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Activation of Erk1/2 and Akt in astrocytes under ischemia

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Abstract

Substantial evidence has shown that extracellular signal-regulated kinases 1 and 2 (Erk1/2) and serine/threonine kinase (Akt) play important roles in regulating cell survival. We examined the activities of these kinases in astrocytes under ischemia in an anaerobic chamber. The level of phosphorylated Erk1/2 in astrocytes began to increase after 1 h ischemia, reached a maximum after 4 h ischemia, before decreasing from 5 to 6 h. Akt was activated later than Erk1/2. It was significantly increased after 4 h ischemia before declining steadily afterwards. Lactate dehydrogenase (LDH) assay and Hoechst nucleic staining indicated that U0126, which inhibits Erk1/2 phosphorylation, enhanced ischemia-induced cell death, whereas LY294002, which inhibits Akt phosphorylation, delayed cell death. These effects were dose-dependent. At 4 and 6 h ischemia, U0126-treated astrocytes expressed a lower level of Bcl-2 than controls. In contrast, LY294002-treated astrocytes expressed a higher level of Bcl-2 than controls as shown by Western blots. Bcl-x_L expression level was not affected by either treatment. These data suggest that activation of the MAPK/Erk1/2 pathway might protect astrocytes from ischemic injury, but activation of the PI3-K/Akt pathway does not. The effect may involve Bcl-2 but not Bcl-x_L expression.

Keywords: U0126; LY294002; Astrocyte; Ischemia; Erk; Akt; Bcl-2; Bcl-x_L

Ischemic injury to brain is a common life threatening neurologic disorder that is associated with high morbidity and mortality. Despite intensive study over many years, data obtained from astrocytes under ischemia are scarce. Previous studies have shown that astrocytes attenuated the neuronal damage induced by glutamate in vitro [1]. In vivo ischemic experiments also showed that the viability of astrocytes was important to the degree of infarction in cerebral ischemia [2,3]. Based on the importance of astrocytes for neuronal survival under pathological conditions, it is important to elucidate the mechanism involved in ischemia-induced cell death in astrocytes. This would provide new insights regarding the future development of novel therapeutic treatment for ischemic insult. We have shown previously that ischemia can induce apoptotic cell death in astrocytes [4]. To further understand the molecular mechanisms involved in ischemic injury in astrocytes, this study examined the activation of MAPK/extracellular signal-regulated kinases 1 and 2 (Erk1/2) and phosphoinositide 3-kinases (PI3-K)/Akt pathways.

The mitogen-activated protein kinase (MAPK) signal pathway is composed of three sequentially activated kinase cascades designated as MAPK kinase kinase (MKKK), MAPK kinase (MKK, MEK), and MAPK [5,6]. Erk1/2, members of the MAPK superfamily, are activated by phosphorylation at threonine and tyrosine residues [7]. The activation of the MAPK/Erk1/2 pathway is known to affect a wide range of cell activities including gene expression, cell proliferation, cell survival, and cell death [6,8,9]. Previous studies demonstrated that Erk1/2 was activated in ischemia model and suggested that the MEK/Erk signal pathway played an important role in deciding the fate of neural cells [10–13]. Although these authors all used an inhibitor of MEK to suppress the phosphorylation of Erk1/2 in

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in vivo or in vitro ischemia models, there were discrepancies in their conclusions of the role of activated Erk in ischemic injury. The molecular mechanisms of any detrimental or beneficial effects of MAPK/Erk1/2 are not fully understood.

The serine/threonine protein kinase, Akt (protein kinase B), is activated by PI3-K [14] and has been known to play an important role in mediating cell survival. The PI3-K/Akt signal pathway is activated in cerebral ischemia in vivo [15]. Suppression of the phosphorylation of Akt by the PI3-K inhibitor, LY294002, increased the number of apoptotic cells in cerebral ischemia [16]. Thus, the PI3-K/Akt pathway was suggested to participate in the neuroprotective mechanism. However, it is not clear how PI3-K/Akt exerts its effects on cerebral ischemia and whether this pathway plays a role in astrocytes during ischemia. In the present study, we examined whether the MAPK/Erk1/2 and PI3-K/Akt pathways are activated in ischemia-injured astrocytes to determine the significance of their roles in ischemic injury through inhibition of their phosphorylation.

Materials and methods

Primary culture of mouse cerebral cortical astrocytes. Primary cultures of cerebral cortical astrocyte were prepared from newborn ICR mice (Animal Care Facility, HKUST) as described by Yu et al. [4]. Briefly, meninges-free cortices were cut into small cubes (<1 mm^3) and suspended in modified Dulbecco's minimum essential medium (DMEM) (GIBCO BRL, Life Technologies, USA). After being mechanically dissociated by vortexing for 90 s, the cell suspension was sieved through a 70 μm and 10 m sterile mesh nylon filter (Spectrum Medical Industries, USA). Then, the cell suspension was mixed with 10% fetal bovine serum (Hyclone, USA) and seeded in a 60-mm Falcon tissue culture dish (Becton–Dickinson Labware, USA), equivalent to 8×10^5 viable cells per dish (approximately one-fifth of a cerebrum). All cultures were incubated in a 37 °C carbon dioxide (CO_2) incubator (Precision Scientific, USA) with 5%/95% CO_2/air (v/v) and 95% humidity. Cultures were used for experiments when they reached 4 weeks.

In vitro ischemia model. Substrate-deprived medium was prepared with glucose- and serum-free modified DMEM. The medium was degassed with 99.95% N_2 for 30 min and then saturated with 5% CO_2 in N_2 for another 20 min. The cultures and anoxic DMEM were then transferred into an anaerobic chamber (Forma Scientific, USA) saturated with 85% N_2/10% H_2/5% CO_2. The residual oxygen in the chamber was removed with palladium. Cultures were washed with the anoxic DMEM and incubated in 2 ml of the same medium. All cultures were kept in a 37 °C incubator inside the anaerobic chamber.

Western blot analysis. After ischemic incubation, astrocytes were washed with ice-cold phosphate-buffered saline (PBS) and the proteins were extracted with 200 μl lysis buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml leupeptin, and 0.5 g/ml pepstatin). After 20 min on ice, the lysate was centrifuged in 4 °C at 14,000 rpm for 10 min. The protein concentration was determined with a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, USA). Equal amounts of proteins from cell lysate were boiled in protein loading buffer for 5 min and separated on a 12% sodium dodecyl sulfate–polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes (Hybond ECL Amersham Pharmacia Biotech, UK). Nonspecific binding was inhibited 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, phospho-Erk1/2, Bcl-2, Bcl-x_L (Santa Cruz, USA), Akt and phospho-Akt (Ser 473) (Cell Signaling, 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 secondary antibodies (Amersham Pharmacia Biotech, UK). After washing, the secondary antibodies were detected with an electrochemiluminescent system (Amersham Pharmacia Biotech, UK).

U0126 and LY294002 treatment. U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] and LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] are MEK1/2 and PI3 kinase inhibitors, which specifically inhibit MAPK/Erk1/2 and PI3-K/Akt signal pathways, respectively [17,18]. They were dissolved in DMSO before use. In this experiment, all cultures were incubated in serum-free media for 1 h and then pre-treated with the respective inhibitor for 45 min before ischemic incubation. During the ischemic incubation two inhibitors always remained in the media. The doses of U0126 and LY294002 were chosen according to the IC_{50} value provided on the data sheet accompanying each compound.

Assessment of viability. Astrocytes were stained with Hoechst 33342 and the nuclear morphology was assessed under phase contrast and fluorescent microscope (Leica DMR, GmbH, Germany). Astrocytes were fixed with pre-chilled 4% (w/v) paraformaldehyde and permeablized with 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate before exposing to 2 mg/ml Hoechst 33342 (Molecular Probes, OR, USA) in PBS for 5 min.

Cytotoxicity Detection Kit (LDH) (Boehringer–Mannheim GmbH, Germany) was used to measure cell death. The degree of cell death was estimated by the change of lactate dehydrogenase (LDH) activity in the culture media.

Statistical analysis. The band intensities of Western blots were quantified by a densitometer (Stratagene, USA) and expressed as relative values to the control. The values of relative phosphoryltion of kinases were shown as the ratios of phosphorylated kinases to the total kinase. All values were expressed as means ± SEM. Statistical significance was evaluated using Student's t test for unpaired data. In all analyses, p value < 0.05 was considered to be statistically significant.

Results

Erk1/2 phosphorylation in astrocytes under ischemia

The phosphorylation of Erk1/2 significantly increased during ischemic incubation (Figs. 1A and B). From the beginning of ischemic incubation to 4 h, the expression level of phosphorylated Erk1/2 (p-Erk1/2) increased rapidly. At 4 h of ischemia, the level of p-Erk1/2 reached a maximum, 22-fold higher than the control (Fig. 1B). The p-Erk1/2 levels began to decrease slightly from 5 to 6 h. The expression of total Erk was measured to examine whether the increase of p-Erk1/2 is derived from the changes of total Erk. Under the same conditions, total Erk expression was significantly lower compared with controls at 2, 3, and 4 h of ischemia (Fig. 1C). Ischemic incubation of 1, 5, and 6 h did not significantly affect the expression levels of total Erk. The ratio of p-Erk1/2 to total Erk closely matched the alterations of p-Erk1/2. At 4 h of ischemia, the ratio also increased to a peak, which was about 26-fold higher than the controls (Fig. 1D).

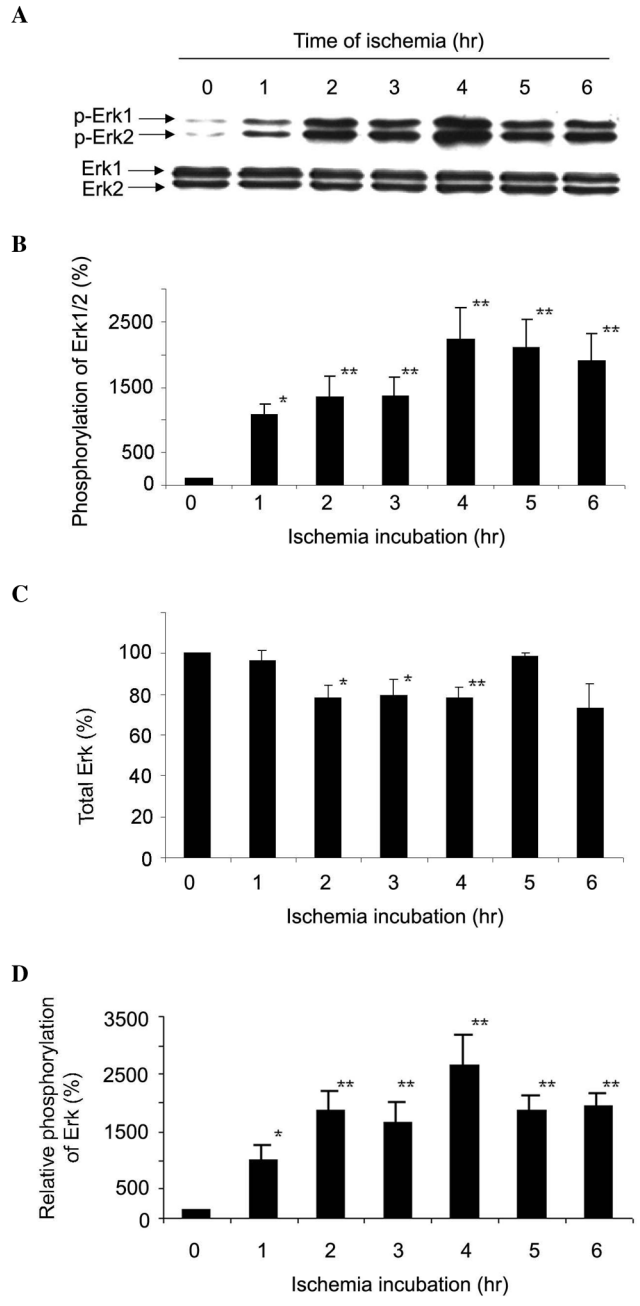

Fig. 1. Phosphorylation of Erk1/2 and total Erk during ischemia. Primary culture of astrocytes was subjected to ischemia for the indicated periods of time. (A) Western blot analysis of the phosphorylation of Erk1/2 and total Erk during ischemia. Blots shown are representative of three independent experiments. B, C, and D indicate the phosphorylation of Erk1/2, total Erk, and relative phosphorylation of Erk1/2 during ischemia, respectively. Astrocytes not subjected to ischemia are used as controls. The relative phosphorylation of Erk1/2 was increased during ischemic incubation with a peak at 4 h (26-fold increase). Data are presented as means ± SEM from three independent experiments. *$p < 0.05$ or **$p < 0.01$ indicates statistical significance compared with control.

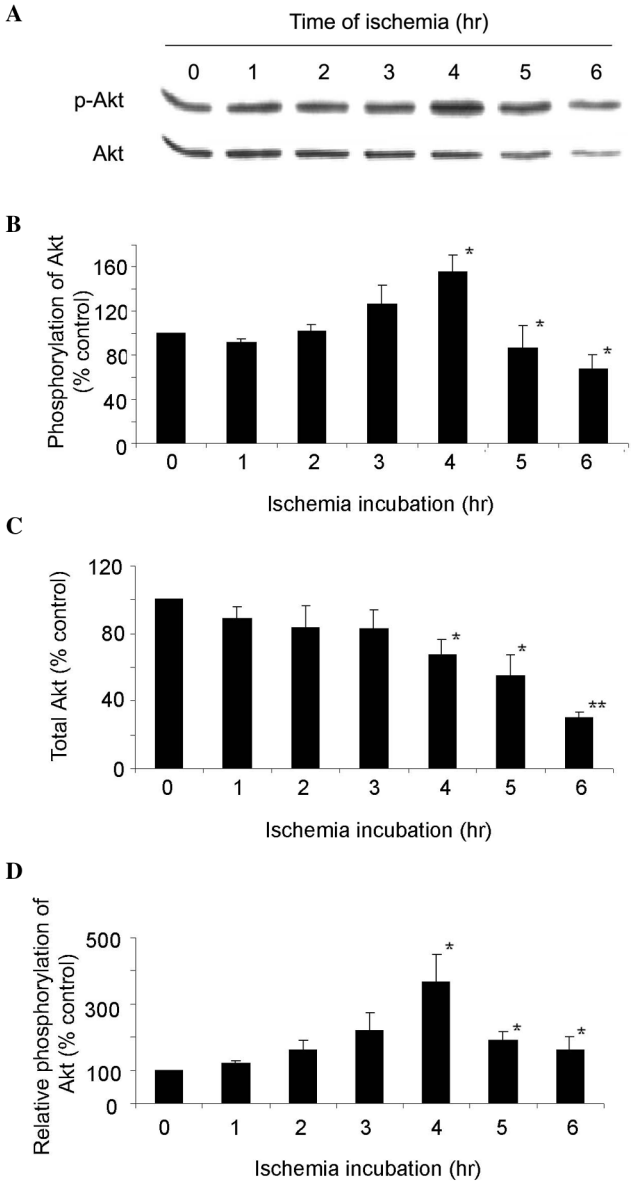

Fig. 2. Phosphorylation of Akt and total Akt during ischemia. Primary culture of astrocytes was subjected to ischemic incubation for the indicated period of time. (A) Western blot analysis of Akt and total Akt phosphorylation. Blots shown are each representative of three independent experiments. B, C, and D indicate the phosphorylation of Akt, total Akt, and relative phosphorylation of Akt during ischemic incubation, respectively. Astrocytes not subjected to ischemia were used as control. The relative phosphorylation of Akt increased during ischemic incubation and reached a peak after 4 h (360% increase). Data are presented as means ± SEM from three independent experiments. *$p < 0.05$ or **$p < 0.01$ indicates statistical significance compared with control.

Akt phosphorylation in astrocytes under ischemia

The phosphorylated Akt (p-Akt) level did not change in the first 3 h of ischemia but increased significantly to 156% of the control after 4 h ischemia. The level decreased rapidly to a level lower than the control from 5 to 6 h ischemia (Figs. 2A and B). The total Akt level in astrocytes under ischemia did not change significantly in the first 3 h of ischemic incubation (Figs. 2A and C), but was reduced significantly at 4 h ischemia compared with the control. The level was reduced to 30% of the control by 6 h. The ratio of p-Akt to total Akt gradually and slowly increased

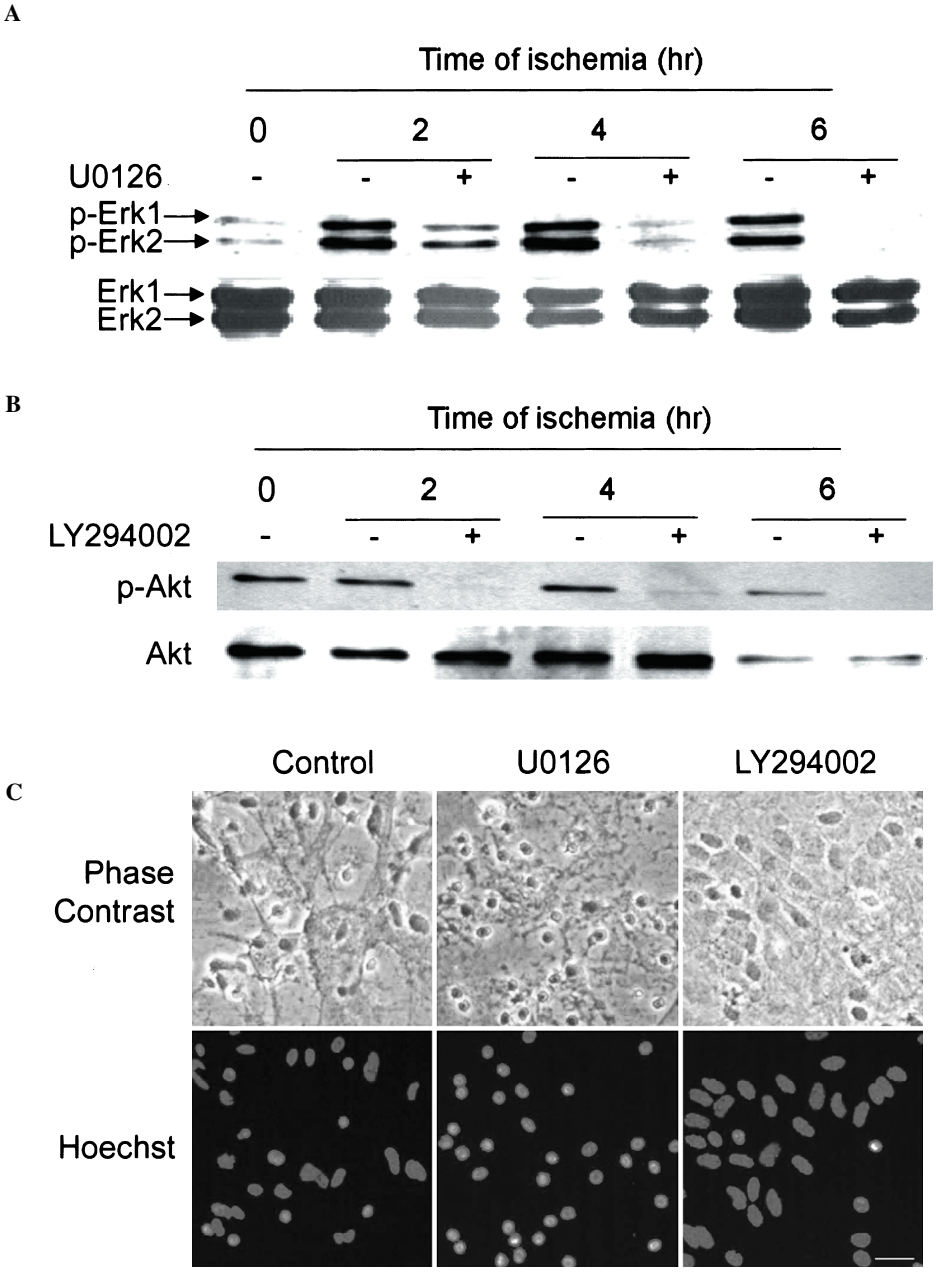

Fig. 3. (A) Inhibition of Erk phosphorylation by U0126. Western blot of p-Erk following treatment of 0, 2, 4, and 6 h ischemia-injured astrocytes with U0126 (10 μM). (B) Inhibition of Akt phosphorylation by LY294002. Western blot of p-Akt following treatment of 0, 2, 4, and 6 h ischemia-injured astrocytes with LY294002 (20 μM). (C) Microscopy studies of 4 h ischemia-injured astrocytes treated with U0126 and Ly294002. Under phase contrast, cell death appears greatest in U0126-treated astrocytes but appears less severe in LY294002-treated astrocytes compared with the control. After Hoechst 33342 staining, the number of condensed nuclei is increased in U0126-treated cells and reduced in LY294002-treated cells compared with control. Scale bar, 20 μm.

and reached a maximum after 4 h ischemia (360% of the control) (Fig. 2D). The phosphorylation ratios at 5–6 h ischemia dropped significantly, but remained higher than the control (191% and 159% of the control, respectively).

U0126 treatment

Western blot showed that 10 μM U0126 effectively blocked Erk phosphorylation at 4 and 6 h of ischemic incubation (Fig. 3A). Phase contrast microscopy showed that astrocytes treated with 10 μM U0126 had greater cellular damage compared with the DMSO control after 4 h ischemia. Hoechst staining showed an increase in the number of condensed nuclei in U0126-treated cultures (Fig. 3C). For astrocytes in 37 °C CO_2 incubator, U0126 treatment for 6 h had no damaging effect (data not shown).

LDH assays demonstrated an increase in cell death at 4 h of ischemia (Fig. 4A). The LDH release level in

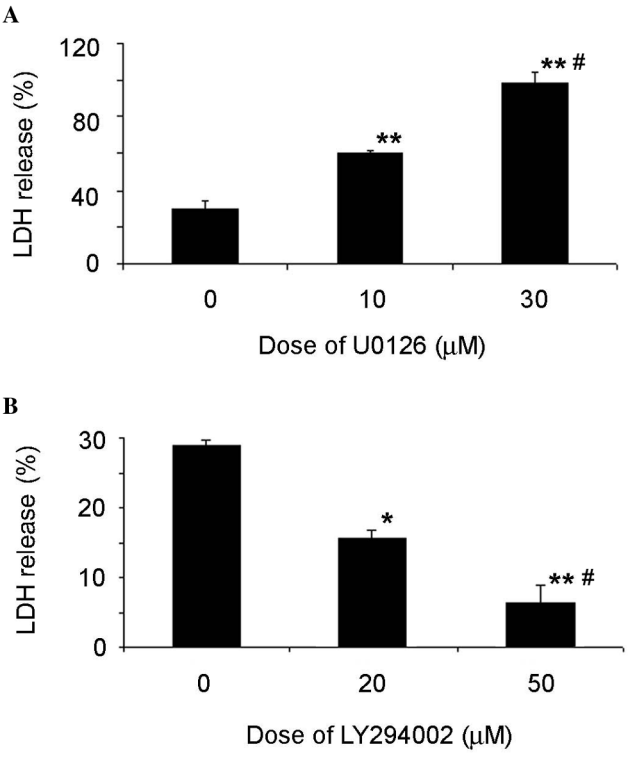

Fig. 4. Effect of phosphorylation inhibitors on LDH release in ischemia-injured astrocytes. Cultured astrocytes were pretreated with U0126 or LY294002 and subjected to 4 h of ischemic incubation. (A) LDH release increased from 30% to 60%, then to 99% of the control level, as the dose of U0126 increased from 0 to 10 μM, and then to 30 μM. (B) LDH was deceased from 28% to 16% of the control level as the dose of LY294002 increased from 0 to 20 μM and decreased further to 6% as the dose was increased to 50 μM. Data are mean values of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical significance compared with control. $^{\#}p < 0.05$ indicates statistical significance when compared with lower dose treatment.

the DMSO control culture was 30% at 4 h ischemia. In cultures treated with 10 μM U0126, the LDH level increased to 60%. Treatment with 30 μM U0126 increased the LDH release level to 99%, indicating total cell death.

LY294002 treatment

Western blot showed that 20 μM LY294002 effectively blocked the phosphorylation of Akt in astrocytes under 2–6 h ischemia (Fig. 3B). Phase contrast microscopy showed that astrocytes treated with 20 μM LY294002 appeared to have less damage than the DMSO control at 4 h ischemia. Hoechst staining showed fewer condensed nuclei in these treated cultures and the nuclei appeared to be less shrunken (Fig. 3C).

The release of LDH in the DMSO control culture was 28% at 4 h ischemia. In cultures treated with 20 and 50 μM LY294002, the LDH release level was significantly reduced to 16% and 6%, respectively (Fig. 4B).

Bcl-2 and Bcl-x_L expression

As shown in Fig. 5, U0126 suppressed Bcl-2 expression in astrocytes. Under 4 and 6 h of ischemic incubation, U0126 treatment significantly decreased the expression of Bcl-2 by about 60% and 44%, respectively. However, the expression of Bcl-x_L was not significantly affected by U0126 at any time compared with control.

Fig. 6 shows the effect of LY294002 treatment on Bcl-2 and Bcl-x_L expression in ischemia-injured astrocytes. In cultures under 4 h and 6 h of ischemic incubation, LY294002 treatment increased the expression of Bcl-2 by 91% and 107%, respectively. In contrast, LY294002 treatment did not affect the expression of Bcl-x_L in astrocytes under ischemic incubation.

Discussion

In the current study, Erk1/2 and Akt phosphorylation in astrocytes under ischemia were measured. At 4 h ischemia, both pathways were activated to their maximal level. However, the dynamics of enzyme activation were different in each case. The p-Erk1/2 level increased significantly only after 1 h ischemia, whereas p-Akt expression increased significantly after 4 h ischemia compared with the control. The rapid activation of Erk1/2 suggested that it might play an important role in regulating gene expression or other signal pathways to limit ischemic damage. The relatively slow activation of Akt indicated that Akt phosphorylation might be a consequence of ischemic injury or be a factor promoting the death of astrocytes. Using this ischemia model in previous studies, massive death of astrocytes has been demonstrated to occur after 4 h of ischemia [4]. Thus, after 4 h ischemia the activated p-Erk1/2 and p-Akt was maximally activated and working at full capacity to resist cell injury or enhance the process of cell death.

To better understand the roles of Erk1/2 activation and PI3-K/Akt activation in astrocytes during ischemic injury, we treated astrocytes under ischemia with a specific MEK1/2 inhibitor (U0126) or a specific PI3-K inhibitor (LY294002). The cultures treated with U0126 showed more severe death and a reduction of Bcl-2 expression under ischemic injury compared with controls. The expression level of Bcl-x_L was not affected. The data suggest that Erk1/2 plays an important role in mediating cell survival, possibly through an elevation of Bcl-2 expression but not Bcl-x_L. We hypothesize that activated Erk1/2 induces Bcl-2 expression through an activation of the transcription factor cAMP-response element-binding protein (CREB), as MAPK/Erk1/2 can also activate pp90 ribosomal S6 kinase (RSK), a potent activator of CREB [19]. Our hypothesis is partially supported by the work of Irving et al. [20], who found that neurons positively stained by p-Erk1/2 and p-CREB

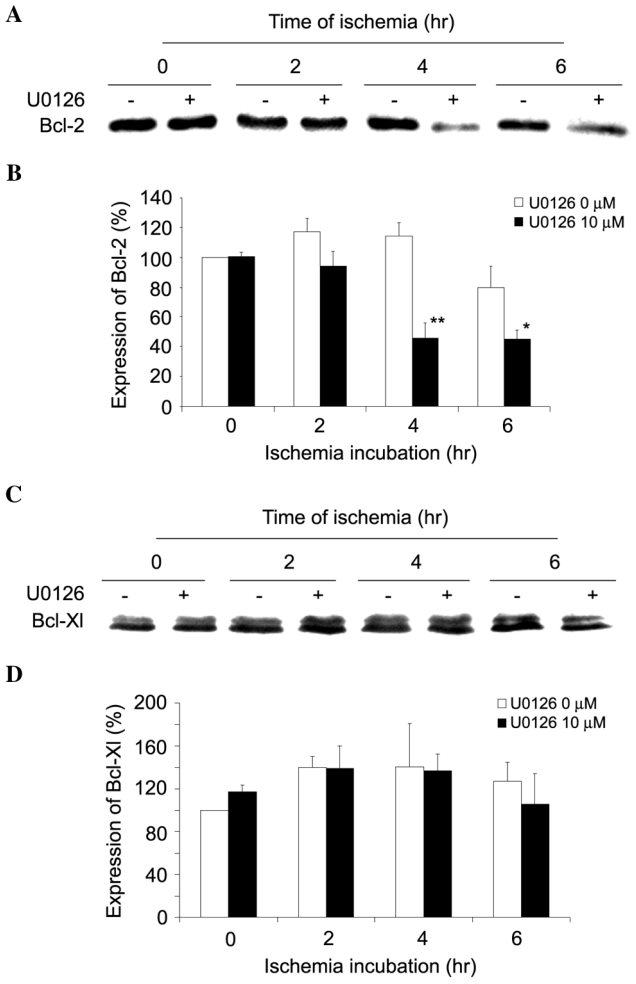

Fig. 5. Effect of U0126 on Bcl-2 and Bcl-x_L expression. (A) and (C) Western blot of Bcl-2 and Bcl-x_L, respectively, in 0, 2, 4, and 6 h ischemia-injured astrocytes pre-treated for 45 min with U0126 (10 μM). (B) and (D) Quantification of Bcl-2 and Bcl-x_L expression, respectively, of the cells treated in (A) (mean of three independent experiments). (C) Western blot analysis of Bcl-x_L expression in 0, 2, 4, and 6 h ischemia-injured astrocytes pre-treated for 45 min with U0126 (10 μM). Under 4 and 6 h of ischemic incubation, U0126 pre-treatment decreased the expression of Bcl-2 by about 60% and 44%, respectively. There was no significant effect of U0126 on Bcl-x_L expression. *$p < 0.05$ and **$p < 0.01$ indicate statistical significance when compared with astrocytes under identical ischemic conditions without inhibitor pre-treatment.

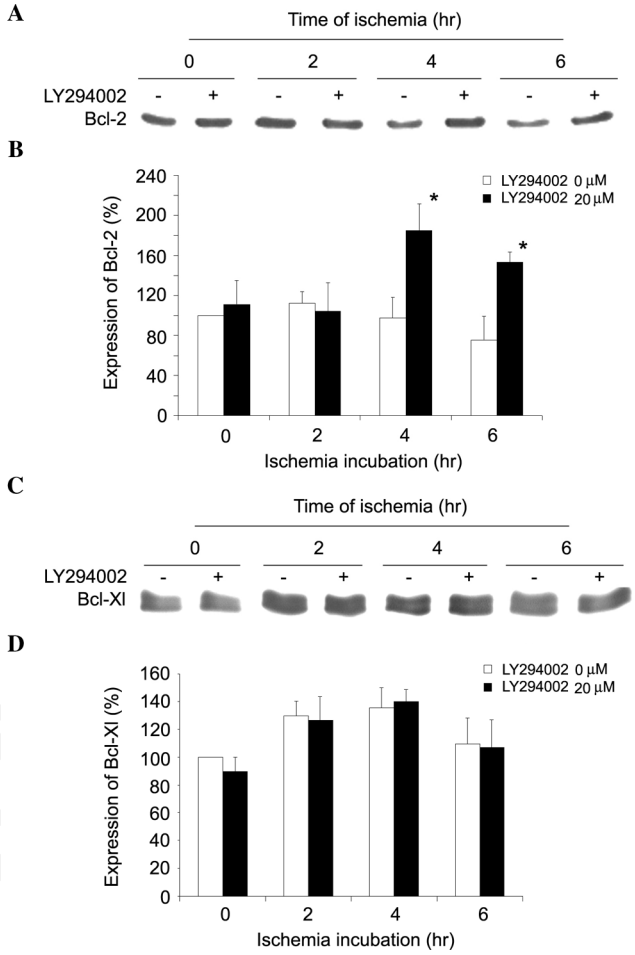

Fig. 6. Western blot analysis showed the effect of LY294002 treatment (20 μM) on expression of Bcl-2 (A) and Bcl-x_L (C) in ischemia-injured astrocytes. After pre-treatment with 20 μM LY294002 for 45 min, primary culture of astrocytes was subjected to ischemic incubation for the indicated times. Experiments were repeated three times with similar results. (B) Quantitative analysis of Bcl-2 expression (mean of three independent experiments). Under 4 and 6 h of ischemic incubation, LY294002 pre-treatment increased the expression of Bcl-2 by about 36% and 57%, respectively. There was no significant effect of LY294002 on Bcl-x_L expression. *$p < 0.05$ indicates statistical significance when compared with astrocytes under identical ischemic conditions without inhibitor pre-treatment.

survived ischemic injury. Although the authors did not provide direct evidence for the phenomena, we propose that the p-CREB-positive neurons survived ischemic injury by elevation of Bcl-2 expression. It is well known that Bcl-2 and Bcl-x_L are important components of the anti-apoptotic system that acts by inhibiting pro-apoptotic members of the Bcl-2 family through heterodimerization [21]. Our results suggest that Bcl-2 rather than Bcl-x_L is involved in the survival of astrocytes from ischemic damage. In a previous study, Kane et al. [22] reported that Bcl-2 protein also has an anti-necrotic effect in neural cell death. We propose that the activation of Erk1/2 kinases in astrocytes under ischemia might protect against necrosis and apoptosis through the elevation of Bcl-2 expression, as astrocytes in our ischemia model are able to undergo necrosis and apoptosis [4]. MAPK/Erk1/2 are considered to provide a neuroprotective mechanism against hypoxic damage in neurons by phosphorylation of Bad [13]. This further demonstrates the protective effect of MAPK/Erk1/2 against cell death and suggests that there are other mechanisms involved in the protective effect besides elevation of Bcl-2 expression. However, Alessandrini et al. [10] and Namura et al. [12] demonstrated that MEK inhibitors provided significant neuroprotection against ischemic injury, suggesting that MAPK/Erk1/2 was involved in ischemia-induced cell death. Thus, the effect of

MAPK/Erk1/2 on ischemic injury remains questionable and may be related to the cell types or ischemia conditions studied. In this paper, we demonstrated that the activation of the MAPK/Erk1/2 pathway under ischemic injury actually protects astrocytes.

The PI3-K/Akt signal pathway is well documented as being involved in supporting various types of cell survival [14]. Previous studies have demonstrated a protective effect of Akt against ischemic insults. For example, the inhibition of the Akt activation by wortmannin or LY294002 increased the number of apoptotic cells in the transient cerebral ischemia and blocked the neuroprotective action of preconditioning in ischemic injury [16,23]. In the present study, we used LY294002 to inhibit Akt activation. Surprisingly, we found that LY294002 elevated the level of Bcl-2 expression and delayed cell death instead of enhancing the death of astrocytes from ischemic insult. We propose that the PI3-K/Akt pathway in ischemic astrocytes does not play a protective role but promotes cell injury. However, considering the fact that Akt was not the only kinase activated by PI3-K, the protective effect of LY294002 might derive from the inhibition of other pathways activated by PI3-K. Aki et al. [24] reported that LY294002 inhibited necrotic cell death in H9c2 cells under hypoxic conditions. In glucose-rich media, LY294002 inhibits the metabolic acidosis induced by PI3-K and protects the cells from hypoxic injury. In the present study, LY294002 also protects cells in glucose-free media, suggesting that other mechanisms are affected by LY294002 to delay cell death such as by elevations in Bcl-2 expression. Carbott et al. [25] observed that LY294002 increased Bcl-2 expression level and attenuated okadaic acid-induced apoptosis in renal epithelial cells, whereas wortmannin enhanced apoptosis. In this study, we could not confirm that the protective effect of LY294002 was derived from an inhibition of the PI3-K/Akt pathway. However, LY294002 can protect astrocytes from ischemic injury by elevating Bcl-2 expression. Thus, it is important to further investigate the effects of the PI3-K/Akt signal pathway on ischemic injury in astrocytes by inhibiting PI3-K with wortmannin.

In conclusion, MAPK/Erk1/2 and PI3-K/Akt pathways were activated in astrocytes under ischemia, but they responded differently; inhibition of the MAPK/Erk1/2 pathway enhances cell death, whereas inhibition of PI3-K protects the astrocytes from ischemic injury. These effects are mediated by changes in Bcl-2 expression but not Bcl-x_L.

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