM) (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA). After being mechanically dissociated by vortex mixing for 90 sec, the cell suspensions were pushed through nylon meshes (Spectrum Medical Industries, Inc., Ranch Dominguez, CA, USA) with pore sizes of 70 μm and 10 μm, respectively. The suspensions were mixed with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and seeded in 35-mm Falcon® tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). All cultures were incubated in a 37°C humidified incubator (Precision Scientific Inc., Chicago, IL, USA) with 5% CO_2/95% air. The culture medium was changed after 2 days and twice weekly thereafter. The cultures were used for experiments after 4 weeks.

Postischemia Model. The primary cultures of astrocytes were incubated in an anaerobic chamber for in vitro ischemia treatment as described previously (10–13). This ischemia model is different from other known in vitro hypoxia models by having the cultures incubated in a reduced volume of incubation medium, thus minimizing the dilution of toxic metabolites by the huge extracellular volume of medium usually experienced in other in vitro models. Therefore the experimental design closely simulates physiological ischemic conditions (14). The duration of ischemia incubation for all the astrocyte cultures in this study was 4 h. For postischemia incubation, cultures were removed from the chamber, fed with serum-free culture medium, and then incubated in a humidified incubator at 37°C with 5% CO_2/95% air.

Hoechst 33342 and TUNEL Double Staining. Double labeling was performed using the method of Whiteside et al. (15) with modifications. Terminal deoxynucleotidyl transferase-mediated DNA nick-end labeling (TUNEL) was carried out using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Germany). Astrocytes were fixed with prechilled 4% (w/v) paraformaldehyde and then permeabilized with 0.1% (v/v) Triton® X-100 in 0.1% (w/v) sodium citrate in phosphate-buffered saline (PBS). The cultures were subsequently incubated in TUNEL mixture at 37°C for 75 min, and then incubated in 2 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eurgene, OR, USA) in PBS for 5 min. The cultures were mounted in Mowiol (Calbiochem-Novabiochem Co., San Diego, CA, USA), and examined under a fluorescent microscope (Axiophot, Zeiss, Germany) with ultraviolet and fluorescein isothiocyanate (FITC) filters for Hoechst 33342 and TUNEL, respectively.

DNA Laddering Analysis. DNA laddering analysis was examined with an Apoptotic DNA Ladder Kit (Boehringer Mannheim, Germany) and Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA) (10). The cell samples were collected in lysis buffer, then the lysate was column-purified and genomic DNA was eluted by centrifugation in an elution buffer (10 mM Tris, pH 8.5). The eluted DNA was further purified with PCR Purification Resin (Promega, USA). The purified DNA was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide and visualized by UV transillumination.

Western Blot Analysis. Cultures were washed with ice-cold PBS and proteins were extracted in 200 μl lysis buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1 mM phenylmethylsulphonyl fluoride, 0.7 μg/ml leupeptin, and 0.5 μg/ml pepstatin) (11). The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was collected. Protein concentration was determined with a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins from cell lysates were denatured in protein loading buffer for 5 min at 100°C and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes (Hybond™ ECL™ Amersham Biosciences UK Ltd, Chalfont St. Giles, UK). Blocking was performed by incubation in TBST (20 mM Tris-buffered saline [pH 7.5] with 0.1% Tween 20) containing 5% nonfat dried milk for 2 h at room temperature. Primary antibodies against Erk1/2, p-Erk1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Akt, and p-Akt (Ser 473) (Cell Signaling Technology Inc., Beverly, MA, USA) were diluted in TBST containing 5% milk and incubated overnight at 4°C. The membranes were washed with TBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated second antibodies (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK). After washing, the second antibodies were detected with an Electrochemiluminescent System (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK).

Statistical Analysis. The band intensities of Western blot were quantified by a densitometer (Stratagene, La Jolla, CA, USA) and expressed as relative values to the controls. All values were expressed as the mean ± SEM from at least 3 separate experiments. Statistical significance was evaluated using Student's t test for unpaired data. In all analyses, P values < 0.05 were considered to be statistically significant.

RESULTS

Hoechst 33342 and TUNEL Staining. Phase contrast microscopy revealed alterations in astrocyte morphology during postischemia incubation (Fig. 1, left hand panels). The control culture contained astrocytes never exposed to ischemia. The 0 h postischemia culture contained astrocytes subjected to 4 h ischemia incubation only. The astrocytes in these ischemic cultures showed no obvious damage apart from minor changes in cell integrity. These changes in cell integrity persisted and detachment of cells began to increase after 6 h of postischemia incubation. Cell integrity decreased further as the length of postischemia incubation increased. Many nuclei formed highly condensed masses and became surrounded by cell debris.

The morphology of astrocytic nuclei during postischemia incubation was examined with Hoechst 33342 staining (Fig. 1, middle panels). The severity of chro-

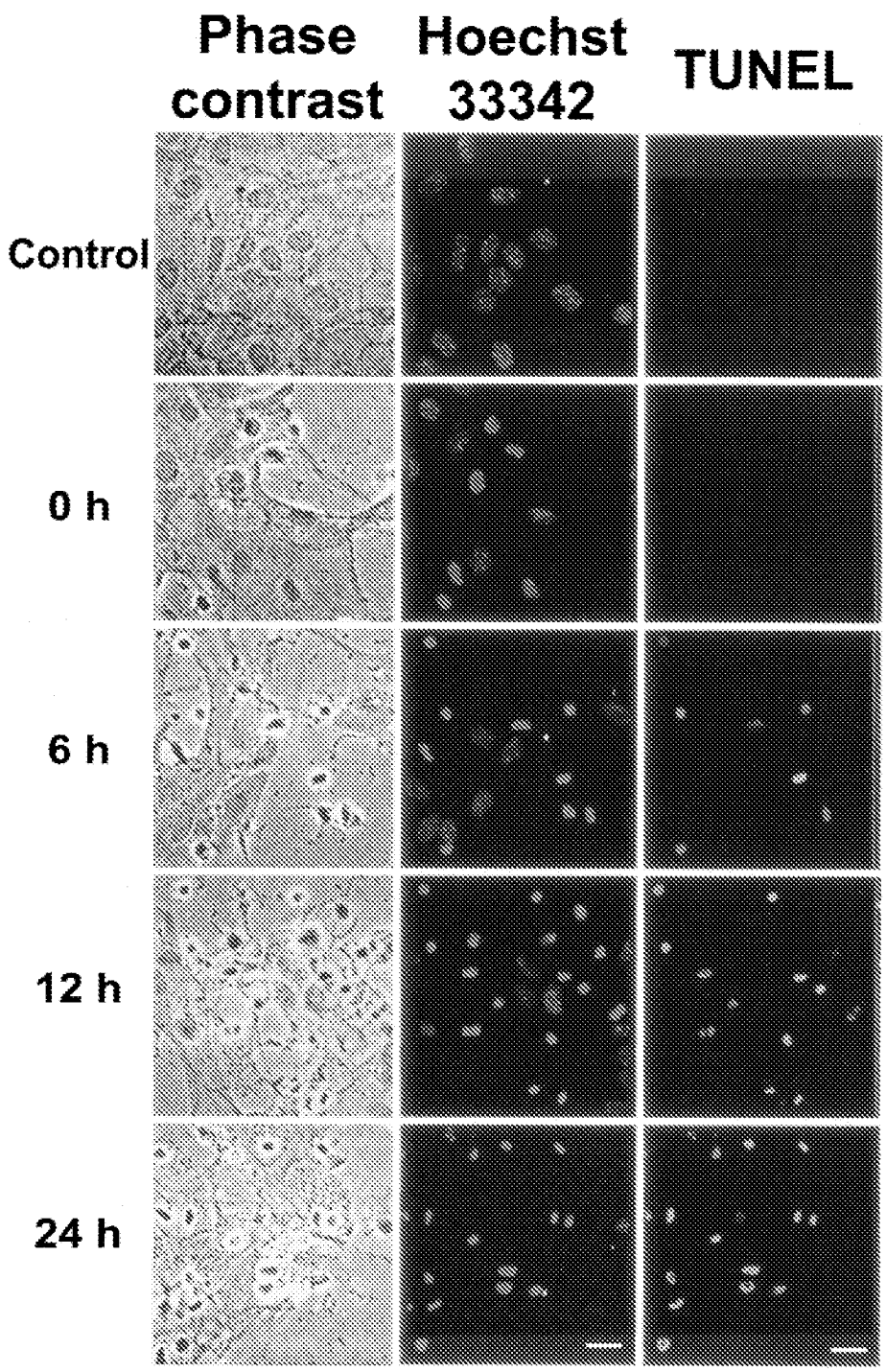

Fig. 1. Cell death during postischemia incubation. Ischemic astrocytes were exposed to postischemia incubation for the designated periods before being fixed and stained. Astrocytes were visualized by phase contrast microscopy (left hand panels). Astrocyte nuclei were visualized with Hoechst 33342 (middle panels). Postischemia incubation induced apoptosis in ischemic astrocytes as revealed by time-dependent changes in TUNEL (right hand panels). Untreated controls displayed no evidence of apoptotic nuclei (upper row). Scale bar, 20 μm.

matin condensation and nuclear shrinkage increased after 6 h of postischemia incubation and increased further as postischemia incubation continued. Most of the nuclei were stained dark-blue after 24 h postischemia incubation.

TUNEL staining produces green fluorescence in nuclei whose DNA is in the process of fragmenting. No green fluorescent nuclei were detected in cultures subjected to 4 h of ischemia only (i.e., 0 h postischemia; Fig. 1, right hand panels). TUNEL positive nuclei were detected in cultures after 6 h postischemia incubation, which increased after 12 and 24 h incubation.

DNA Laddering Analysis. The internucleosomal cleavage of genomic DNA was visualized as an oligonucleosomal ladder in agarose gel electrophoresis analysis (Fig. 2). Laddering was detected in samples from astrocytes under postischemia incubation for 6 h, and it became more obvious afterward (Fig. 2).

p-Erk and Total Erk Measurement. The level of p-Erk1/2 in astrocytes increased to 1900% of the control after 4 h of ischemia (Fig. 3A and 3B). Its level was slightly lowered when the cell cultures were transferred to normoxic (postischemia) incubation conditions, but remained 1750% and 1730% of the control at 6 h and 12 h postischemia, respectively. The amount of p-Erk1/2 declined rapidly to 5.9 times that of the control after 24 h (Fig. 3B), and remained significantly higher than controls 48 h postischemia. Under similar incubation conditions, the total Erk level in cultures

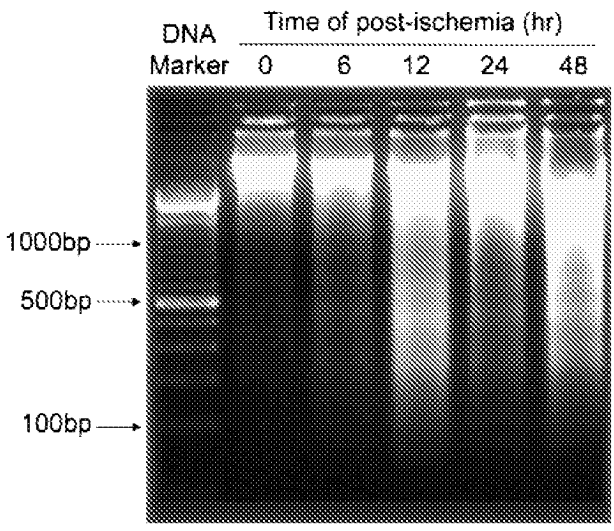

Fig. 2. Postischemia induces DNA laddering in ischemic astrocytes. Degraded chromosomal DNA was isolated from ischemic astrocytes under postischemia and subjected to agarose gel electrophoresis with ethidium bromide. Significant internucleosomal DNA fragmentation in ischemic astrocytes was first observed in 6 h postischemia and was sustained for at least 48 h.

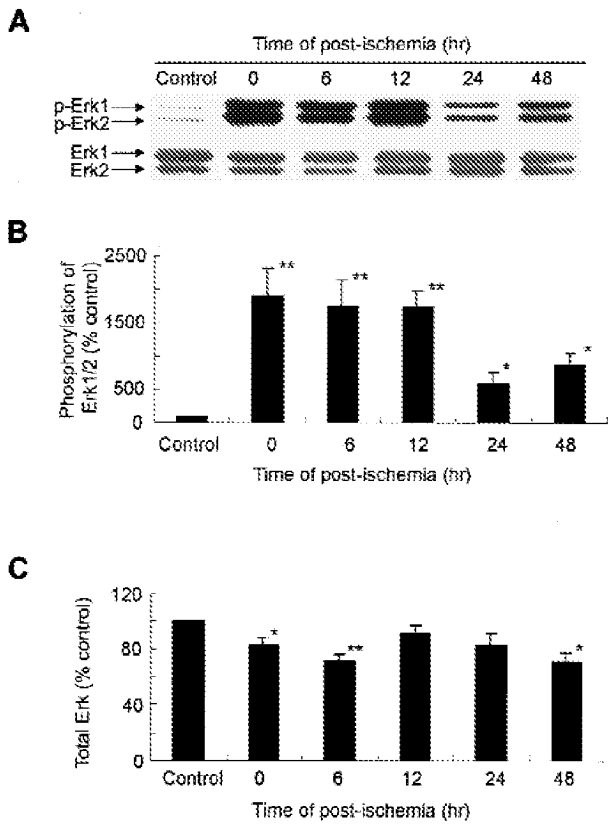

Fig. 3. Activation of Erk1/2 during postischemia. Primary cultures of astrocytes were subjected to ischemia for 4 h followed by postischemia incubation for the indicated periods of time. Experiments were repeated three times with similar results. **A,** Western blot analysis indicating changes in Erk1/2 and p-Erk1/2 during ischemia and postischemia incubation. **B,** The relative phosphorylation of Erk1/2 during postischemia incubation (mean of three independent experiments). During postischemia incubation, p-Erk1/2 level was elevated during the first 12 h and decreased from 24 h. C) Changes in total Erk. Comparisons were considered significant if $^*P < 0.05$, $^{**}P < 0.01$.

subjected to 4 h of ischemia was significantly lower than controls (Fig. 3A and 3C). Six hours after entering postischemia incubation, the total Erk level was 72% of the control. The total Erk level recovered slightly and was not significantly different from the control after 12 h and 24 h postischemia incubation but again became significantly lower than the control at 48 h, when it was 71% of the control level (Fig. 3C).

p-Akt and Total Akt Measurement. The level of p-Akt in astrocytes increased significantly to 159% of the control value after 4 h ischemia (Fig. 4A and 4B). The level of p-Akt in cultures increased further after 6 and 12 h post-ischemia incubation, to 270% and 245% of the control value, respectively (Fig. 4B). At 24 and 48 h postischemia, the p-Akt levels declined significantly, reaching 150% and 166% of the control

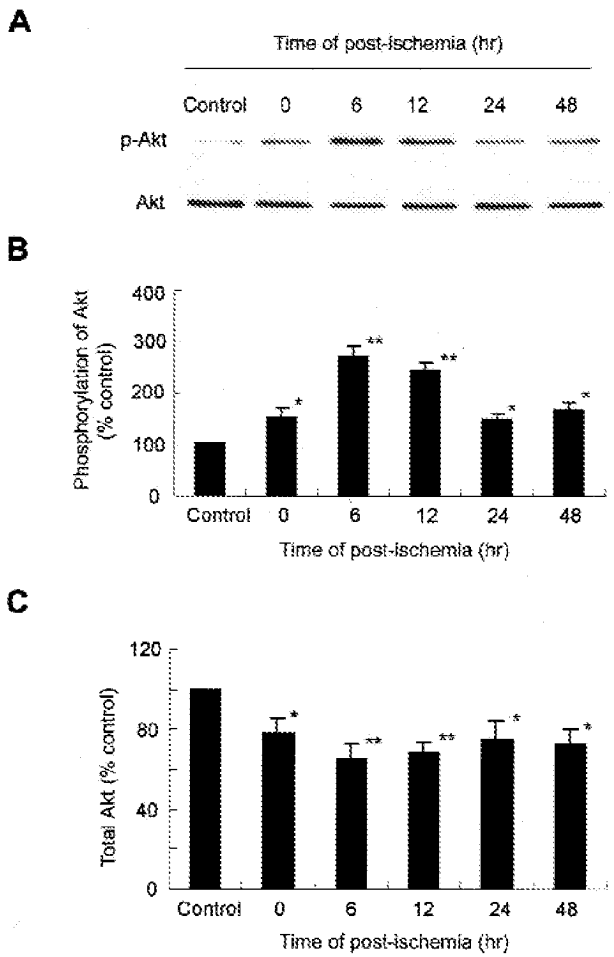

Fig. 4. Activation of Akt during postschemia. Primary culture of astrocytes was subjected to ischemia for 4 h and postischemia incubation for the indicated periods of time. Experiments were repeated three times with similar results. **A,** Western blot analysis indicating changes in Akt and p-Akt during ischemia and postischemia incubation. **B,** The relative phosphorylation of Akt during postischemia (mean of three independent experiments). Akt activity is elevated during ischemia incubation. During postischemia incubation, Akt activity further increases, reaching a maximum (270%) after 6 h, before decreasing significantly from 12 h. **C,** Changes in total Akt. Comparisons were considered significant if $^*P < .05$, $^{**}P < .01$.

value, respectively (Fig. 4B). The total Akt level was slightly but significantly reduced to 78% of the control after 4 h of ischemia (0 h postischemia). Throughout the postischemic incubation, the level of total Akt remained lower than the control. After 6 h of postischemia incubation, the total Akt expression reached its lowest level at 65% of control (Fig. 4C).

DISCUSSION

During apoptosis, the genomic DNA of programmed cells is cleaved into oligonucleosomal fragments by endonucleases (16) that form a characteristic laddered DNA pattern during agarose gel electrophoresis. This DNA fragmentation is considered the "hallmark" of apoptosis. TUNEL and DNA laddering techniques have been used to detect apoptotic cells (10,17,18). In this study, TUNEL-positive astrocytes in culture could be detected after 6 h postischemia incubation, during which the number of TUNEL-positive cells increased as the incubation time progressed. DNA laddering analysis confirmed the presence of DNA fragmentation. During postischemia incubation, reactive oxygen species including superoxide, hydroxyl, and nitric oxide (NO) radicals can be induced by reperfusion, promoting apoptosis and further tissue damage (19,20). In our ischemia and postischemia in vitro models, astrocytes underwent cellular damage throughout the incubations. This phenomenon might be related to the restoration of oxygen during the postischemia process resulting in the production of locally high concentrations of reactive oxidative species that induce apoptosis and DNA fragmentation.

Wieloch et al. (21) reported that ischemia and postischemic reperfusion could turn on some intracellular signal pathways. Wu et al. (22) showed that permanent middle cerebral artery occlusion (MCAO) induced p-Erk1/2 in neurons and astrocytes. However, Irving et al. (23) demonstrated that p-Erk1/2 was mainly localized in neurons but not in astrocytes after transient and permanent MCAO. In the present study we found that p-Erk1/2 levels were elevated in astrocytes under ischemic incubation. In addition, p-Erk1/2 remained elevated after 6 and 12 h postischemia incubation. Erk1/2 activation was believed to promote cell survival in ischemic injury, an effect related to antiapoptosis, as inhibition of p-Erk1/2 by PD98059 increased the number of apoptotic cells by 33.4% (24). In our previous study, we demonstrated the beneficial roles of Erk1/2 in astrocytes in primary culture of astrocytes under in vitro ischemia incubation (11). Erk1/2 protected ischemic astrocytes through the elevation of Bcl-2 expression (11). Jin et al. (25) also found that Erk1/2 protected hypoxic neurons via phosphorylation of Bad. These studies confirm the protective and antiapoptotic roles of Erk1/2 in ischemic injury. In this study, p-Erk1/2 levels were reduced significantly after 12 h of postischemia incubation, during which greater cellular damage appeared and the number of apoptotic cells increased. We propose that p-Erk1/2 exerts its protective effect against injury at the early stage of postischemia incubation when the injury to astrocytes is slight. At the later stages of postischemia incubation, cells died because the p-Erk1/2 level declined.

The relationship between ischemia, reperfusion injury and Akt was discussed by Kitagawa et al. (26), who detected phosphorylation of Akt in neurons under permanent MCAO. Akt was suggested to have an antiapoptotic function in cerebral neurons (27). Noshita et al. (28) showed that transient cerebral ischemia induced the phosphorylation of Akt and that inhibition of Akt with LY294002 increased the incidence of apoptotic cells. This suggested that Akt might inhibit apoptosis. In this study, the p-Akt level increased in astrocytes during the early stages of postischemia incubation but decreased as cell death became more severe. Consistent with our findings, Ouyang et al. (29) demonstrated an activation of Akt during the early stages of reperfusion in rat brain. During the later stages of reperfusion, activation of Akt decreased, while cytochrome *c* and caspase-3–like activity increased. Akt may exert a protective role in ischemic injury (29). However, this finding was not supported in a previous study (11). Furthermore, we found that inhibition of Akt with LY294002 could delay cell injury and increased Bcl-2 expression. Aki et al. (30) also reported that inhibition of P13-K/Akt signal pathway reduced necrotic cell death in H9c2 cells under hypoxic conditions. Thus the change in p-Akt concentration in astrocytes might be related to the onset of apoptosis.

In conclusion, changes in p-Erk and p-Akt during postischemia incubation correlated well with the appearance of apoptosis, indicating a potential direct relationship of these signal pathways to ischemia- and postischemia–induced damage to astrocytes in primary culture.

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