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Ischemia Preconditioning Protects Astrocytes From Ischemic Injury Through 14-3-3γ

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Stroke is a leading cause of death and disability, and new strategies are required to reduce neuronal injury and improve prognosis. Ischemia preconditioning (IPC) is an intrinsic phenomenon that protects cells from subsequent ischemic injury and might provide promising mechanisms for clinical treatment. In this study, primary astrocytes exhibited significantly less cell death than control when exposed to different durations of IPC (15, 30, 60, or 120 min). A 15-min duration was the most effective IPC to protect astrocytes from 8-hr-ischemia injury. The protective mechanisms of IPC involve the upregulation of protective proteins, including 14-3-3γ, and attenuation of malondialdehyde (MDA) content and ATP depletion. 14-3-3γ is an antiapoptotic intracellular protein that was significantly upregulated for up to 84 hr after IPC. In addition, IPC promoted activation of the c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK)−1/2, p38, and protein kinase B (Akt) signaling pathways. When JNK was specifically inhibited with SP600125, the upregulation of 14-3-3γ induced by IPC was almost completely abolished; however, there was no effect on ATP or MDA levels. This suggests that, even though both energy preservation and 14-3-3γ up-regulation were turned on by IPC, they were controlled by different pathways. The ERK1/2, p38, and Akt signaling pathways were not involved in the 14-3-3γ upregulation and energy preservation. These results indicate that IPC could protect astrocytes from ischemia injury by inducing 14-3-3γ and by alleviating energy depletion through different pathways, suggesting multiple protection of IPC and providing new insights into potential stroke therapies.

Key words: ATP; astrocytes; ischemia preconditioning; 14-3-3γ; JNK

Because of the short therapeutic time window for stroke treatment, more than 95% of stroke patients do not receive effective, successful treatment (Fonarow et al., 2011; Iadecola and Anrather, 2011). New and efficient interventions are required to extend the therapeutic time window for stroke patients to minimize the severity of any resulting disabilities. Ischemia preconditioning (IPC) is a promising candidate because exposure to short episodes of nonlethal ischemia protects against a subsequent fatal ischemia. IPC can induce ischemia tolerance, which confers robust neuroprotection against ischemia damage (Liu et al., 1992; Fan et al., 2011; Pan et al., 2014). Most studies of brain IPC have been limited to neuronal injury and protection; however, glial cells, in particular

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astrocytes, make up the majority of brain cells (Nedergaard and Dirnagl, 2005) and can resist greater amounts of ischemic damage than neurons (Trendelenburg and Dirnagl, 2005). To understand the mechanisms of IPC fully, astrocytes must also be studied. Upon activation during ischemia, astrocytes show enhanced expression of numerous protective proteins and factors (Li et al., 2008; Yu et al., 2008). Furthermore, astrocytes may also be activated under ischemia tolerance to protect themselves and/or other cells (Liu and Alkayed, 2005; Trendelenburg and Dirnagl, 2005). It should be emphasized that the role of astrocytes in IPC-induced ischemia tolerance has garnered increasing attention (Trendelenburg and Dirnagl, 2005; Li et al., 2008). The protective mechanisms of ischemia tolerance induced by IPC might provide new insights into future neural-protective clinical treatments.

IPC induces a complex series of protective mechanisms (Dirnagl et al., 2003) that includes slowing of ATP depletion, preservation of mitochondrial function (Murry et al., 1990; Dave et al., 2001; Brucklacher et al., 2002), and promotion of prosurvival pathways through induction of endogenous protective proteins (Rizvi et al., 1999; Dong et al., 2010), such as the highly conserved 14-3-3 proteins (Chen et al., 2007). The γ member of the 14-3-3 family exerts pleiotropic effects during various physiological processes, such as cell division and cell proliferation, and has an antiapoptotic role (Freeman and Morrison, 2011; Zhao et al., 2011; Shimada et al., 2013). We have previously demonstrated that, in astrocytes, 14-3-3γ is upregulated during ischemia through the c-Jun N-terminal kinase (JNK)/c-Jun/AP1 pathway and promotes cell survival by binding to p-Bad (Chen et al., 2005; Dong et al., 2009). In neurons, the 14-3-3γ/p-β-catenin Ser37/B-cell lymphoma 2-associated X protein (Bax) axis controls cell survival under ischemia injury (Lai et al., 2014). These findings suggest that 14-3-3γ is cytoprotective under ischemia and might also be involved in IPC's protective mechanisms.

From our primary astrocyte cultures, we establish an in vitro IPC model, detect how long the cells could resist ischemia damage after IPC, and measure mortality. We also show that 14-3-3γ is involved in IPC-induced neuroprotection and determine that the JNK but not the extracellular signal-related kinase (ERK)-1/2, p38, or protein kinase B (Akt) signaling pathway controls 14-3-3γ expression under IPC. ATP preservation and malondialdehyde (MDA) reduction, which were also induced by IPC to protect astrocytes, were controlled by different pathways but not the JNK pathway. Elucidation of the early induction mechanisms of 14-3-3γ upregulation and energy preservation under IPC could provide a crucial method to alleviate ischemia injury. Clarifying the signaling pathways responsible for 14-3-3γ upregulation could provide a therapy for patients who have a high risk of stroke by preactivating 14-3-3γ.

MATERIALS AND METHODS

Primary Astrocyte Culture

Primary cultures of cerebral cortical astrocytes were prepared from newborn ICR mice as described elsewhere (Dong et al., 2009; Zhou et al., 2010; Chai et al., 2013; Gao et al., 2013). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/Life Technologies, Grand Island, NY) containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) for the first 2 weeks and 7% (v/v) FBS for the next 2 weeks. All cultures were incubated at 37°C in 5%/95% CO_2/air and 95% humidity (Thermo Fisher Scientific, Waltham, MA).

Ischemia and IPC Treatments

Ischemia was induced as described elsewhere (Dong et al., 2009; Zhou et al., 2010; Chai et al., 2013). Ischemia medium was prepared from glucose/serum-free DMEM and equilibrated with anaerobic gas (85% N_2, 10% H_2, and 5% CO_2) for 30 min to displace the dissolved oxygen. The cultures were washed twice and incubated in 800 μl ischemia medium in a 35-mm culture dish. Cultures were subsequently wrapped with parafilm to prevent evaporation and were kept in an anaerobic chamber at 37°C.

For IPC, primary astrocyte cultures were incubated under transient sublethal ischemia (15, 30, 60, or 120 min) in an anaerobic chamber as described above. After IPC, the ischemia medium was removed, and the cultures were incubated with normal DMEM in the normal incubator for a designated after-IPC interval (1, 2, 6, or 12 hr). The cultures were then subjected to ischemia for a period long enough to cause obvious astrocytic death in control cell cultures (4, 6, and 8 hr) to measure the protective effects of IPC. Lactate dehydrogenase (LDH) release was measured in the cell medium to detect cell injury with the Promega diagnostic LDH reagent (Promega, Madison, WI).

Hoechst/Propidium Iodide Staining and Cell Counting

Cultures were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at room temperature. After cultures had been rewashed with PBS, 2 μg/ml Hoechst33342 (Sigma-Aldrich, St. Louis, MO) and 4 μg/ml propidium iodide (~~PI~~; Sigma-Aldrich) in PBS were added to each culture, and cultures were incubated for 15 min. The cultures were observed under a fluorescence microscope (Olympus, Tokyo, Japan). For quantification of cell survival, nine photographs of random visual fields from each culture were taken, and at least three cultures were chosen from each group. Quantification of cells was facilitated in Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Automated counting of cells was performed with the "analyze particles" function by specifying the color of stained nuclei. Hoechst33342 stained nuclei blue, whereas the nuclei of dead cells were stained red with PI.

Signaling Pathway Studies

U0126 (40 μM), SB203580 (40 μM), LY294002 (20 μM), and SP600125 (40 μM; Sigma-Aldrich) are inhibitors for ERK1/2, p38, PI3K/Akt, and JNK, respectively. Primary astrocyte cultures were washed twice and pretreated with serum-free DMEM with inhibitors for 30 min under normal conditions before IPC treatment.

Western Blotting

Astrocytes in primary culture, after specific incubation conditions, were lysed in radioimmunoprecipitation assay buffer

(50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 7.4). Cells were centrifuged at 12,000 rcf for 20 min, and the protein contents of the supernatants were determined spectrophotometrically in triplicate with the Lowry et al. (1951) method according to Chai et al. (2013). Briefly, total proteins were separated on 10% SDS-PAGE. After electrophoretic transfer onto PVDF membranes (GE Healthcare, Piscataway, NJ) and blocking with 5% bovine serum albumin solution, membranes were incubated with primary antibodies (1:1,000 for 14-3-3γ, p-ERK1/2, ERK1, ERK2 [Santa Cruz Biotechnology, Santa Cruz, CA], p-Akt-Ser473, Akt, p-p38-Thr180/Tyr182, p38, p-JNK-Thr183/Tyr185, and JNK [Cell Signaling Technology, Danvers, MA] and 1:5,000 for β-actin [Sigma-Aldrich]) at 4°C overnight. Goat anti-rabbit and goat anti-mouse secondary antibodies (Santa Cruz Biotechnology) were used at 1:2,000. The band densities were quantified in Quantity One software (Bio-Rad, Hercules, CA).

MDA and ATP Measurements

The MDA content of astrocytes was measured as an index of the amount of lipid peroxidation in the cell membrane and carried out according to Xu et al. (2009) and Yu et al. (1989). ATP levels were determined with an ATP assay kit (Beyotime, Beijing, China) via the luciferin/luciferase method. The standard curve was obtained with defined ATP concentrations from which the levels of ATP were calculated.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by one-way factorial ANOVA combined with Tukey's multiple-comparisons test or Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of IPC on Ischemia-Induced Changes in LDH Release

With primary astrocyte cultures, we investigated whether an IPC of 0, 15, 30, 60, or 120 min administered 1, 2, 6, or 12 hr before ischemia treatment (4, 6, or 8 hr) had any protective effects in our experimental model (Fig. 1A). In 0-min IPC groups, LDH release increased significantly to 43.5% at 4 hr of ischemia injury, further increased to 60.1% at 6 hr, and reached 71.2% at 8 hr. In the 1-hr after IPC group, a 15-min IPC caused significant reduction of LDH release from 26.3% at 4 hr to 47.8% at 8 hr of ischemia (Fig. 1B). After a 2-hr post-IPC interval, a 15-min IPC significantly decreased LDH release to 19.3% at 4 hr of ischemia and 50.6% at 8 hr of ischemia. Thirty minutes of IPC also caused a significant reductive effect at 4 hr (25.5%) and 8 hr (59.7%; Fig. 1C) of ischemia. After a 6-hr post-IPC interval, all four IPC times induced a significant decrease of LDH release. Fifteen minutes of IPC caused the most significant reduction of LDH release to 21.1% and 36.6% at 4 hr and 8 hr of ischemia, respectively. After a 30-min IPC, LDH release decreased to 23.4% at 4 hr and 40.1% at 8 hr. LDH release was significantly reduced (from 25.9% at 4 hr to 43.4% at 8 hr) in the 60-min IPC group, and it was reduced to 51.8% (8 hr) in the 120-min IPC group (Fig. 1D). After a 12-hr post-IPC interval, only 15 min of IPC decreased LDH release, to 25% at 4 hr and 51% at 8 hr; 30-, 60-, and 120-min IPC did not have protective effects under ischemia incubation (Fig. 1E). These data show that IPC protected astrocytes from ischemia-induced death in vitro. The 6-hr post-IPC interval, after which all four IPC (15, 30, 60, and 120 min) groups displayed obvious and consistent protection, was chosen to assess cell viability and energy metabolism.

Effects of IPC on Ischemia-Induced Morphological Changes and Cell Death

Cell morphological changes were observed, revealing the protective levels of different IPC treatments. After exposure to 4 hr of ischemia, the structure of astrocytes began to change; the nuclei were shrunken and cells had detached from the culture dish, as we have described previously (Yu et al., 2002). In contrast, astrocytes pretreated with IPC (15, 30, 60, or 120 min) exhibited no obvious structural changes or cell detachment under 4 hr of ischemia. Under 6 hr of ischemia, more than half of the cells lost their normal cellular structure. After IPC treatments (15, 30, 60, or 120 min), about 80% of the cells in the dish survived. Under 8 hr of ischemia, viable cells were further reduced to about 20% of control, whereas surviving cells were increased to about 50% after different IPC durations. It is noteworthy that, in the four different IPC groups, no significant differences in cellular morphology were observed (Fig. 2).

We next confirmed the protective effects of different IPC treatments with Hoechst/PI double staining and calculations of astrocytic mortality. Under 4 hr of ischemia, cultures pretreated with 15 or 30 min of IPC had less cell death (6.5% and 6.7%, respectively) compared with 0-min IPC (25.1%); 60 and 120 min of IPC also significantly reduced cell death to 10.9% and 10.5%, respectively (Fig. 3A,B). Under 6 hr of ischemia, 15, 30, 60, and 120 min of IPC all produced a marked attenuation of cell death (24.0%, 27.9%, 26.4%, and 26.2%, respectively) compared with 0-min IPC (55.7%; Fig. 3A,C). Under 8 hr of ischemia, 93.1% of astrocytes were PI positive and dead. Fifteen and thirty minutes of IPC significantly attenuated the cell death rate to 61.7% and 65.6%, respectively, and 60- and 120-min IPC reduced the rate to 69.7% and 68.9%, respectively (Fig. 3A,D).

Effects of IPC on 14-3-3γ Protein Expression

After 15 min of IPC, 14-3-3γ protein levels were significantly upregulated by 84.8% compared with control at 4 hr. 14-3-3γ continuously increased to a peak of expression at 12 hr (208.8% of control). The high expression of 14-3-3γ was maintained until 60 hr and then began to decrease. The upregulation of 14-3-3γ was still detectable at 84 hr after IPC (Fig. 4A). Thirty minutes of IPC resulted in a 200% increase in 14-3-3γ protein levels

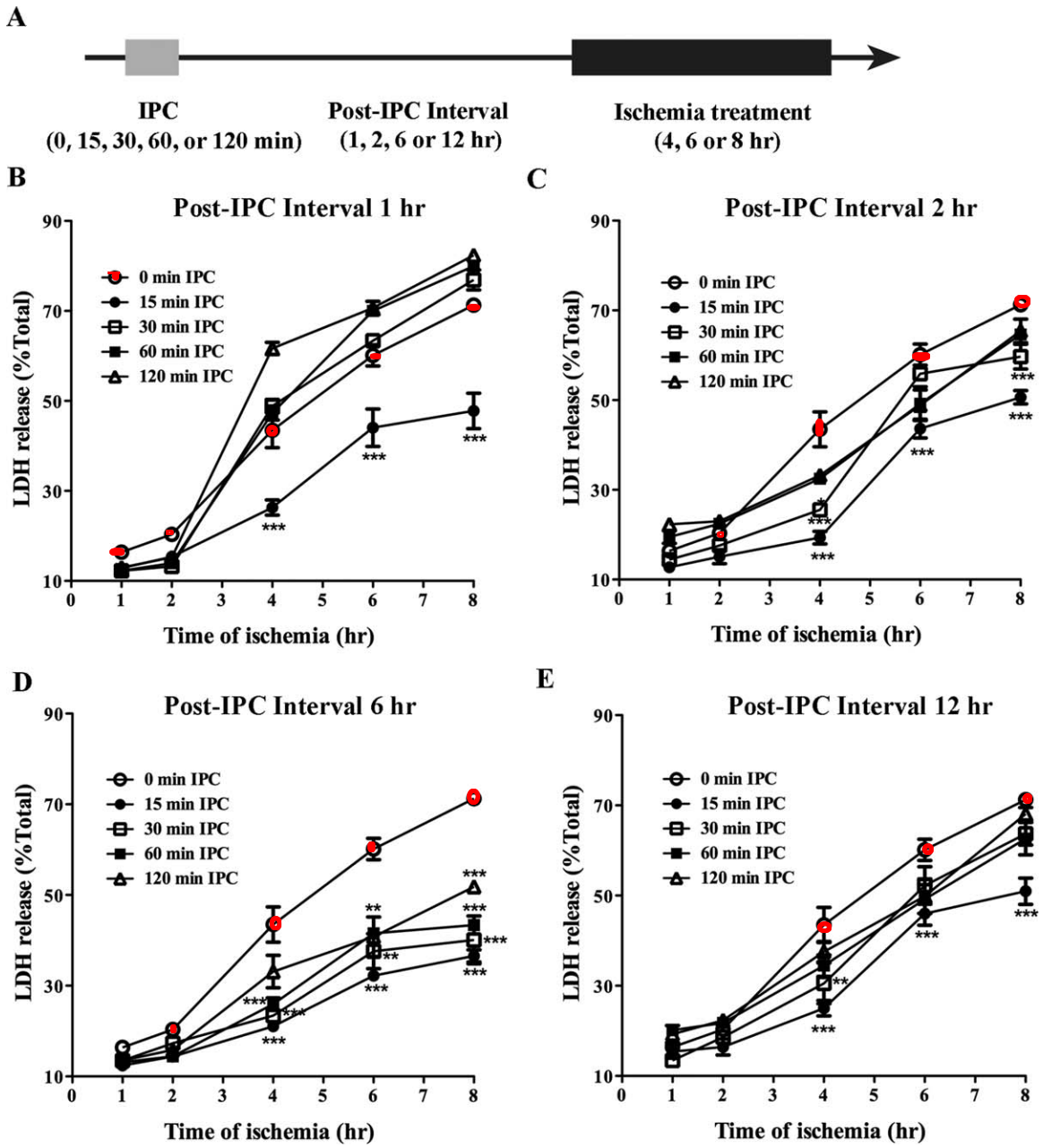

Fig. 1. Effects of IPC on ischemia-induced changes in LDH release. **A:** Time frame for IPC in primary astrocytic models. Cells were exposed to IPC (15, 30, 60, or 120 min), followed by a 1 (**B**)-, 2 (**C**)-, 6 (**D**)-, or 12 (**E**)-hr post-IPC interval before ischemia treatment (1, 2, 4, 6, or 8 hr). LDH release of each group was compared with the 0-min IPC group. Data are a ratio of control to LDH; n = 3. $^{**}P < 0.01$, $^{***}P < 0.001$.

at 6 hr, and the upregulation was attenuated but still significantly higher than control between 12 and 36 hr. At 48 hr, 14-3-3γ protein levels had increased by 192.2% of control and reached a second peak after 30 min of IPC stimuli (Fig. 4B). Sixty minutes of IPC induced a 99.8% increase in 14-3-3γ expression at 4 hr, and 14-3-3γ further increased to a peak at 36 hr (an increase of 212.3%; Fig. 4C). After 120 min of IPC, 14-3-3γ was increased by 97.3% at 6 hr and reached a 145.6% peak at 60 hr (Fig. 4D). There were no statistical differences in 14-3-3γ protein levels among the four IPC groups.

Effect of IPC on the Activities of the MAPK and PI3K/Akt Pathways

We previously have shown that the MAPK and PI3K/Akt pathways are activated with different temporal patterns and degrees of increase in ischemic astrocytes

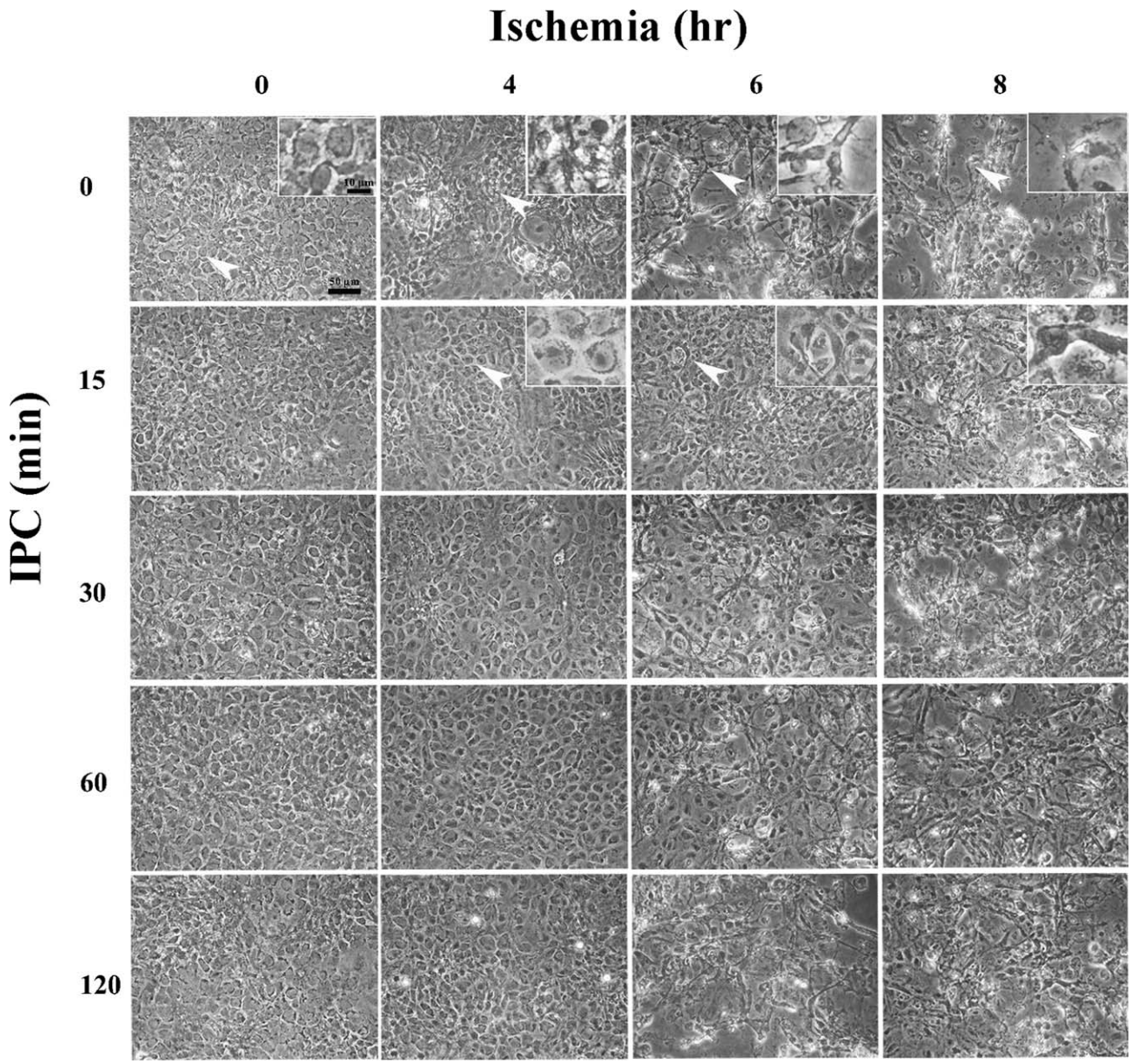

Fig. 2. Phase-contrast microscopy shows the effects of IPC on ischemia-induced cellular morphological changes. Cells pretreated with IPC (15, 30, 60, or 120 min) were compared with the normal control group and the 0-min IPC group. Arrowheads indicate sections that are shown enlarged in the top right corners of select panels. Scale bars = 50 μm; 10 μm in insets.

(Dong et al., 2009), and we have investigated whether either pathway was involved in the paradigm of IPC. JNK phosphorylation was increased significantly at 2 hr after 15 min of IPC (168.2%; Fig. 5A). At 4 hr, p-JNK had gradually increased to a peak of 230.2%. Between 6 and 8 hr, p-JNK remained significantly higher than control (205.1% vs. 197.0%; Fig. 5A). The level of p-ERK1/2 increased to 191.4% at 2 hr after 15 min of IPC and reached a peak at 4 hr (193.9%; Fig. 5B). The level of p-p38 did not differ significantly from the control level during the first 2 hr after IPC but had risen to 161% at 4 hr (Fig. 5C). The p-Akt level showed a slight transient decrease at 0 hr after IPC and then dramatically rose to 203% of control at 2 hr. The high level of p-Akt was maintained to 6 hr (196.6%) and then began to decrease gradually (Fig. 5D).

All four signaling pathways were activated after 15 min of IPC in astrocytes, although the time courses and degrees of increase were different. Over an 8-hr time frame, the activation of the JNK (from 2 to 8 hr) and the Akt (from 2 hr to 6 hr) pathways happened earlier than the activation of the ERK (4 hr) or the p38 (4 hr) signaling pathways, indicating that JNK and Akt might be involved in the early induction of 14-3-3γ.

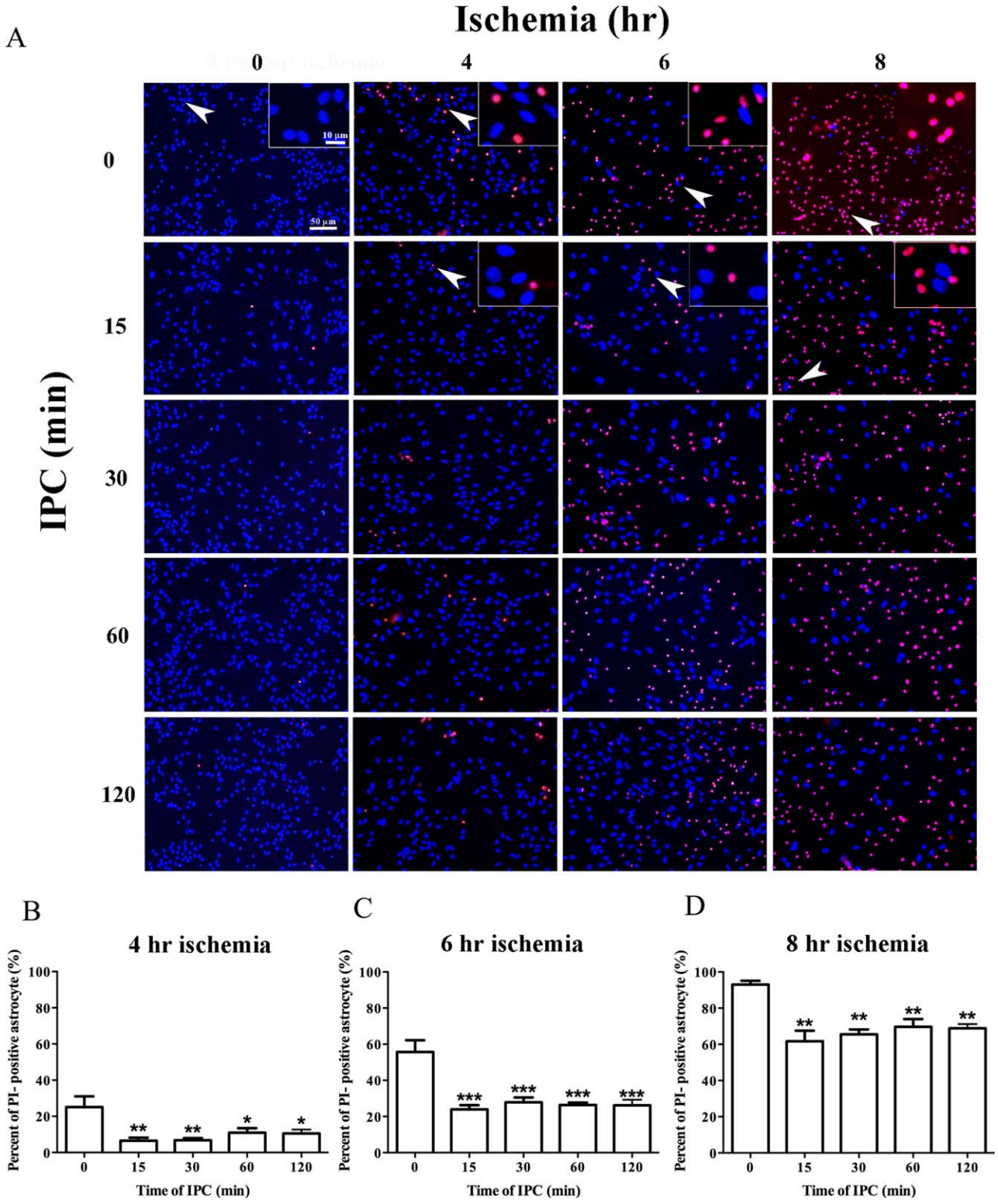

Fig. 3. Effects of IPC on ischemia-induced cell death. **A:** Cells pretreated with IPC (15, 30, 60, or 120 min) were compared with the normal control group and the 0-min IPC group. Arrowheads indicate sections that are shown enlarged in the top right corners of select panels. Percentages of PI-positive astrocytes under different IPC were counted after 4 (**B**), 6 (**C**), or 8 (**D**) hr of ischemia injury. The percentage of each group was compared with the 0-min IPC group; $n > 3$. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$. Scale bars = 50 μm; 10 μm in insets. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

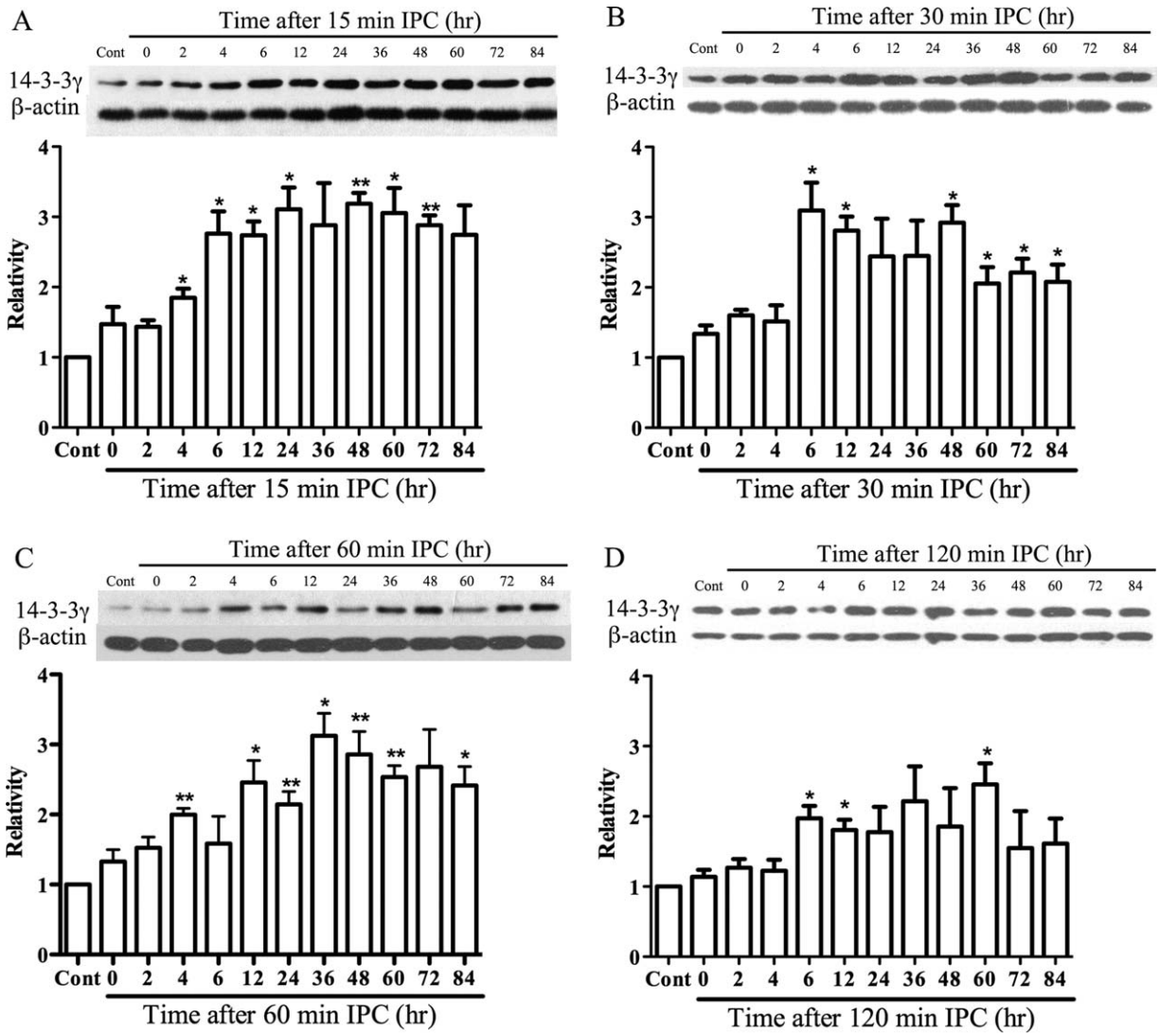

Fig. 4. 14-3-3γ changes after 15 (**A**)-, 30 (**B**)-, 60 (**C**)-, or 120 (**D**)-min IPC treatments. Protein levels of 14-3-3γ were detected until 84 hr after IPC treatments. The values of 14-3-3γ protein were normalized to β-actin and presented as a ratio of that to control; n = 3. $\star P < 0.05$, $\star\star P < 0.01$.

Relationship Between the MAPK and PI3K/Akt Pathway Activation and 14-3-3γ Expression

14-3-3γ protein levels in astrocytes were significantly upregulated after 15 min of IPC. Blocking ERK1/2 (U0126), p38 (SB203580), or PI3K/Akt (LY294002) did not affect the IPC-induced 14-3-3γ protein levels (Fig. 6A). SP600125, an inhibitor of JNK, caused an inhibition of 14-3-3γ protein elevation to control levels (Fig. 6B). This result suggests that the JNK pathway was responsible for the IPC-induced 14-3-3γ upregulation in astrocytes.

Effects of IPC on MDA Content and ATP Concentration

Under 4 hr of ischemia, the MDA content was significantly increased to 5.95 μmol/g protein (0-min IPC) from the normal level (1.01 μmol/g) protein. After 15 and 30 min of IPC, the MDA content was significantly lower, 1.49 and 1.76 μmol/g protein, respectively, whereas 60 and 120 min of IPC attenuated the MDA content to 2.64 and 2.71 μmol/g protein, respectively. To determine whether the JNK-induced upregulation of 14-3-3γ affected the MDA levels, we blocked the JNK pathway (with SP600125); however, there was no effect on MDA content under IPC. Inhibition of ERK1/2, p38, or PI3K/Akt also had no effect on MDA content (Fig. 7A).

Under 3 hr of ischemia, the ATP concentration decreased significantly to 20.53 μmol/liter from the normal level (34.38 μmol/liter). Fifteen or thirty minutes of IPC significantly prevented the decline of ATP concentrations that were induced by ischemia to 30.16 and 26.24 μmol/liter, respectively. After 60 and 120 min of IPC, the depletion of ATP concentrations was significantly alleviated to 26.21 and 27.08 μmol/liter, respectively.

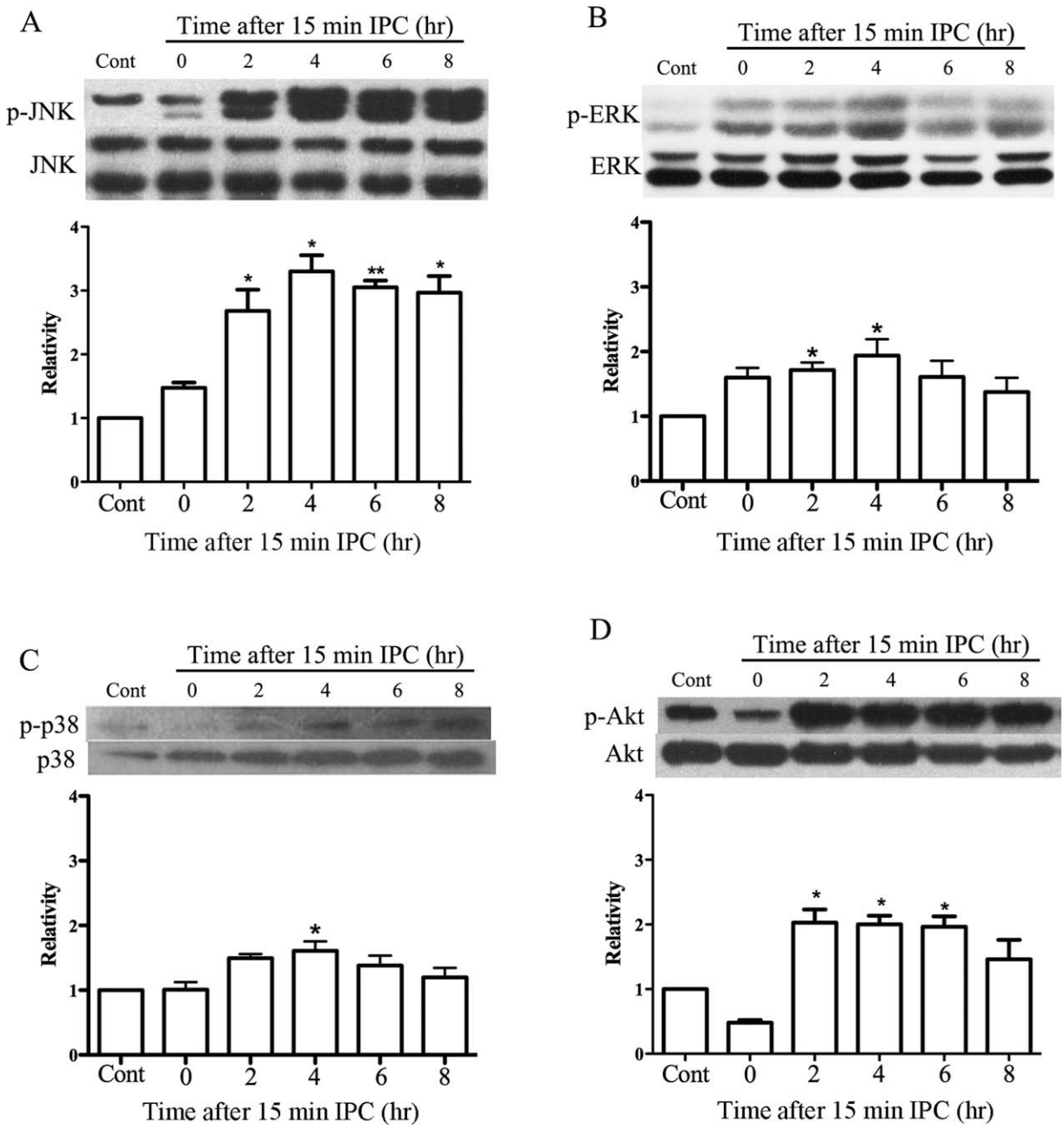

Fig. 5. The activation of JNK, ERK, p38, and Akt after 15 min of IPC treatment. **A:** Representative results of the Western blots (top). The values of p-JNK were normalized with the total JNK and presented as a ratio of that to control (bottom). **B:** ERK activation. **C:** p38 activation. **D:** Akt activation; $n \geq 3$. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$.

Inhibition of the JNK pathway by SP600125 did not have any effect on ATP preservation induced by IPC. Blocking the ERK1/2, p38, or PI3K/Akt pathways also did not have any effect on the ATP concentration (Fig. 7B). Under 4 hr of ischemia, 15, 30, 60, or 120 min of IPC also significantly alleviated ATP depletion to 13.52, 17.79, 13.28, and 13.52 μmol/liter, respectively, from 8.99 μmol/liter (0-min IPC; data not shown).

DISCUSSION

This study establishes an in vitro astrocytic model of IPC in which mouse cortical astrocytes preconditioned with a sublethal exposure to ischemia were rendered resistant to injury induced by a subsequent longer exposure to ischemia. All four IPC times utilized (15, 30, 60, and 120 min) appeared to have protective effects during subsequent ischemia injury, and 15 min of IPC provided the most

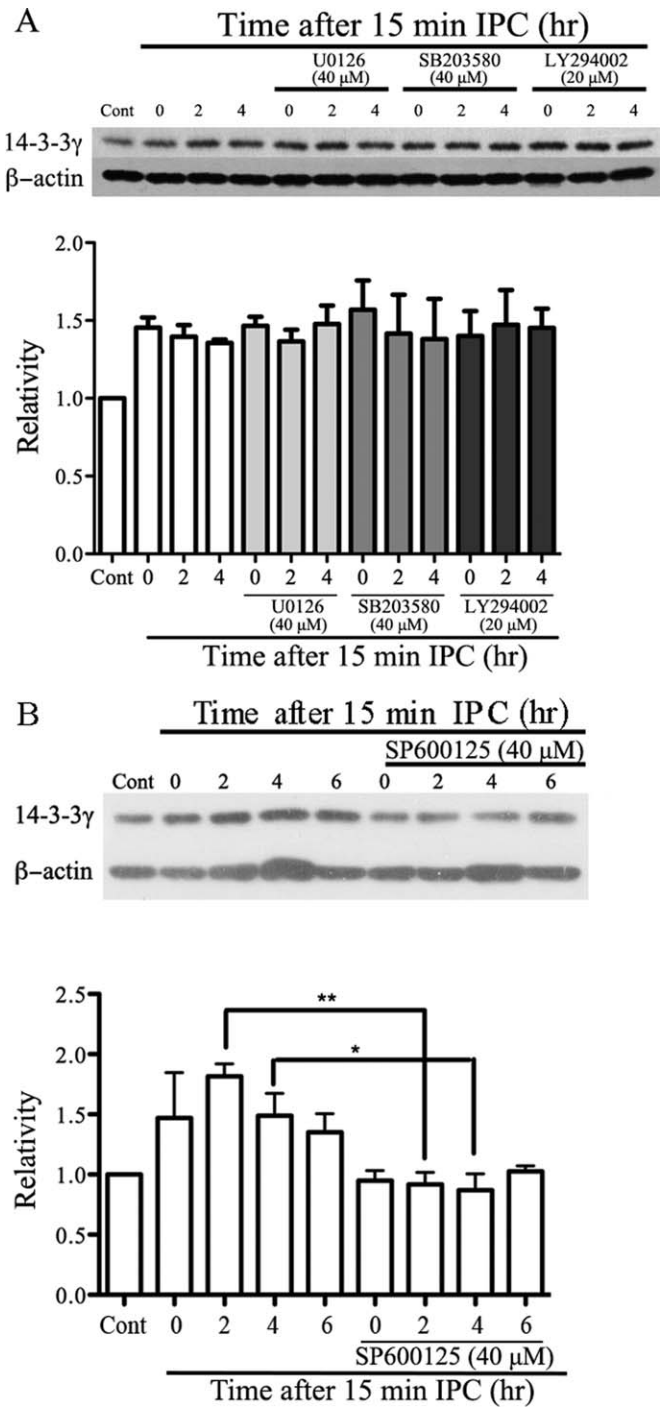

Fig. 6. Effects of PI3K/Akt and MAPK pathway inhibitors on IPC-induced 14-3-3γ upregulation in astrocytes. **A:** 14-3-3γ protein levels under IPC show no observable inhibition with U0126 (ERK inhibitor), SB203580 (p38 inhibitor), or LY294002 (Akt inhibitor). **B:** However, SP600125 (JNK inhibitor) inhibits IPC-induced upregulation of the 14-3-3γ protein. The values of 14-3-3γ were normalized to β-actin and presented as a ratio of that to control; $n \geq 3$. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$.

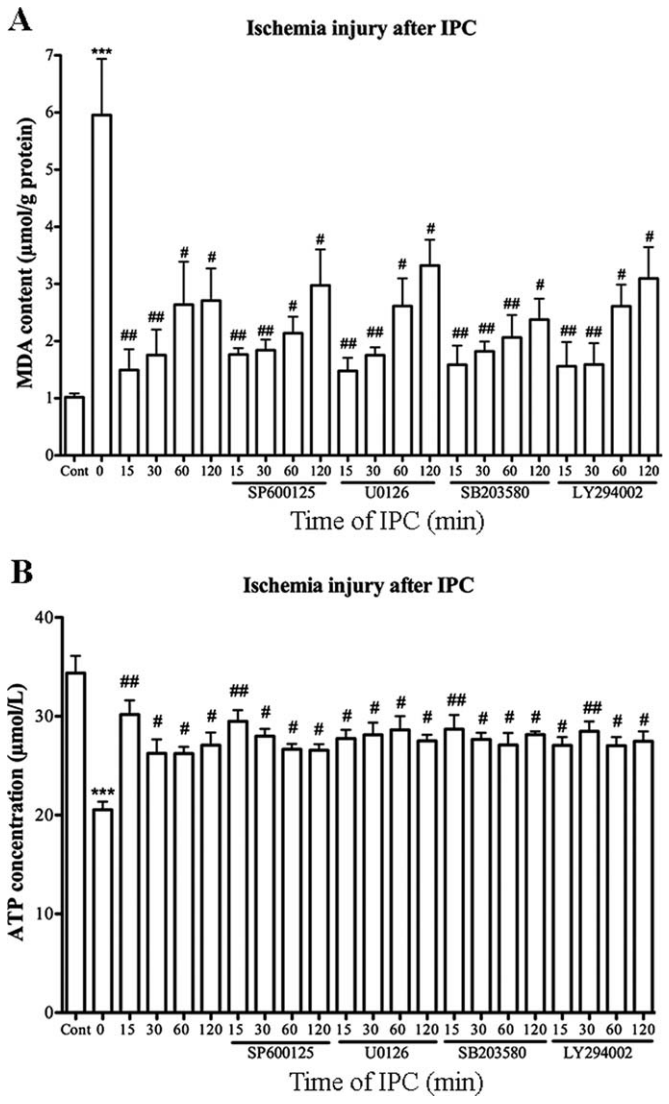

Fig. 7. Effects of IPC on ischemia-induced changes in MDA content and ATP concentration. **A:** Changes of MDA content under 4-hr ischemia with different IPC durations. **B:** Changes of ATP concentration under 3-hr ischemia with different IPC durations. SP600125 (JNK inhibitor), U0126 (ERK inhibitor), SB203580 (p38 inhibitor), or LY294002 (Akt inhibitor) were used to detect the roles of different signaling pathways. Control groups were the normal conditions for cells; 0-, 15-, 30-, 60-, and 120-min IPC groups were subjected to ischemia injury. Zero-minute IPC groups were compared with the control groups. $^{\star\star\star}P < 0.001$. Changes of 15-, 30-, 60-, or 120-min IPC were compared with the 0-min IPC group; $n \geq 3$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

significant protection. Other researchers have found that sublethal 60-min oxygen–glucose deprivation (OGD) preconditioning protected astrocytes from subsequent OGD-induced injury (Pan et al., 2014), which supports our results. The ischemia model in the current study, which might be more similar to physiological ischemia (Yu et al., 1995; Chen et al., 2003, 2005; Dong et al., 2009), caused 95% astrocytic death at 8 hr, whereas the 12-hr OGD model reduced cell viability by 40%. These

differences in viability might explain why the most effective IPC time that we found was different from other in vitro OGD models.

The post-IPC interval between IPC and ischemia treatment is important in determining the amount of protection offered by the IPC. In vivo and neuronal culture studies have shown that there is rapid and delayed ischemia tolerance after IPC (Dong et al., 2010). Rapid ischemia tolerance develops within minutes after the IPC; this is followed by an unprotected period and then a delayed ischemia tolerance that can last for several days (Liu et al., 1992; Cardenas et al., 2002). The unprotected period in vivo and in neuronal cultures appears between several and 24 hr after IPC (Stetler et al., 2014). In our in vitro astrocytic model, IPC had protective effects between 6 and 12 hr, and a 6-hr post-IPC interval was the best interval for cell recovery, implying that, after IPC, astrocytes might have a longer tolerance time to resist ischemia injury. This study also demonstrates that, under ischemia, cells survive more than 4 hr under 15 min of IPC in in vitro experiments, which suggests that the mechanistic information obtained from these and future IPC studies might lead to new therapeutic approaches for clinical interventions.

Protection develops slowly over hours after IPC, indicating that the gene expression of protective proteins might be involved (Grabb and Choi, 1999). 14-3-3γ is a multifunctional protein that has been shown to be important in promoting cell survival under ischemia (Pirim, 1998; Jang et al., 2009). It also serves as a general survival protein by enhancing prosurvival signaling while suppressing proapoptotic pathways under ischemia (Chen et al., 2005; Dong et al., 2009; Lai et al., 2014). 14-3-3γ is expressed mainly in the brain and was originally thought to exist only in neurons; however, we previously have demonstrated that 14-3-3γ is also expressed in primary cortical astrocytes (Chen and Yu, 2002). Our in vitro ischemia model in astrocytes demonstrates that the expression of 14-3-3γ mRNA and protein, but not those of other subtypes of the 14-3-3 family, increases under ischemia, indicating that the γ subtype is specifically upregulated to protect astrocytes (Yu et al., 2001; Chen et al., 2003). Other insults, such as heat shock and scratch wound, do not upregulate the expression of 14-3-3γ (Chen et al., 2003). The current results in cultured astrocytes may not completely reflect what would happen to 14-3-3 levels in vivo or if multiple cell types were present. However, in human cerebrovascular ischemic lesions, 14-3-3 proteins are also upregulated in astrocytes in the infarct lesions and are particularly abundant in infarcts at the chronic stage (Kawamoto et al., 2006). Moreover, in vitro and in vivo studies in neurons have shown that 14-3-3γ is upregulated in OGD and in middle cerebral artery occlusion models and prevents cell death (Lai et al., 2014). Furthermore, we found that increased 14-3-3γ promoted cell survival by binding to p-Bad and kept Bad from entering mitochondria to induce apoptosis (Chen et al., 2005). In addition to the protection observed during ischemia, 14-3-3γ might also be induced as an intrinsic protective factor for cell survival under IPC. In the spinal cord, IPC suppresses the dissociation of ASK1 from 14-3-3 proteins induced by ischemia and prevents apoptotic cascades (Yang et al., 2008). IPC also plays a neuroprotective role in rat hippocampus by decreasing 14-3-3 protein phosphorylation and Bax translocation to mitochondria (Du et al., 2009). In cardiomyocytes, 14-3-3 proteins are induced by anoxic preconditioning (APC) and play a cardioprotective role (Chen et al., 2007). However, which subtype of protective 14-3-3 protein is induced by IPC in astrocytes and the pathways controlling its protective effects have not been clarified. We found that IPC dramatically enhanced 14-3-3γ expression in astrocytes in parallel with a reduction of LDH release, increased cell viability, and energy metabolism changes. Our results suggest that the protection of IPC could be associated with apoptosis suppression by 14-3-3γ in cerebral cortical astrocytes. 14-3-3 proteins are involved in the protective effects of IPC in different kinds of cells. In astrocytes, the γ subtype is upregulated to protect from ischemia injury, indicating that clarifying the protective effects of specific 14-3-3 subtypes in different cell types might provide more effective treatments for ischemia.

The MAPK family (JNK, ERK1/2, and p38) and the PI3K/Akt pathway control a diverse array of cellular processes implicated in cell growth and survival (Hausenloy and Yellon, 2006). We previously have demonstrated that the JNK signaling pathway plays a critical role in the upregulation of 14-3-3γ under ischemia incubation in primary astrocytes (Dong et al., 2009). During IPC, activation of the PI3K/Akt pathway during the preconditioning phase was first studied in the heart, and the cardioprotective effects of IPC are partially eliminated when PI3K/Akt is blocked (Tong et al., 2000). In cortical neurons, activation of ERK1/2 is induced by IPC and results in neuroprotection (Kim et al., 2010). Inactivation of the p38 pathway abrogates the protective effects of IPC on CA1 neurons (Guan et al., 2014). In our primary astrocyte cultures, the JNK, ERK1/2, p38, and Akt pathways were all activated after IPC, suggesting that IPC stimulates not only one but multiple pathways and that the different pathways might turn on different protective mechanisms. JNK and Akt were activated early from 2 hr after IPC, indicating that they might be related to the early upregulation of 14-3-3γ. Subsequently, we found that only the JNK pathway but not ERK1/2, p38, or Akt took part in 14-3-3γ upregulation in primary astrocyte cultures under IPC. During hydrogen peroxide preconditioning, another type of preconditioning, in PC12 cells the ERK and the p38 pathways but not JNK were involved in 14-3-3 protein upregulation (Su et al., 2008), whereas in cardiomyocytes 14-3-3 protein induction by APC might involve activation of the ERK1/2 signaling pathway (Chen et al., 2007). This implies that 14-3-3 proteins could be induced by IPC to protect different tissues but through activating different signaling pathways. Clarifying these upregulating 14-3-3 pathways during IPC could reveal the differences in cellular protection that occur in different cell types and allow more effective protection from ischemia injury.

Although many cellular and molecular pathways or mechanisms induced by IPC have been identified, it is believed that IPC might exist in some unknown cell types and turn on as yet undiscovered protective cascades (Trendelenburg and Dirgnal, 2005). Cells cannot generate energy without oxygen and glucose, and a major cause of cell death during ischemia is a failure to generate sufficient energy. As early as 1964 it was known that short periods of global hypoxia can preserve brain energy metabolism during longer hypoxia (Dahl and Balfour, 1964). Subsequent studies have demonstrated that improved preservation of energy metabolism and mitochondrial function might be involved in the protective mechanism of IPC (Dave et al., 2001; Liu et al., 2002). Here, the results show that, in primary astrocyte cultures, IPC significantly reduces MDA content and alleviates ATP depletion, indicating that preserving energy metabolism is another important mechanism by which IPC attenuates ischemia injury. Because IPC stimulated 14-3-3γ upregulation through JNK, it was important to clarify whether IPC also preserved energy metabolism by activating the same signaling pathway. SP600125, an inhibitor of the JNK pathway, did not have any effect on MDA or ATP under IPC, indicating that IPC preserved energy and induced protective proteins through different signaling pathways. ERK1/2, p38, and Akt also did not take part in MDA or ATP changes during IPC. Other pathways involved in energy metabolism during IPC include protein kinase C (PKC)-α and PKC-ε; PKC-ε was upstream and PKC-α was downstream of mito-KATP channels in the signaling transduction (Hassouna et al., 2004). In addition, during IPC the phosphatase and tension homolog/phosphodiesterase 4 signaling pathway is also activated and inhibits the mitochondrial permeability transition pore opening to resist cell ischemia–reperfusion injury (Zheng et al., 2014). These results suggest that multiple pathways are utilized by IPC to acquire its protective effects, providing redundancy in case any of the pathways is absent or damaged.

In conclusion, this study demonstrates that IPC reduces the cell injury induced by ischemia in primary astrocytes and reveals that the most effective IPC time is 15 min. This study also provides evidence that IPC turns on multiple pathways to play protective roles while inducing 14-3-3γ through the JNK signaling pathway and preserving energy metabolism through other pathways. Therefore, the use of drugs or other therapies to upregulate 14-3-3γ and alleviate energy depletion could serve as a prophylactic treatment to mimic the protection of IPC and extend the therapeutic time window, which would provide more opportunities for stroke patients to receive effective treatments.

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