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Ischemia activates JNK/c-Jun/AP-1 pathway to up-regulate 14-3-3γ in astrocyte

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Abstract

Ischemia occurs in the brain as the result of stroke and other related injuries and few therapies are effective. If more is understood then potential treatments could be investigated. It was previously reported that 14-3-3γ could be up-regulated by ischemia in astrocyte to protect cells from ischemia-induced apoptosis. In this study, we attempted to uncover the mechanism responsible for this 14-3-3γ up-regulation in primary culture of astrocytes under ischemic-like conditions. It was found that *in vitro* ischemia may activate PI3K/Akt and MAPK signaling pathways. Astrocyte cultures were treated with LY294002 (PI3K inhibitor), U0126 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor). Only SP600125 could inhibit the ischemia-induced 14-3-3γ up-regulation in astrocytes. At the same time, we observed an ischemia-induced nuclear translocation of p-c-Jun, a major downstream component of JNK. Inhibition of AP-1 with curcumin also inhibited 14-3-3γ up-regulation indicating that ischemia-induced up-regulation of 14-3-3γ in astrocyte involves activation of the JNK/p-c-Jun/AP-1 pathway.

Keywords: AP-1, astrocytes, c-Jun, JNK, ischemia, 14-3-3γ.

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The 14-3-3 family is a group of acidic proteins originally identified by Moore and Perez (1967). 14-3-3γ is one of the seven subtypes (β, ε, η, γ, σ, τ, and ζ) and was thought to be expressed mainly in neurons of the CNS (Aitken *et al.* 1992; Watanabe *et al.* 1993). Others have shown elevated 14-3-3γ protein levels in several brain regions of patients with Alzheimer's disease and Down's syndrome (Burkhard *et al.* 2001), and in the cerebrospinal fluid in patients with Creutzfeldt–Jakob disease (Wiltfang *et al.* 1999), indicating a possible involvement of 14-3-3γ in neurodegenerative diseases. Interestingly we previous showed that among the five subtypes of 14-3-3 (γ, β, ε, η, and ζ) expressed in the CNS, ischemia would specifically induce an up-regulation of 14-3-3γ in astrocytes (Chen *et al.* 2003), indicating that 14-3-3γ was not neuronal specific. These observations were confirmed in human brain by Kawamoto *et al.* (2006). We also showed 14-3-3γ being neuroprotective and crucial in protecting astrocytes from ischemic induced apoptosis with the mechanism through binding to p-112 Bad to prevent Bad from getting into mitochondria (Chen *et al.* 2005).

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Abbreviations used: PI3K, Phosphoinositide 3-kinase; ERK, Extracellular signal-regulated kinase; JNK, c-Jun NH(2)-terminal kinase; AP-1, activator protein 1; SAPK/JNK, Stress-activated protein kinase/c-Jun NH(2)-terminal kinase.

The failure of many therapies for CNS ischemic diseases including stroke is because of the lack of effective means to prevent cell death, neuronal and astrocytic. A considerable body of evidence supports a hypothesis of 'ischemic tolerance'–a condition of transiently increased resistance to ischemic injury as a result of the activation of innate endogenous protective mechanisms. Up-regulation of 14-3-3γ in astrocyte under ischemia appears to be early (Yu *et al.* 2001). Hence, it is interesting to elucidate the mechanism for this endogenous neuroprotective protein being regulated, especially in the early stage of ischemia insult. Autieri (2004) reported that 14-3-3γ in human vascular smooth muscle cells could be up-regulated by fetal bovine serum, platelet-derived growth factor and T-cell conditioned media. Yet, no investigation on its regulatory mechanism has been reported.

Here we try to elucidate which signaling pathways are involved in the up-regulation of 14-3-3γ in primary cultured astrocytes under an ischemia conditions. These are essential for understanding regulation and may provide some new insight in ischemia tolerance and the potential in development of novel therapeutic strategies.

Materials and methods

Cerebral cortical astrocytes primary cultures were prepared from newborn Institute of Cancer Research mice according to Yu *et al.* (2007).

In vitro ischemia was induced using an anaerobic chamber (model 1029, Forma Scientific, Marietta, OH, USA) (Yu and Lau 2000). The cultures were saturated with 85% N_2/10% H_2/5% CO_2, with glucose/oxygen-free Dulbecco's modified Eagle's medium. The extracellular incubation medium volume is reduced to only 0.8 mL for a 35-mm culture dish during the ischemia incubation to create the accumulation of toxic metabolites, an important component contributing to physiological ischemia (Yu *et al.* 1995). The controls were incubated in 2 mL serum-free Dulbecco's modified Eagle's medium under normoxia. All results were obtained from three to four cultures repeated two to three times.

LY294002, U0126, SB203580, SP600125 (Promega, Madison, WI, USA) and curcumin (Sigma Chemicals, St Louis, MO, USA) were phosphorylation inhibitors for Phosphoinositide 3-kinase (PI3K), Extracellular singal-related kinase (ERK), p38, c-Jun NH(2)-terminal kinase (JNK), and activator protein 1 (AP-1), respectively. All cultures were pretreated with inhibitors for 30 min under normal condition before ischemia treatment.

Total RNA was extracted by TRIzol reagent according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). For RT-PCR, RNA was reverse-transcribed using Moloney Murine Leukemia Virus (M-MLV) Reverse-Transcriptase (Promega) and random primers (Invitrogen Corporation). cDNA was amplified by PCR (Promega). Forward and reverse primers and PCR were *18S rRNA*: 5′-AGTACGCACGGCCGGTACAGTG-3′ and 5′-GGGTC-GGGAGTGGGTAATTTGC-3′; *14-3-3γ*: 5′-GTTGGTCTG-GCTCTTCATCAT-3′ and 5′-AGGTGCAGAGTAGACTTG-GGTG-3′ (28 cycles; annealing temperature 59°C). *18S rRNA* was used as internal control. Semi-quantification was performed with TotalLab software (v. 201; Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

Protein contents were determined photometrically in triplicate samples using Lowry's method (Lowry *et al.* 1951). Western blot results were expressed as a ratio of 14-3-3γ to β-actin. The same membrane was probed with antibodies to total ERK and Akt after stripping.

Cytoplasmic and nuclear proteins were extracted according to Griffin *et al.* (1967) and Hattori *et al.* (1990). Cultures of astrocytes were incubated in 100 μL hypotonic buffer for 15 min. Then centrifuged at 10 000 *g* for 5 min at 4°C, where the supernatant was the cytoplasm components. The collected nuclear pellet was incubated in 50 μL dialysis buffer on ice for 30 min, then centrifuge in 12 000 *g*, 4 min, 4°C to collect the supernatant as nuclear protein.

Statistical analysis was performed by one way factorial analysis of variance test for all comparison pairs (ANOVA). Results were expressed as mean ± SEM with p-values < 0.05 considered significant.

Results

Up-regulation of 14-3-3γ was previously reported (Chen *et al.* 2003). Here, we measured the changes of mRNA in astrocytes at 2 h of ischemia and protein at 4 h of ischemia in the following studies.

Effect of Ischemia on PI3K/Akt and MAPKs Pathways

Akt, ERK1/2, p38, and JNK were all detected in astrocytes under ischemia conditions by western blot analysis. The level of total Akt did not change throughout the 4 h ischemia incubation (Fig. 1a). Level of phosphorylated Akt (p-Akt) was low in the control. Between 1 and 4 h of ischemia, the p-Akt/Akt ratio gradually increased and reached 1.75 times of the control at 4 h.

The level of total ERK1/2 did not change but p-ERK1/2 began to increase after 0.5 h ischemia and it continued to increase until at 2 h, it was three times the control where it remained this high (Fig. 1b). The ratio of p-ERK1/2 to total ERK1/2 also increased gradually and was significantly higher than control at 2 and 4 h.

The level of total p38 in astrocytes remained unchanged throughout the 4 h ischemia incubation. The level of p-p38 began to increase after 1 h ischemia but did not reach any statistical significant level. The ratio increased gradually and reached a significant higher level of 2.72 times of the control by 4 h of ischemia (Fig. 1c).

The total JNK level showed a slight transient rise between 0.5 and 1 h of ischemia (Fig. 1d). However, the level of

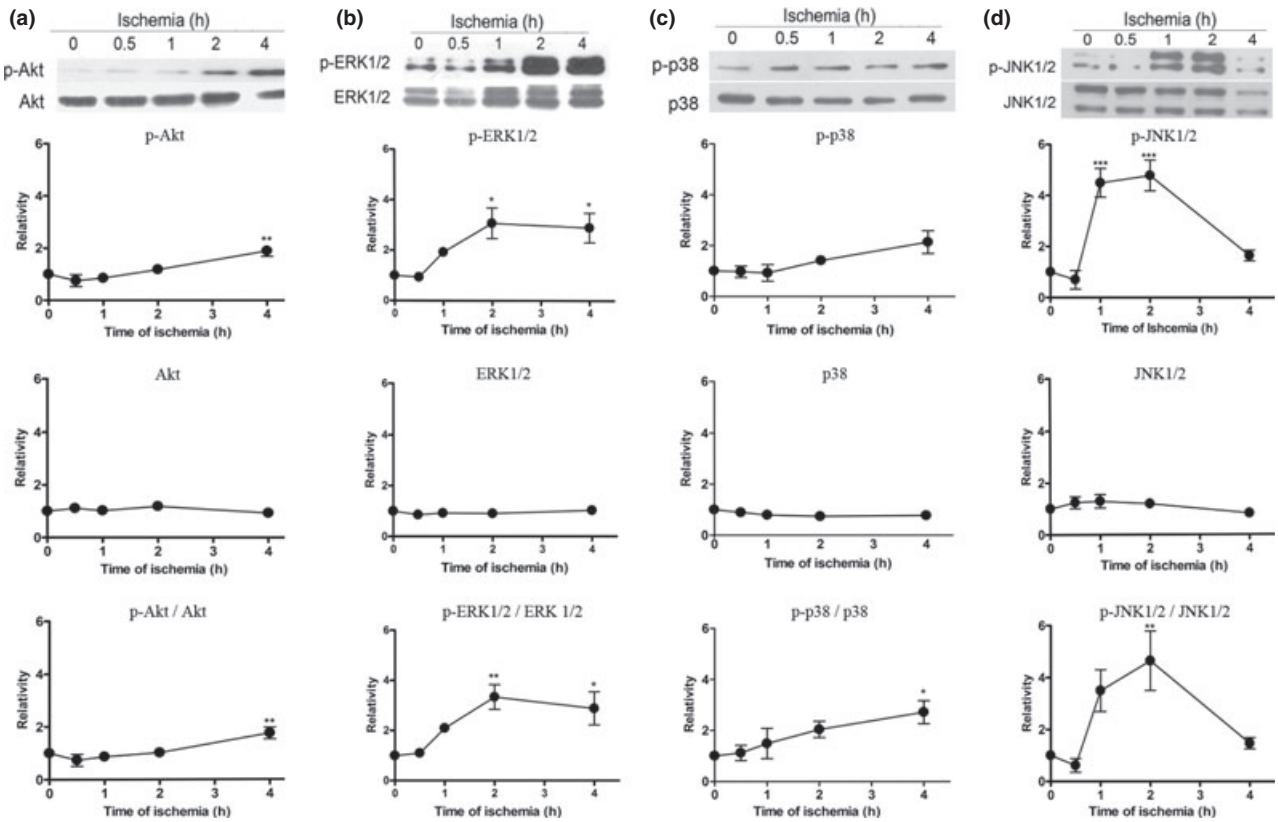

Fig. 1 The activation of Akt, p38, ERK and JNK under ischemia treatment. (a) Representative results of the western blot at the top. Upper graph: phosphorylated Akt (p-Akt) activation relative to control for different times of ischemia by statistical analysis. Middle graph: total Akt activation. Lower graph: ratio of p-Akt/Akt (b) p38 activation. (c) ERK activation. (d) JNK activation. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, versus 0 h control.

p-JNK slightly decreased at 0.5 h of ischemia, and then dramatically rose to 4.50 times of the control at 1 h of ischemia. This high level of p-JNK was maintained to 2 h of ischemia and then began to gradually decrease. The ratio of p-JNK to total JNK followed the transient expression pattern of p-JNK except the highest level reached 5.41 times of the control at 2 h. At 4 h, the p-JNK and the ratio returned to about 1.50 times of the control.

All four signaling pathways were activated in astrocytes by ischemia, although with a different temporal pattern and degree of increase. The activation of ERK1/2 (from 1 to 4 h) and JNK (from 1 to 2 h) appeared earlier than those of Akt (4 h) and p38 (4 h). Based on these results on the time course of activation of these pathways in astrocytes under ischemia, only ERK1/2 and JNK pathways appeared likely to be involved in the 14-3-3γ up-regulation.

Role of PI3K/Akt and MAPKs on 14-3-3γ under ischemia

Significant up-regulation of 14-3-3γ mRNA levels in astrocytes at 2 h of ischemia was observed and dimethyl sulfoxide (DMSO) as a vehicle control did not exert any additional effects on the 14-3-3γ mRNA level (Fig. 2a). Treatment with inhibitor LY294002 for PI3K/Akt, U0126 for ERK1/2, and SB203580 for p38 did not affect the ischemia-induced 14-3-3γ mRNA up-regulation. JNK inhibitor SP600125 exerted a dose dependent inhibition of 14-3-3γ mRNA levels at 2 h ischemia and was further analyzed for protein levels at 4 h (Fig 2b and c, respectively). At 40 μM for both times the ischemia-induced 14-3-3γ mRNA up-regulation was almost completely inhibited. Thus, suggesting the JNK pathway is largely responsible for the ischemia-induced 14-3-3γ up-regulation in astrocytes.

Role of c-Jun in 14-3-3γ up-regulation under ischemia

c-Jun protein is an immediate early response gene and known to be regulated by JNK pathway. Astrocytes under ischemia showed an early but transient increase in p-c-Jun and total c-Jun level from 0.5 to 2 h of ischemia returning to the control level at 4 h (Fig. 3a). The level of c-Jun in astrocytes decreases further at 6 h, a time when cell death began. The p-c-Jun levels in cytoplasm of astrocyte increased from 0.5 to 1 h under ischemia and the total c-Jun did not change (Fig. 3b). In the nucleus, both p-c-Jun and total c-Jun were increased from 1 h and peaked at 2 h of ischemia (Fig. 3c).

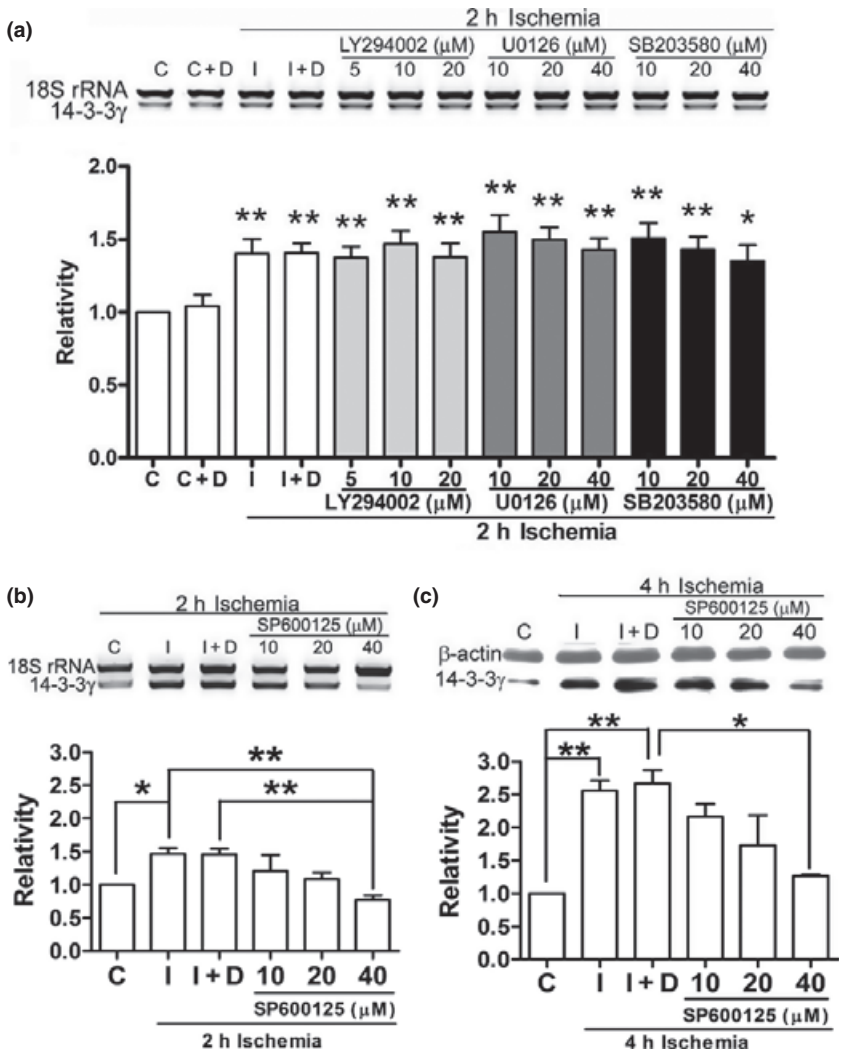

Fig. 2 Effects of PI3K/Akt and MAPK pathway inhibitors on the up-regulation of 14-3-3γ in astrocytes. 'C', control, 'D', DMSO, and 'I', ischemia. The values of 14-3-3γ were normalized to the internal controls. (a) RT-PCR analysis of 14-3-3γ mRNA levels under ischemia showed no observable inhibition with dosages (5 μM, 10 μM, 20 μM) of LY294002 (PI3K inhibitor), U0126 (ERK inhibitor), and SB203580 (p38 inhibitor). However, SP600125 (JNK inhibitor) exerted a dose–dependent inhibition in ischemia induced up-regulation of (b) 14-3-3γ mRNA (c) and protein. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, versus 0 h control.

This increase appeared later than those in the cytoplasm indicating a translocation of p-c-Jun from the cytoplasm to nucleus in astrocytes.

Role of AP-1 in 14-3-3γ up-regulation under ischemia

We searched the transcription factor binding sites in the upstream sequence of mouse 14-3-3γ gene and located two AP-1 binding sites that the p-c-Jun could bind to and promote gene transcription (data not shown). AP-1 inhibitor curcumin was used to clarify the relationship between AP-1 and ischemia-induced up-regulation of 14-3-3γ in astrocytes. Curcumin dose dependently inhibited ischemia-induced 14-3-3γ mRNA up-regulation (Fig. 4a). At 5 μM it did not show any detectable inhibition on the mRNA level. At concentrations of 10 and 20 μM, curcumin could completely abolish the ischemia-induced up-regulation in 14-3-3γ mRNA. Curcumin at 4 h also had a significant effect on the protein level, a 10 μM dose could completely inhibit up-regulation and 20 μM further reduced it to 0.272 times of the control (Fig. 4b).

Discussion

Ischemia is the major cause of brain injury in stroke victims, and currently lacks proper and effective treatment. The *in vitro* ischemia model for this study has also been previously used to demonstrate ischemia-induced astrocyte death and an early and specific up-regulation of 14-3-3γ in astrocytes under ischemia was previously identified (Chen *et al.* 2003). Using over-expression experiments and antisense treatment, we showed that an elevation of 14-3-3γ protein in astrocytes promotes survival and a decrease enhances apoptosis in astrocytes under ischemia conditions. Its eluded neuroprotective mechanism is thought to be through binding to phosphorylated Bad to attenuate injury (Chen *et al.* 2005). Hence, 14-3-3γ makes a good

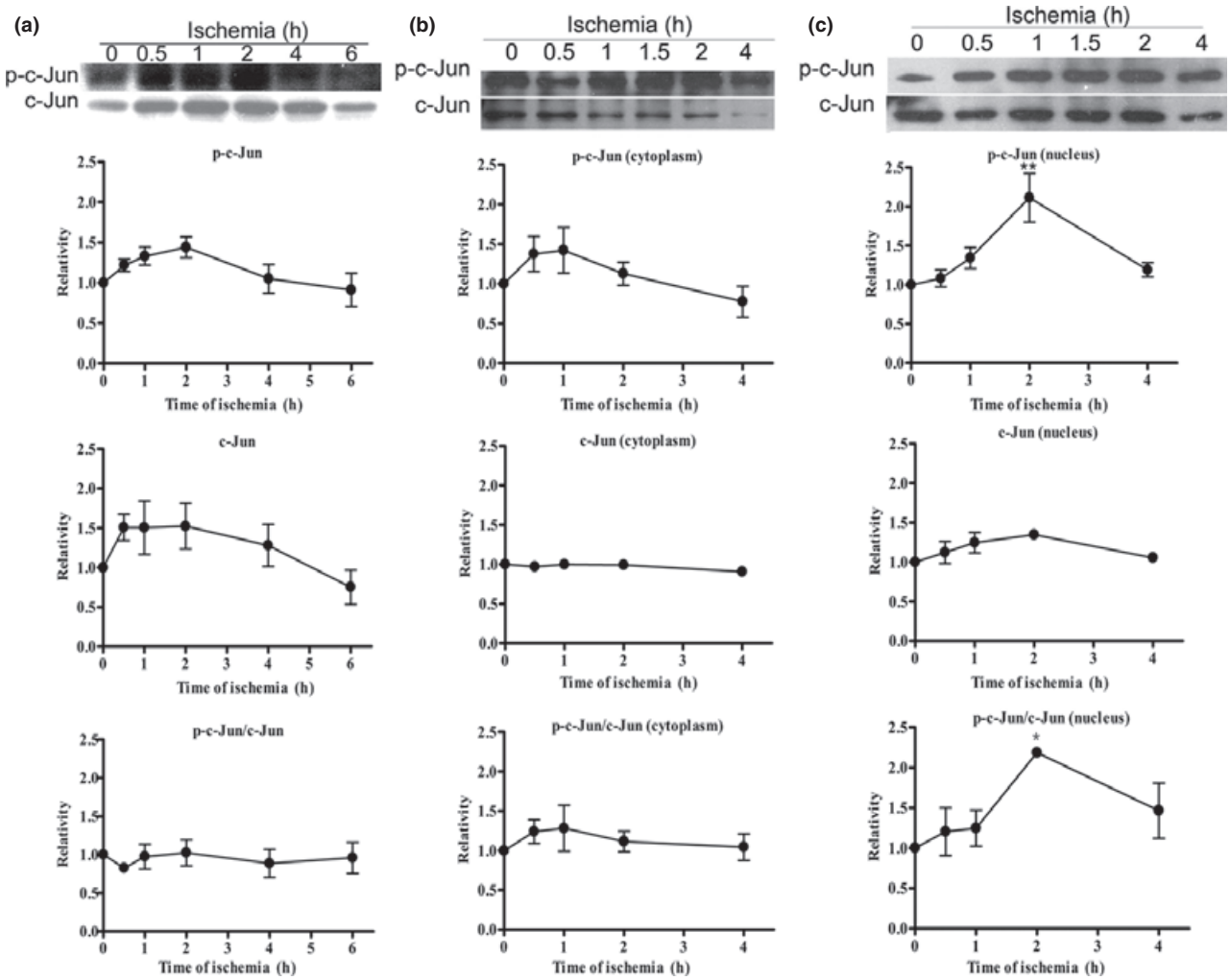

Fig. 3 p-c-Jun and c-Jun expression in astrocytes under ischemia. The proteins were isolated from whole astrocytes (a), their cytoplasm (b) and nucleus (c) after 0–4 h of ischemia. Note the earlier increased level in cytoplasm (b) than in the nucleus (c). *$p < 0.05$ and **$p < 0.01$, versus 0 h control of ischemia.

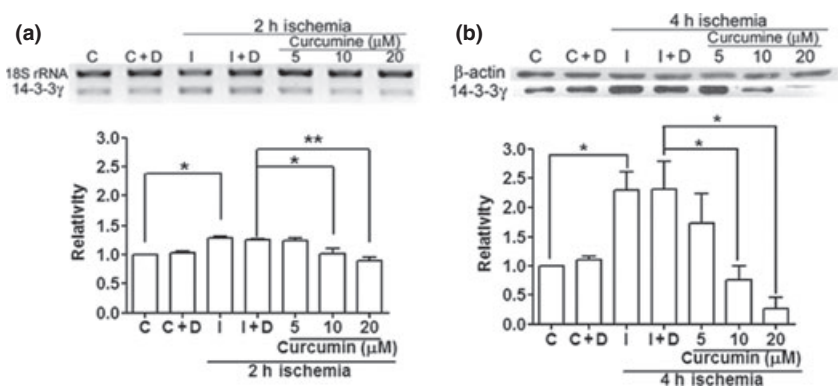

Fig. 4 Effect of AP-1 inhibitor curcumin on the up-regulation of 14-3-3γ in astrocytes under ischemia. 'C', control, 'D', DMSO, and 'I', ischemia. RNA levels under 2 h of ischemia (a) and protein levels at 4 h (b) both showed dose-dependent inhibition. Moreover, curcumin at 40 μM reduced the level of 14-3-3γ protein below the control. *$p < 0.05$ and **$p < 0.01$, versus 0 h control.

candidate as an innate endogenous neuroprotective protein involved in 'ischemia tolerance' and holds promise for identifying unique stroke therapeutics. We provide the first evidence for the JNK/c-Jun/AP-1 pathway to be responsible for up-regulation of 14-3-3γ in astrocytes under ischemia.

The PI3K/Akt and MAPK pathways, major survival pathways in a variety of systems (Cantley 2002; Irving and Bamford 2002), were investigated. MAPK pathways composed of extracellular signal-related kinase pathways (MAPK/ERK1/2, ERK5) and stress activated kinase pathways [Stress-activated protein kinase/c-Jun NH (2)-terminal kinase (SAPK/JNK) p38 MAPK]. In previous studies the change of ERK1/2 and Akt in astrocytes under ischemia and post-ischemia showed the activation of ERK1/2 increased after 1 h ischemia, reached a maximum at 4 h, and decreased from 5 to 6 h (Jiang *et al.* 2003). Akt was activated later than ERK1/2, and was significantly increased after 4 h ischemia before declining steadily afterwards. U0126 inhibition of ERK1/2 enhanced ischemia-induced cell death and LY294002 inhibition of PI3K/Akt delayed cell death. These effects were dose-dependent, suggesting the activation of MAPK/ERK1/2 pathway might protect astrocytes from injury, but activation of PI3K/Akt pathway did not. The activation of ERK1/2 and Akt is specific in astrocytes, not in neurons, has also been demonstrated before (Chen *et al.* 2005).

The stress activated kinase pathways (SAPK/JNK, p38 MAPK) were included in this study in order to cover all the pathways possibly involved in the up-regulation of 14-3-3γ. All four signaling pathways were activated in astrocytes under ischemia. The pattern of activation of ERK1/2 and Akt were the same as previous reported (Jiang *et al.* 2003). The ERK1/2 and JNK pathways were found to be activated earlier than the p38 and Akt pathways. The activation of JNK and ERK1/2 began to significantly increase at 1 h of ischemia until it peaked at 2 h while ERK1/2 persisted throughout. The activation of p38 and Akt only significantly increased at 4 h of ischemia. The temporal activation of these signaling pathways in astrocytes cultures were in the same sequence as reported in *in vivo* ischemia. ERK1/2 activated after 2 min and JNK activated after 30 min in neurons under ischemia (Gu *et al.* 2001); Akt activated after 3–12 h in ischemic brain and p38 activated after 2–4 days (Piao *et al.* 2002; Choi *et al.* 2005) Comparing the temporal activation profiles with 14-3-3γ up-regulation, only the JNK activation pattern matched. The inhibitor experiments clearly confirmed this speculation.

The JNK pathway could be involved in expressions of many genes related to cell survival or death in ischemic brain injury, through the phosphorylation of its most important downstream c-Jun protein and translocation of p-c-Jun to the nucleus. An early increase of p-c-Jun in their cytoplasm under ischemia and a later increase of p-c-Jun in nucleus which indicated a translocation of p-c-Jun from cytoplasm into nucleus were shown. Previously, JNK pathway was shown to involve gene expressions of heme oxygenase, vascular endothelial growth factor and nerve growth factor (Semkova and Krieglstein 1999; Aggeli *et al.* 2006; Lee *et al.* 2006). These genes have a common characteristic of having AP-1 binding sites in their promoter sequences. After analyzing the upstream DNA sequence of 14-3-3γ gene, we successfully located two AP-1 binding site at −725∼−719 and −1054∼−1048 (relative to ATG) (data not shown). Therefore, curcumin was used to inhibit the binding capacity of AP-1 (Cho *et al.* 2006), and it was found that both 14-3-3γ gene and protein up-regulation were being significantly inhibited. This clearly demonstrates that the JNK/c-Jun/AP-1 pathway is responsible for ischemia-induced up-regulation of 14-3-3γ. It is controversial to think that JNK has been involved in both apoptosis and neuroprotection because many immediate early genes that are up-regulated were AP-1 related components (Akins *et al.* 1996). However, the exact signaling pathways leading to JNK activation in response to ischemia/stroke are still not clear. Most studies reported that JNK activation lead to cell death under ischemia, and inhibitors to JNK pathway could protect cell death against cerebral ischemia (Borsello *et al.* 2003). Moreover, Wang *et al.* (2006) reported that the JNK pathway mediated an important role in anti-apoptosis in brain via Protease-activated receptor 1 (PAR-1) to preserve astrocytes from some toxic insult. The latest evidence showed in glucose deprivation activated JNK with biphasic kinetics that the early phase of JNK activation promoted cell survival, whereas the late phase of JNK activation induced apoptosis (Yun *et al.* 2008). Interestingly, in our *in vitro* ischemic-like condition model, we observed two peaks of JNK activation when astrocytes were subjected to ischemic injury (data not shown). The first peak was mostly consistent with the up-regulation of 14-3-3γ. Whether the second peak of JNK activation at 6 h contributes to cell death or not requires further experimental confirmation.

Protein kinase, transcription factor, and immediate early gene appear to transduce the external signals into a tolerant response. Although the mechanism of ischemic tolerance remains uncertain, its discovery provides the focus for further understanding of the mechanism of innate endogenous neuroprotection. 14-3-3γ up-regulation appeared to involve JNK (protein kinase), AP-1 (transcription factor), and c-Jun (immediate early gene). Previous reports have shown that 14-3-3γ can act as an endogenous pro-survival factor. Whether it involved the 'programmed cell survival' pathway, in ischemic tolerance (Obrenovitch 2008) needs further investigation. Our findings identified the regulatory pathway of 14-3-3γ expression and provide new avenues for future investigation into the prevention and treatment of stroke and related diseases.

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Conflicts of interest

All authors declare no conflicts of interests.

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