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Cycloheximide and Actinomycin D Delay Death and Affect bcl-2, bax, and Ice Gene Expression in Astrocytes Under In Vitro Ischemia

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An in vitro ischemia model was established and the effect of the metabolic inhibitors cycloheximide (CHX) and actinomycin D (ActD) on apoptosis in astrocytes under ischemia studied. CHX decreased by 75% the number of cells dying after 6 hr of ischemia compared with control cultures. TdT-mediated dUTP nick end labelling (TUNEL) staining of comparable cultures was reduced by 40%. ActD decreased cell death by 60% compared with controls. The number of TUNEL-positive cells was reduced by 38%. The nuclear shrinkage in TUNEL-positive astrocytes in control cultures did not occur in ActD-treated astrocytes, indicating that nuclear shrinkage and DNA fragmentation during apoptosis are two unrelated processes. Expression of bcl-2 (α and β), bax, and Ice in astrocytes under similar ischemic conditions, as measured by quantitative reverse transcription-polymerase chain reaction, indicated that ischemia down-regulated bcl-2 (α and β) and bax. Ice was initially down-regulated from 0 to 4 hr, before returning to control levels after 8 hr of ischemia. ActD decreased the expression of these genes. CHX reduced the expression of bcl-2 (α and β) but increased bax and Ice expression. It is hypothesized that the balance of proapoptotic (Bad, Bax) and antiapoptotic (Bcl-2, Bcl-Xl) proteins determines apoptosis. The data suggest that the ratio of Bcl-2/Bad in astrocytes following ActD and CHX treatment does not decrease as much in untreated cells during ischemia. Our data indicate that it is the ratio of Bcl-2 family members that plays a critical role in determining ischemia-induced apoptosis. It is also important to note that ischemia-induced apoptosis involves the regulation of RNA and protein synthesis.

Key words: actinomycin D; apoptosis; astrocytes; cycloheximide; Bcl-2; Bax; Ice; ischemia

Astrocytes provide structural and nutritive support to neuronal cells and maintain a homeostatic environment in the brain (Kimelberg et al., 1995; Longuemare and Swanson, 1995; Ciccarelli et al., 2001), which is essential for neuron survival, especially during injury. Other studies have also revealed the essential function of astrocytes in maintaining normal neuronal activity. Reducing the number of astrocytes in neuronal-astrocytic cocultures (from 90% to 30%) increases the sensitivity of neurons to glutamate toxicity by 100-fold (Rosenberg and Aizenman, 1989). We have demonstrated previously that astrocytes die from apoptosis under in vitro ischemia in the absence of other neural cells (Yu et al., 2001). The astrocytes exhibited typical apoptotic features, including annexin IV staining, chromatin condensation, nuclear shrinkage, and DNA fragmentation and laddering (Yu et al., 2001). The expression of proinflammatory cytokines [interleukin (IL)-1α, tumor necrosis factor (TNF)-α, IL-6] was up-regulated at both transcriptional and translational levels (Yu and Lau, 2000; Lau and Yu, 2001). We have also found that ischemia induced changes in the Erk1/2 and Akt signalling pathways (Jiang et al., 2002). Apoptosis can be induced in astrocytes by exposure to a variety of molecules, including CHX, platelet-activating factor, and purine nucleosides, especially adenosine (Tsuchida et al., 2002; Hostettler et al., 2002; Di Iorio et al., 2002). Apoptosis in astrocytes can be prevented by exposure to piracetam and vinpocetine under simulated hypoxia (Gabryel et al., 2002).

The mechanism of apoptosis is highly conserved across a variety of cell types and is related to the up- and

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down-regulation of many genes, including bcl-2, bax, and IL-1β-converting enzyme (Ice; Ceretti et al., 1992; Hengartner and Horvitz, 1994). Bcl-2 has been demonstrated to inhibit apoptosis, whereas Ice induces or facilitates apoptosis (Allsopp et al., 1993; Reed, 1994; Strasser et al., 1994; Nicholson, 1996; Patel et al., 1996). Among the members of the Bcl-2 family, bcl-2 and bax have been studied extensively in the nervous system (Pinon et al., 1997; Parsadanian et al., 1998; Hata et al., 1999; Stadelman et al., 2001; Roucou et al., 2002). It is known that their interaction plays an important role in regulating apoptosis during developmental and pathologic conditions (Poommipanit et al., 1999; Middleton et al., 2001; Krajewska et al., 2002). Evidence has accumulated that Bcl-2, Bax, and Ice modulate neuron survival and death (Sato et al., 1998; Stadelman et al., 2001; Roucou et al., 2002) under different conditions. Thus, it would be interesting to investigate the expression of these genes in astrocytes under ischemia. Using actinomycin D (ActD) and cycloheximide (CHX) as tools, we could further elucidate whether these processes involve RNA and protein synthesis, respectively.

MATERIALS AND METHODS

Primary Culture of Cerebral Cortical Astrocytes

Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice (Animal Care Facility, HKUST, Hong Kong) as previously described (Yu et al., 1986, 1993, 2001), with minor modifications. Briefly, meninges-free cortices were cut into small cubes (<1 mm^3) and suspended in modified Dulbecco's modified essential medium (DMEM; Gibco BRL, Life Technologies Inc., Grand Island, NY). After being mechanically dissociated by vortexing for 90 sec, the cell suspensions were sieved though 70-μm and 10-μm sterile mesh nylon filters (Spectrum Laboratories, Inc., CA). The filtrate was mixed with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) and seeded in approximately one-thirteenth of a cerebrum in a 35-mm Falcon tissue culture dish (Becton Dickinson, NJ). All cultures were incubated in a 37°C Napco CO_2 incubator (Precision Scientific Inc., VA) with 5%/95% CO_2/air (v/v) and 95% humidity. Cultures reached confluence after about 2 weeks. They were used for experiments when they were 4 weeks old.

Experimental cultures were preincubated with the inhibitor ActD (25 μg/ml; Gibco BRL) or CHX (10 μg/ml; Sigma Chemical Co., St. Louis, MO) in serum-free modified DMEM for 24 hr before ischemia incubation, whereas the controls were incubated in serum-free modified DMEM only. These concentrations of ActD and CHX were nontoxic to astrocytes under our preincubation conditions (data not shown).

In Vitro Ischemia Model

An anaerobic-chamber-induced ischemia model has been reported before from many studies in our laboratory (Yu and Lau, 2000; Ho et al., 2001; Lau and Yu, 2001; Yu et al., 2001; Chen and Yu, 2002; Jiang et al., 2002; Chen et al., 2003). In brief, the substrate-deprived medium comprised modified DMEM free of glucose and serum. This medium was degassed with 99.95% N_2 gas for 30 min and then saturated with 5% CO_2 in N_2 for another 20 min. The cultures with anoxic DMEM were then transferred into an anaerobic chamber (model 1029; Forma Scientific Inc., OH). The atmosphere of the entire unit was saturated with 85% N_2/10% H_2/5% CO_2; residual oxygen inside the chamber was removed with palladium. Four- to five-week-old astrocytic cultures were washed three times with the anoxic medium and then incubated with 0.8 ml/dish with the same medium. The cultures were wrapped with Parafilm and kept in the 37°C incubator within the anaerobic chamber. After different times, cultures were removed for further studies.

Cell Viability Assay

The viability assay was performed using a Live/Dead Eukolight Viability/Cytotoxicity Kit (Molecular Probes Inc., Eugene, OR; Yu et al., 2001). The ischemic cultures were incubated in labeling solution containing 40 μM calcein-AM and 40 μM ethidium homodimer in DMEM for 1 hr in the 37°C incubator. Thereafter, cell death could be examined under a fluorescent microscope (Zeiss Axiophot).

TdT-Mediated dUTP Nick End Labelling

The TdT-mediated dUTP nick end labelling (TUNEL) technique was used to detect nuclear DNA fragmentation with an In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany; Yu et al., 2001). The ischemic cultures were fixed with precooled 4% paraformaldehyde (Sigma Chemical Co.) in phosphate-buffered saline (PBS) for 30 min and permeabilized with 0.1% Triton X-100 (Sigma Chemical Co.) in 0.1% sodium citrate (Sigma Chemical Co.) with PBS for 2 min at 4°C. Cultures were incubated in the TUNEL reaction mixture in a moist-air chamber for 75 min at 37°C before examination under a fluorescent microscope. For each experiment, nonspecific labelling was assessed by omitting TdT during the first step of the labelling procedure. Positive controls were obtained by pretreating the cultures with 200 U/ml DNase I (Boehringer Mannheim) at 37°C for 10 min prior to TUNEL labelling.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total cellular RNA was extracted from the cultures with Trizol reagent (Gibco BRL). The expression of apoptotic-related genes was determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the "primer dropping" method (Wong et al., 1994) with an endogenous internal control, 18S rRNA.

RT was performed using 1 μg of total RNA. The reaction mixtures contained 20 μl 1× first strand buffer (Gibco BRL), 5 μM random hexamer (Gibco BRL), and 1 μg RNA in RNase-free water and were denatured at 70°C for 15 min together. Afterward, 1 U/μl RNase inhibitor (Promega Corp., Madison, WI), 10 mM dithiothreitol (DTT; Gibco BRL), and 1 mM dNTPs (Pharmacia Biotech, Piscataway, NJ) were added to the reaction mixture and incubated at room temperature for 10 min, followed by at 42°C for 2 min. Finally, 10 U/μl SuperScript II RNase H$^-$ Reverse Transcriptase (Gibco BRL) was added and incubated at 42°C for 50 min. The reaction mixture was then incubated at 70°C for 15 min to stop the reaction. After the RT reaction, 0.15 U/μl RNase H (Gibco

BRL) was added to the reaction mixture and incubated for 20 min at 37°C to remove the RNA strand of the DNA-RNA hybrid.

PCR was performed in a thermocycler (Robocycler; Stratagene, Amsterdam, Holland), using a single master mix in a reaction volume containing 17× PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 200 μM dNTP (Pharmacia Biotech), 400 nM forward and reverse target gene primer, 400 nM forward and reverse internal control primer, and 0.04 U/μl *Taq* DNA polymerase (Gibco BRL). The PCR cycle conditions were 1.5 min at 94°C, followed by 94°C for 1 min, 55°C for 40 sec, and 72°C for 1 min, and further extension at 72°C for an additional 10 min. The number of amplification cycles varied between 22 and 38. PCR products were subjected to analysis on 2% agarose gel electrophoresis. The relative intensity value of DNA bands was quantified with a densitometer (Stratagene), and these are expressed as relative values.

The primer pairs for the target genes were the following: bcl-2α forward, 5′GTCGCTACCGTCGTGACTTC3′; bcl-2α reverse, 5′ACAGCCAGGAGAAATCAAAC3′; bcl-2β forward, 5′GTCGCT ACCGTCGTGACTTC3′; bcl-2β reverse, 5′TCAACCAGACATGCACCTAC3′; Ice forward, 5′CAGAGCACAAGACTTCTGAC3′; Ice reverse, 5′CTCTATATGGGCCTTCTTAA3′; bax forward, 5′ATGGACGGGTCCGGGGAGCAGCTT3′; bax reverse, 5′CGGGCACTTTAGTGCACAGGGCCT3′; internal control 18S rRNA forward, ′AGTACGCACGGCCGGTACAGTG3′; internal control 18S rRNA reverse, ′GGGTCGGGAGTGGGTAATTTGC3′.

Statistical Analysis

All data are presented as the means ± SEM from six separate experiments. Data were analyzed using an unpaired Student's *t*-test. A confidence interval of 95% (i.e., $P \leq .05$) was considered statistically significant.

RESULTS

Metabolic Inhibitors Delay Apoptosis in Astrocytes

The effect of metabolic inhibitors on the viability of cultured astrocytes is shown in Figure 1. Figure 1A shows phase-contrast micrographs of cultures taken after different periods of ischemia (Fig. 1Aa, 4 hr; Fig. 1Ab, 6 hr; Fig. 1Ac, 8 hr). An increase in the number of shrunken astrocytes is evident after 4–8 hr of ischemia. Figure 1Ad shows astrocytes pretreated with ActD after 6 hr of ischemia. Figure 1Ae shows astrocytes pretreated with CHX after 6 hr of ischemia. The number of shrunken astrocytes in the equivalent control culture (Fig. 1Ab) is much greater than that in astrocytes treated with either metabolic inhibitor.

Figure 1B shows the number of viable astrocytes in control and ActD- and CHX-treated cultures. The total number of viable astrocytes in each culture was estimated from multiple samplings and expressed as a percentage of the total number of astrocytes. In controls, cell viability declined rapidly during prolonged ischemic insult. In contrast, the majority (82.2%) of astrocytes pretreated with ActD remained viable after 6 hr of ischemia compared with control (18.2%). However, after 8 hr of ischemia, both ActD-treated and control astrocytes had a similar proportion of viable cells (<10%).

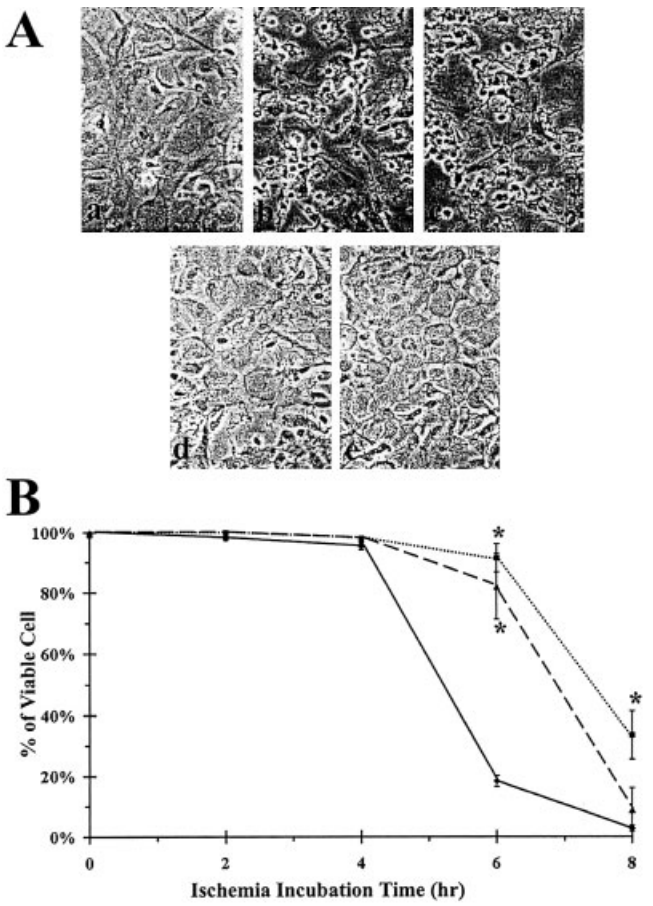

Fig. 1. Viable cells under ischemia after inhibitor pretreatment. A: Phase-contrast micrographs of ischemia in astrocytes. **Aa–Ac:** control culture after 4 hr, 6 hr, and 8 hr of ischemia, respectively. **Ad:** ActD-pretreated culture after 6 hr of ischemia. **Ae:** CHX-pretreated culture after 6 hr of ischemia. **B:** Percentage of viable cells in culture of astrocytes under ischemia in the presence of ActD or CHX. Circles, control; triangles, 25 μg/ml ActD; squares, 10 μg/ml CHX. The numbers of viable cells are expressed as the percentage of the total number of cells. Data represent means ± SEM from six separate experiments; nine fields were counted per culture. $^{\star}P \leq .05$ compared with the appropriate control cultures.

The survival of cells following CHX treatment was more pronounced. After 6 hr of ischemia, 91.6% of CHX-pretreated cells remained viable. This decreased to 33.4% after 8 hr of ischemia. The effect of ActD and CHX on apoptosis in ischemic astrocytes was measured by TUNEL staining. No positively stained nuclei were detected after 4 hr of ischemia in the control, ActD-treated, or CHX-treated cultures (data not shown). After 6 hr of ischemia, many cells in the control culture were positively stained following TUNEL. In the ActD- and CHX-treated cultures, the number of TUNEL-positive nuclei after 6 hr of ischemia was lower than in the control.

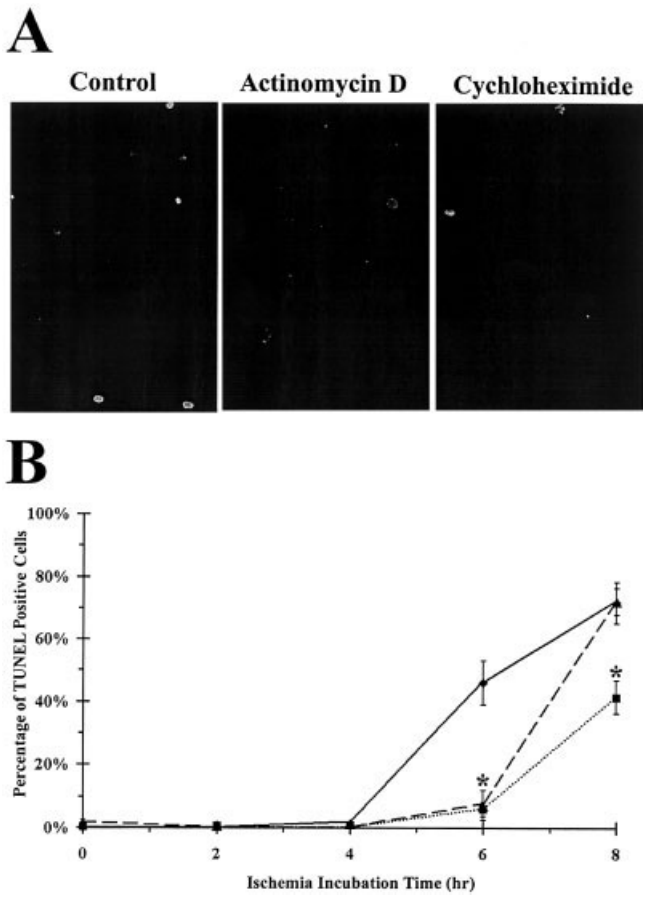

Fig. 2. TUNEL staining of cultured astrocytes. **A:** TUNEL staining of nuclei in control, ActD-pretreated (25 μg/ml), and CHX-pretreated (10 μg/ml) primary astrocytic cultures following 6 hr of ischemia. **B:** Percentage of TUNEL-positive cells in astrocytes culture following ischemia and in the presence of ActD and CHX. Circles, control; triangles, 25 μg/ml ActD; squares, 10 μg/ml CHX. The numbers of TUNEL-positive nuclei are expressed as a percentage of the total number of cells. Data represent means ± SEM from six separate experiments; nine fields per culture were counted. $^{\star}P \leq .05$ compared with the appropriate culture controls.

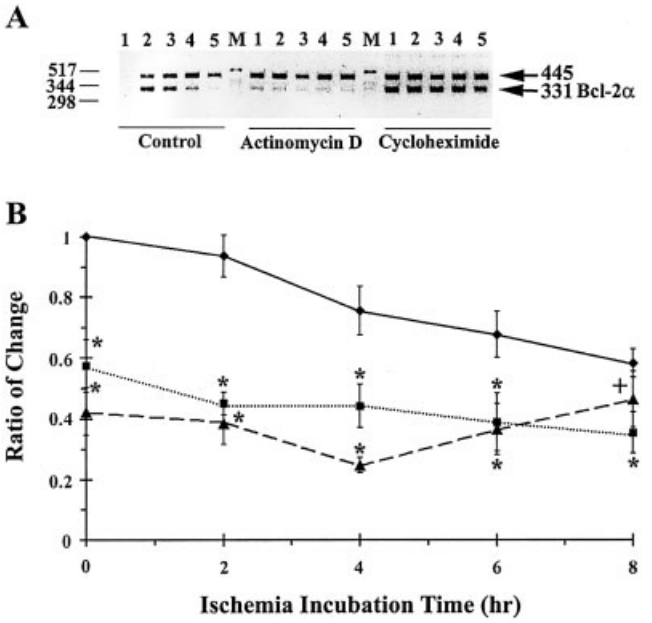

Fig. 3. Expression of bcl-2α in control (circles), 25 μg/ml ActD-treated (triangles), and 10 μg/ml CHX-treated (squares) astrocytes following different periods of ischemia. Quantitative RT-PCR was used to quantify gene transcription. **A:** Agarose gel analysis. M, 1-kb DNA marker; lanes 1, 0 hr (control); lanes 2, 2 hr of ischemia; lanes 3, 4 hr of ischemia; lanes 4, 6 hr of ischemia; lanes 5, 8 hr of ischemia. The labels 445 and 331 indicate the size of the 18S rRNA and bcl-2α PCR products, respectively. **B:** Statistical analysis. Data represent the mean ± SEM from six different experiments. $^{\star}P \leq .05$, $^{\dagger}P \leq .1$ compared with the expression observed in appropriate control cultures.

The number of TUNEL-positive nuclei was counted in the control, ActD-treated, and CHX-treated cultures (Fig. 2B). The total number of TUNEL-positive nuclei in representative samples was expressed as a percentage of the total number of cells. The data show that only 7.2% of ActD-treated cells were TUNEL-positive after 6 hr of ischemia. However, there was no significant difference between the number of TUNEL-positive nuclei in the control and ActD-treated cultures after 8 hr of ischemia. In contrast, the number of TUNEL-positive nuclei in CHX-treated cultures after 6 hr of ischemia was 6.9%, but this increased to 41.4% at 8 hr of ischemia.

Metabolic Inhibitors Alter the Expression Profile of Apoptosis-Related Genes

In the presence of ActD, the expression of bcl-2α under ischemia following preincubation with the inhibitor for 24 hr decreased to about 43% of the control value (Fig. 3B). The effect of ischemia on these pretreated astrocytes was studied. As the duration of ischemia increased, the expression of bcl-2α decreased in both the control and the inhibitor-treated cultures. After 8 hr of ischemia, bcl-2α expression in ActD-pretreated cultures was 12.2% lower than that in controls. After a 24-hr pretreatment with CHX, bcl-2α expression was reduced 58% compared with control cultures. After 8 hr of ischemia, bcl-2α expression was reduced 23% compared with controls (Fig. 3).

The expression of bcl-2β following various treatments is shown in Figure 4. Bcl-2β expression decreased to 19.1% of the control value after 24 hr of preincubation with ActD. After 8 hr of ischemia, bcl-2β expression was reduced to 40.7% of the control value. After 24 hr of pretreatment with CHX, bcl-2β expression decreased to 22.7% compared with control value. After 8 hr of ischemia, bcl-2β expression was reduced to 62.5% of the control value.

Figure 5 shows the expression of bax following various treatments. After a 24-hr preincubation with ActD, bax expression was reduced to 25.5% compared with controls. During 8 hr of ischemia, its expression did not change significantly and remained below the control value. After 8 hr of ischemia, bax expression was reduced

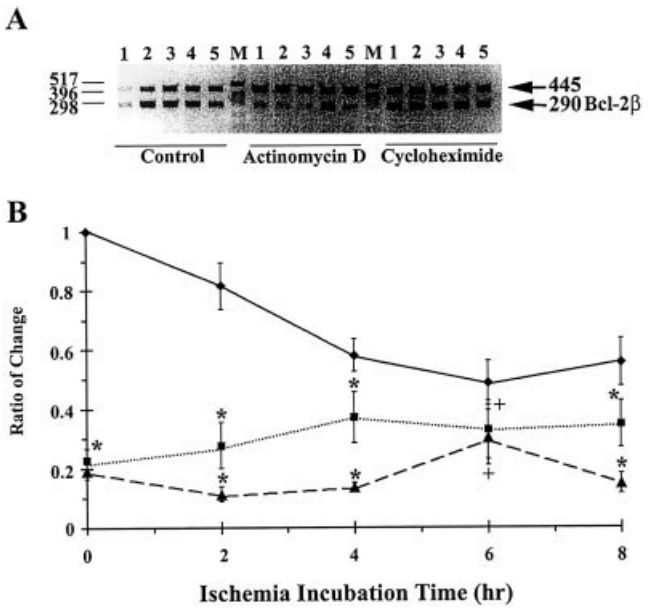

Fig. 4. Expression of bcl-2β in control (circles), 25 μg/ml ActD-treated (triangles), and 10 μg/ml CHX-treated (squares) astrocytes following different periods of ischemia incubation. Quantitative RT-PCR was used to quantify the gene transcription. **A:** Agarose gel analysis. M, 1-kb DNA marker; lanes 1, 0 hr of ischemia (control); lanes 2, 2 hr of ischemia; lanes 3, 4 hr of ischemia; lanes 4, 6 hr of ischemia; lanes 5, 8 hr of ischemia. The labels 445 and 290 indicate the size of the 18S rRNA and bcl-2β PCR products, respectively. **B:** Statistical analysis. Data represent the mean ± SEM from six different experiments. $^{\star}P \leq .05$ and $^{\dagger}P \leq .1$ compared with the expression observed in appropriate control cultures.

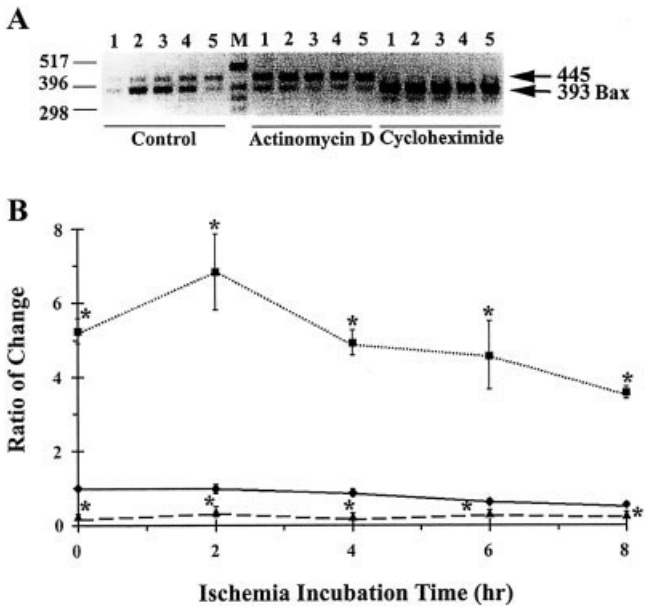

Fig. 5. Expression of bax in control (circles), 25 μg/ml ActD-treated (triangles), and 10 μg/ml CHX-treated (squares) astrocytes following different periods of ischemia incubation. Quantitative RT-PCR was used to quantify gene transcription. **A:** Agarose gel analysis. M, 1-kb DNA marker; lanes 1, 0 hr of ischemia (control); lanes 2, 2 hr of ischemia; lanes 3, 4 hr of ischemia; lanes 4, 6 hr of ischemia; lanes 5, 8 hr of ischemia. The labels 445 and 393 indicate the size of the 18S rRNA and bax PCR products, respectively. **B:** Statistical analysis. Data represent the mean ± SEM from six different experiments. $^{\star}P \leq .05$ compared with the expression observed in appropriate control cultures.

to 27.3% of the control value. However, 24 hr of pretreatment with CHX produced accumulation of bax gene transcripts relative to the control culture. After a 24-hr preincubation, bax expression increased to 524% of the control value. Bax expression fluctuated significantly between 0 and 4 hr of ischemia but remained greatly in excess of the control value. From 4 to 8 hr of ischemia, bax expression remained constant. The amount of bax expression after 8 hr of ischemia was significantly lower than the 0-hr-ischemia culture but still 303% greater than the control value. Bax expression was higher than that in the control throughout the 8-hr-ischemia incubation.

Figure 6 shows the variation in Ice expression in astrocyte cultures following various treatments. Figure 6A shows the electrophoretic separation of the PCR products on agarose gel, and Figure 6B quantifies the change in Ice gene expression. After 24 hr of preincubation with ActD, the amount of Ice produced by astrocytes was reduced to 23.9% compared with controls (Fig. 6). After a 24-hr pretreatment with ActD, expression of Ice was consistently lower than in the control throughout the 8-hr-ischemia incubation. After 8 hr of ischemia, Ice expression was reduced to 23.6% compared with the control culture, which was not significantly different from the preischemia level. CHX pretreatment for 24 hr produced an accumulation of Ice genes transcripts. Gene expression was elevated to 313% of the control value. The expression of Ice did not change significantly from 0 to 4 hr of ischemia but decreased significantly from 4 to 6 hr and remained at this level from 6 to 8 hr. The expression of Ice after 8 hr of ischemia was not significantly different from the control value (Fig. 6).

DISCUSSION

In vitro studies can only mimic ischemia as experienced in vivo. As the volume of medium bathing the cells was reduced, this created not just hypoxia but also substrate limitation, byproduct accumulation, and increases in biologically derived stressors. "In vitro ischemia" is a closer definition of the conditions experienced by the cultured cells than "simple" and/or "severe hypoxia." This terminology has been used and accepted widely in previously published accounts (Chen et al., 1996, 2003; Yu and Lau, 2000; Yu et al., 2001; Lau and Yu, 2001). Apoptosis is a process dependent on the synthesis of a variety of macromolecules (Wyllie et al., 1984). Inhibiting the synthesis of RNA or protein prevents or delays apoptosis following various apoptotic stimuli (Serghini et al., 1994; Schulz et al., 1996). In our in vitro studies on cultured astrocytes, cell death following ischemic insult could be delayed but not prevented following 24 hr of

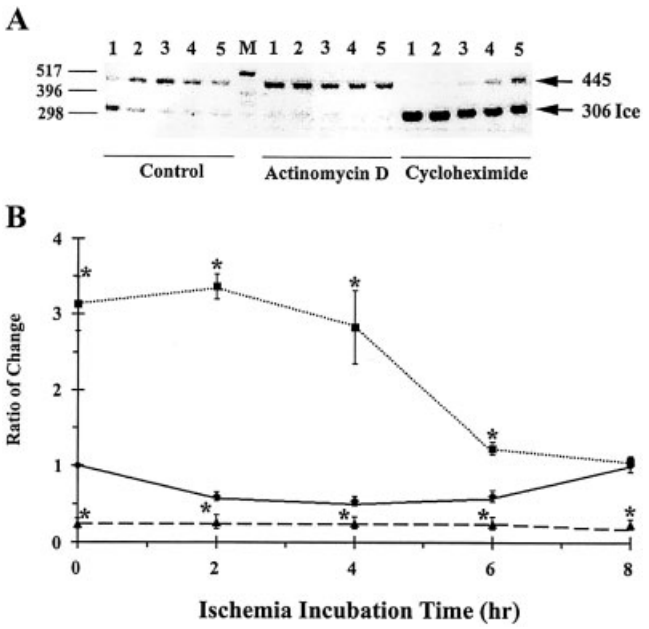

Fig. 6. Expression of Ice in control (circles), 25 μg/ml ActD-treated (triangles), and 10 μg/ml CHX-treated (squares) astrocytes following different periods of ischemia incubation. Quantitative RT-PCR was used to quantify gene transcription. **A:** Agarose gel analysis. M, 1-kb DNA marker; lanes 1, 0 hr of ischemia (control); lanes 2, 2 hr of ischemia; lanes 3, 4 hr of ischemia; lanes 4, 6 hr of ischemia; lanes 5, 8 hr of ischemia. The labels 445 and 306 indicate the size of the 18S rRNA and Ice PCR products, respectively. **B:** Statistical analysis. Data represent the mean ± SEM from six different experiments. $\star P \leq .05$ compared with the expression observed in appropriate control cultures.

pretreatment with either ActD or CHX. The protective effect of these inhibitors was most obvious after 6 hr and diminished after 8 hr of ischemia. Supporting data were reported following global ischemia in CA1 neurons treated with protein synthesis inhibitors (Chen et al., 1996).

The mechanism of the delay and continuation of apoptosis in cultured cells following treatment with a variety of effectors is unclear. The protective effect of ActD and CHX treatment against induction of apoptosis in cultures of astrocytes under ischemia was not observed in cultures treated for less than 24 hr (data not shown). A hypothesis involving constitutive expression of inactive "killer proteins" under normal physiological conditions is useful in this context. Caspases, the executors of apoptosis, exist as inactive zymogens under normal physiological conditions but are converted to an active form during apoptosis. The RNA and protein synthesis inhibitors ActD and CHX would block the synthesis of caspases such that their level might decrease after 24 hr of pretreatment. Existing intracellular pools of "killer proteins" would play their role in the induction of the cell death process as normal. However, preincubation with inhibitors would allow existing intracellular stores of killer proteins to degrade, enhancing the apparent protective effect. It was reported by Furukawa et al. (1997) that CHX (50–500 μM) had neuroprotective effects on hippocampal neurons that was not the result of inhibition of protein synthesis. No information related to the protection of astrocytes was provided. Tsuchida et al. (2002) reported that CHX (20 μg/ml) induced apoptosis in astrocytes. The mechanism for the dual effects of CHX is not understood. The CHX concentration (10 μg/ml) and preincubation period (24 hr) used in this study ensured that astrocyte protein synthesis was affected before the ischemic insult was applied. It also avoided the neuroprotective and apoptotic effects of CHX alluded to previously (Furukawa et al., 1997; Tsuchida et al., 2002). Thus, the effect of blocking protein synthesis could be elucidated in astrocytes during ischemia and apoptosis.

Delayed cell death may provide a window in which to prevent the death-mediated proteins, such as proteolytic proteases, from executing their actions in the cell death process. It is important to note that the ischemia model utilized in this study was a terminal-death model; i.e., the injury was continuous, was accumulative, and allowed no break for cells to recuperate. From the data presented here, it is not possible to determine whether the delay in cell death observed was a protective delay or merely a slowing of apoptosis. Returning the ischemic astrocytes to normal incubation conditions after 6–8 hr of ischemia could help to elucidate this.

The bcl-2 family plays regulatory roles in apoptosis. Ischemia affects the expression of bcl-2 family members' mRNA and protein in various tissues and cells (Gillardon et al., 1996; Isenmann et al., 1998; Kaushal et al., 1998). In our model, the expression of bcl-2α and bcl-2β mRNA was suppressed in astrocytes under ischemia. Insofar as bcl-2 is a critical antiapoptotic protein, the decrease in protective bcl-2 might be a critical factor in the release of cytochrome c (data not shown) in ischemic astrocytes. We have reported previously that MAPK/Erk inhibitors accelerate cell death and block Bcl-2 protein expression (Jiang et al., 2002). This evidence supports the idea that a decrease in Bcl-2 might be an important factor in ischemia-induced apoptosis. This supports a similar observation of suppressed bcl-2 mRNA expression in rat brain after global ischemia (Gillardon et al., 1996). The expression of Bcl-2 protein was not elevated in the lesion core of focal ischemic rat brain cortex (Isenmann et al., 1998).

The proapoptotic member of the bcl-2 family, Bax, has been shown to be elevated in CA1 neurons after global ischemia (Chen et al., 1996), indicating that it might contribute to apoptosis under ischemia. However, Bax has also been reported in nonvulnerable regions after global ischemia (Gillardon et al., 1996). This suggests a critical role for Bax under ischemia.

The expression of the apoptotic gene Ice was also suppressed under ischemia. Unusually, the expression of this gene returned to initial levels following an 8-hr-ischemia insult. The increase of Ice from 6 to 8 hr of ischemia relative to controls might be due to a decrease in

mRNA degradation, a reduction or termination of translation, or an increase in mRNA synthesis. Further studies will be required to clarify the mechanism of the Ice increase. In contrast, expressions of Ice mRNA and protein were induced in gerbil hippocampus after ischemia (Bhat et al., 1996). Ice does not act directly as an executor of programmed cell death. Its usual role is activation of other caspases from their zymogens, e.g., caspase 3 from procaspase 3 (Tewari et al., 1995). Because the intracellular amount or concentration of Ice was not measured in this study, the cause of the early decrease in Ice is unknown, but it might be due to the inhibition of translation of its gene into protein or a reduction of a protein that suppresses Ice translation. Alternatively, Ice mRNA may be degraded more rapidly under ischemia. The increase in Ice mRNA at a later stage of ischemia confirmed the previous observation of an increase in caspase 3 in ischemic astrocytes (Yu et al., 2001).

Pretreatment with both ActD and CHX decreased the expression of all members of the bcl-2 family that we examined compared with untreated samples, indicating the general effect of suppression of mRNA and protein synthesis. We found that, after ActD and CHX treatment, the Bcl-2 decrease during ischemia was not as evident as that without treatment. ActD and CHX treatment did not prevent the decrease of Bax and Ice during ischemia. Insofar as the overall effect of ActD and CHX treatment was protective, our data further suggest that Bcl-2, but not bax or Ice, plays a critical role in preventing ischemia-induced apoptosis.

It should be noted that treatment with inhibitors for either RNA or protein synthesis would have general effects and would not be confined to apoptotic cell death. It is the general consensus that reduced metabolic activity, as occurs during hibernation, for example, protects cells from hypoxia. In applying this general observation to the current study, the effects of ActD and CHX might be attributable not to the Bcl-2/Bax ratio but to other nonspecific changes in the cell.

It is hypothesized that the balance of proapoptotic (Bad, Bax) and antiapoptotic (Bcl-2, Bcl-Xl) elements determines apoptosis. During ischemia, Bcl-2 was decreased significantly, whereas Bax was not. The decrease in the Bcl-2/Bax ratio might be an important factor in ischemia-induced apoptosis. ActD and CHX treatments suppressed the expression of both bcl-2 and bax genes, and the total amount of Bcl-2 family genes was decreased. However, ActD and CHX prevented the decrease of Bcl-2 gene expression during ischemia. This suggests that the ratio of Bcl-2/Bax in astrocytes following ActD and CHX treatment does not decrease as much as with untreated cells during ischemia. Although these may not be the direct effects of ActD and CHX treatment, our data provide the first evidence that ischemia-induced apoptosis in astrocytes is a process involving RNA and protein synthesis; and the ratio of Bcl-2 family members might play a critical role in determining ischemia-induced apoptosis in astrocytes.

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