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Research Report

The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signaling pathway by PI3K/Akt activation

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Abstract

Current studies demonstrated that cell survival is determined by a balance among signaling cascades, including those that recruit the Akt and JNK pathways. In our present work, the relationship between Akt1 and JNK1/2 was evaluated after cerebral ischemia–reperfusion in the hippocampus in a four-vessel occlusion model of Sprague–Dawley rats. This paper was based on our present and previous studies. Firstly, Akt1 had one active peak during reperfusion following 15 min ischemia. Secondly, two peaks of JNK1/2 activation occurred during reperfusion, respectively. Thirdly, the phosphorylation of JNK substrates c-Jun and Bcl-2, and the activation of a key protease of caspase-3 were detected. They only had one active peak, respectively, during reperfusion. To clarify the mechanism of Akt1 activation and further define whether JNK1/2 activation could be regulated by Akt1 through PI3K pathway, LY294002 and insulin were, respectively, administrated to the rats prior to ischemia. Our research indicated that LY294002, a PI3K inhibitor, significantly suppressed Akt1 activation. Furthermore, LY294002 significantly strengthened both peaks of JNK1/2 activation, c-Jun activation, Bcl-2 phosphorylation, and the activation of caspase-3 during reperfusion. In contrast, insulin, a PI3K agonist, not only obviously activated Akt1 during early and later reperfusion, but also inhibited phosphorylation of JNK1/2, c-Jun, and Bcl-2 and attenuated the activation of caspase-3. In addition, pretreatment of insulin significantly increased the number of the surviving CA1 pyramidal cells at 5 days of reperfusion. Consequently, our results indicated that the cross-talk between Akt1 and JNK1/2 could be mediated by insulin receptor through PI3K in rat hippocampus during reperfusion. This signaling pathway might play a neuroprotective role against ischemic insults via inhibition of the JNK pathway, involving the death effector of caspase-3.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Cerebral ischemia/reperfusion (I/R); Akt1; Phosphatidylinositol 3-kinase (PI3K); JNK1/2; c-Jun; Bcl-2; Caspase-3; LY294002; Insulin; Hippocampus

1. Introduction

Insulin is a polypeptide hormone that consists of 51 amino acids and promotes a variety of anabolic enzymatic pathways and inhibits many catabolic enzymatic pathways involved in energy storage, as well as in synthesis of structural tissue proteins. In addition, insulin serves as a growth factor, modulating mitogenesis, growth, and differentiation. Insulin and insulin-like growth factor-1 (IGF-1) are two structurally related hormones which produce similar biological activities such as metabolic and growth promoting actions. Their receptors, insulin and IGF-1 receptors, also share similarities in both structure and functions such as tyrosine-specific protein kinase. The two receptors phos-

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phorylate many of the same substrates and activate the same signaling modules, including phosphatidyl inositol 3′kinase (PI3K) signaling pathway. In transient global ischemia, insulin has a direct neuroprotective effect on CNS parenchyma. Insulin-like growth factor-1 (IGF-1) had a similar but lesser effect in ischemic neuroprotection [1].

Phosphatidylinositol 3-kinase (PI3K) is a key anti-apoptotic effector in the growth factor signaling pathway. Akt/PKB, a 57-kDa protein–serine/threonine kinase [6], serves a key role in mediating anti-apoptotic actions of growth factors on cell [5]. Mammalian genomes contain three genes encoding Akt/PKBs (termed PKBα/Akt1, PKBβ/Akt2 and PKBγ). Akt1 and Akt2 are widely expressed in brain. Stimulation of tyrosine kinase growth factor receptors activates PI3K, which leads to Akt activation. Akt activation is correlated with phosphorylation of Thr-308 at its catalytic domain and of Ser-473 at the C terminus. It has been reported that Akt phosphorylation at Ser-473 has obvious relation with global brain ischemia [6], and Akt plays an important role in neuronal protection. Recent studies reveal that IGF-I binding IGF-IR prevents neuronal death after transient forebrain ischemia [8,24,31].

c-Jun N-terminal kinase (JNK), a member of mitogen-activated protein kinase (MAPK) family, is important in inducing neuronal death. The JNKs are encoded by three genes: JNK1, JNK2, and JNK3. Recent studies show that JNK is involved in apoptosis. For instance, overexpression of a constitutively activated JNK kinase potentiates apoptosis of PC12 cell in response to nerve growth factor (NGF) deprivation [27]. Activated JNK, in turn, phosphorylates a number of transcription factors, especially the c-Jun of component of AP-1, and cellular proteins, particularly those associated with apoptosis (for example, Bcl-2, P53, and so on) [17].

The downstream mechanism of JNK-mediated apoptosis may include the induction of c-Jun, Bcl-2 families, and the mitochondrial release of cytochrome *c* [10,18,28]. Since apoptosis is a form of cell death, it should be associated with alteration of gene expression, which is regulated by immediately early genes (IEGS) such as c-Jun. Recently, some studies revealed that c-Jun plays important roles in neuronal cell death under in vitro and in vivo conditions. Activated JNK specifically phosphorylates the N-terminal activation domain of transcription factor c-Jun at serine 63 and 73 thereby increasing transcriptional activity of c-Jun [18]. The Bcl-2 family proteins have one or more Bcl-2 homology domains and play a crucial role in intracellular apoptotic signal transduction by regulating permeability of the mitochondrial membrane [29]. Recent studies proved that Bcl-2 serves as a non-nuclear substrate of JNK [21,23,28]. Most data indicate that Bcl-2 phosphorylation decreases its anti-apoptotic function [3,11]. Caspase-3 is a potent effector of apoptosis triggered via several different pathways in a variety of mammalian cell types and is one of the most important caspases activated downstream of cytochrome *c* in the cytochrome *c*-dependent apoptosis pathways. Caspase-3 activates caspase-activated DNase (CAD) and leads to DNA damage. Recent in vivo studies demonstrate that cerebral ischemia induces the release of cytochrome *c* and the activation of caspase-like proteases that result in delayed cell death [15,20].

Apoptotic and proliferative pathways are separated but may be activated by common signals and the intracellular balance between these different pathways decides the ultimate fate of the cells. PI3K/Akt and JNK pathways have been known to correlate with neuronal survival and apoptosis, but not until recently have these specific pathways associated with the prevention of apoptosis been well described. Therefore, we decided to investigate whether they could cross-talk in cerebral ischemia and the cross-talk could have effects on ischemic injury. We addressed the question of whether the neuroprotective action of insulin was related to modulation of Akt and JNK signaling pathways in cerebral ischemia and insulin plays a neuroprotective role in ischemic injury via inhibition of the JNK pathway, involving the death effector of caspase-3. In addition, by blocking PI3K activation with LY294002, a PI3K inhibitor, we wanted to test whether Akt was activated via PI3K and activated Akt could affect JNK and its downstream during ischemia–reperfusion (I/R) in rat hippocampus.

2. Materials and methods

2.1. Animal surgical procedures

Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of science) weighing 250–300 g were subjected to 15 min of brain ischemia by four-vessel occlusion methods as previously described [22]. Briefly, under anesthesia with chloral hydrate (300–350 mg/kg, i.p.), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. Ischemia was induced by occluding the common arteries with aneurysm clips. An EEG was monitored to ensure isoelectricity. Carotid artery blood flow was restored by releasing the clips. During ischemia, animals were elected to match the following criteria: completely flat electroencephalographs, maintenance of dilated pupils, absence of a cornea reflex when exposed to strong light stimulation, and maintenance of rectal temperature at 36.5–37.5 °C. Those not matching these criteria or with seizures were all excluded. Sham controls underwent the same surgical procedures without carotid arteries occlusion. Surgical procedures were conducted under guidelines and the terms of all relevant local legislation.

2.2. Brain tissue and drug treatment

Rats were decapitated at 0, 30 min, 3, 6, 12 h, 1, and 3 days of reperfusion following 15 min of ischemia to verify

the time courses of Akt1 activation and expression. When necessary, rats were given insulin (20 μg, 10 μl in 0.9% NaCl) and LY294002 (25 μg, 5 μl in 100% DMSO) by left cerebral ventricle injection (from the bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; depth, 3.5 mm). The drugs were injected 20 min prior to occlusion. Control rats received equal volume of 0.9% NaCl or DMSO.

2.3. Tissue preparation

Rats were decapitated at various times of reperfusion following 15 min of ischemia and then hippocampi were immediately separated and frozen in nitrogen then stored at −80 °C. The samples were homogenized in ice-cold homogenization buffer containing 50 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 10 mM β-phosphoglycerol, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 1 mM benzamidine and enzyme inhibitors: 5 μg/ml each of phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, aprotinin (Sigma). The homogenates were centrifuged at 800 × *g* for 15 min at 4 °C and then supernatants were stored at −80 °C until use. Protein concentrations were determined using Lowry method. The nuclear pellets were extracted with 20 mM HEPES, pH 7.9, 20% glycerol, 420 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and enzyme inhibitors for 30 min at 4 °C with constant agitation. After, they were centrifuged at 12,000×*g* for 15 min at 4 °C. Supernatants as nuclear parts were collected and protein concentrations were determined by the method of Lowry et al. Samples were stored at −80 °C and were thawed only once.

2.4. Western blot analysis

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membrane (pore size 0.45 μm) which were blocked 3 h with 3% BSA and then probed with primary antibody at 4 °C overnight. The membranes were washed and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:10,000), goat anti-mouse IgG (1:5000), or donkey anti-goat IgG (1:3000) in PBST for 2 h. Immunoreactivity was detected by NBT/BCIP assay kit (Promega, Madison, WI). The bands on the membrane were scanned and analyzed by an image analyzer (Labworks Software, UVP Inc., Upland, CA).

2.5. Histology

Rats were perfusion-fixed with 4% paraformaldehyde under anesthesia after 5 days of reperfusion. Paraffin sections (5 μm) were prepared and stained with Cresyl violet. The sections were examined with light microscopy and the number of surviving hippocampal CA1 pyramidal cells per 1 mm length was counted as the neuronal density. An initial dissector frame was positioned randomly in hippocampal sector and cells in every 10th section throughout the entire hippocampus. The cell numbers in hippocampus were assessed by means of previously published unbiased stereological techniques. In brief, cell counts were performed at × 400 magnification with the use of an Olympus BH-2 microscope connected to a Sony charge-coupled device video camera, a motorize stage system, and commercial stereology software. The optical dissector technique was used to avoid double counting of cells [4,26].

2.6. Reagents

Anti-Akt1 antibody, anti-p-c-Jun antibody, anti-c-Jun antibody, and anti-Bcl-2 antibody were brought from Santa Cruz Biotechnology. Anti-p-Akt1 antibody and LY294002 were from Upstate. Insulin and anti-JNK1/2 antibodies were from Sigma. Anti-active (diphosphorylated) JNK1/2 was from Promega. Anti-p-Bcl-2 antibody and anti-caspase-3 antibody were purchased from Cell Signaling Biotechnology.

2.7. Statistics

Values were expressed as means ± SD from four independent animals. Statistical analysis of the results was carried out by one-way analysis of the variance followed by the Duncan's new multiple range method or Newman–Keuls test. $P < 0.05$ was considered significant.

3. Results

3.1. The neuroprotective effects of insulin against I/R-induced neuronal loss in hippocampal CA1 region

To explore the neuroprotective effects of insulin against I/R-induced neuronal loss, Cresyl violet staining was used to examine the survival of CA1 pyramidal neurons. Normal cells showed round and pale stained nuclei. The shrunken cells with pyknotic nuclei after ischemia were counted as dead cells. Transient brain ischemia followed by 5 days of reperfusion induced severe cell death. Administration of insulin (20 μg/10 μl) 20 min before ischemia significantly decreased neuronal degeneration (Fig. 1, Table 1).

3.2. Effects of insulin on activation of Akt1 following cerebral ischemia

To examine the possible role of PI3K/Akt1 pathway mediated by insulin receptor, firstly, we examined Akt1 activation at 0, 30 min, 3, 6, 12 h, 1 and 3 days of reperfusion following 15 min of ischemia. As shown in Figs. 2A and B, after Akt1 activation firstly decreased at 0 min of reperfusion, phospho-Akt increased rapidly over the control level from 30 min of reperfusion, peaked at 3 h of

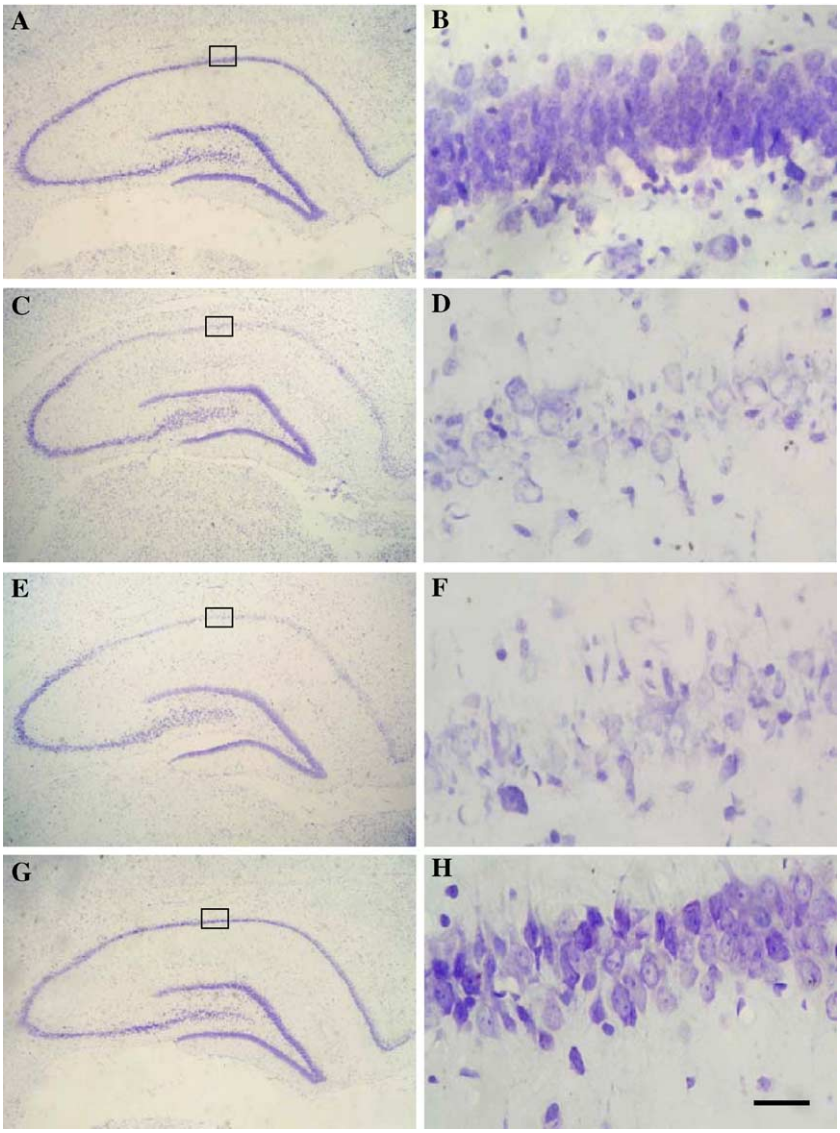

Fig. 1. Example of Cresyl violet-strained sections of the hippocampi of sham operated rats (A, B) and rats subjected to 15 min of ischemia followed by 5 days of reperfusion (C, D), and rats subjected to 15 min of ischemia followed by 5 days of reperfusion, which is the administration of the vehicle (E, F), and 20 μg/10 μl of insulin 20 min before ischemia (G, H). Data were obtained from seven independent animals in each experiment group and the results of a typical experiment are presented. Scale bars: (A, C, E, G) 250 μm; (B, D, F, H) 20 μm.

reperfusion, and decreased generally from 6 h and then returned to the control level at 1 day, with no secondary increase from 1 day to 3 days. Total Akt1 protein levels were unchanged.

Table 1
Quantitative analysis of the protective effects of insulin against transient ischemia followed by reperfusion

Group	Neuronal density (mean ± SD)
Sham	212.0 ± 18.2
I/R5d	19.7 ± 6.9
Pre-saline I/R5d	25.1 ± 8.4
Pre-insulin I/R5d	126.8 ± 13.7*

Neuronal density is expressed as the number of surviving neurons per 1 mm length of CA1 pyramidal cells counted under light microscopy. Data are mean ± SD (n = 7).

* $P < 0.05$ vs. vehicle-treated group.

Secondly, to further investigate the effects of insulin and LY294002 on Akt1 activation, we administrated 4-VO rats with LY294002, a PI3K inhibitor. As shown in Figs. 2C and D, LY294002 inhibited Akt1 activation at 3 h of reperfusion after 15 min of ischemia compared with vehicle controls. Then, we pretreated rats with insulin 20 min prior to ischemia. As shown in Figs. 2E and F, insulin strengthened Akt1 activation during early and later period of reperfusion. Level of total Akt1 protein was unaffected by insulin or LY294002.

3.3. Effects of insulin on JNK1/2 activation following cerebral ischemia

To examine whether activation of Akt1 after ischemia was related with JNK1/2 and to test the effects of those two drugs on JNK1/2, we selected two active peaks of JNK1/2,

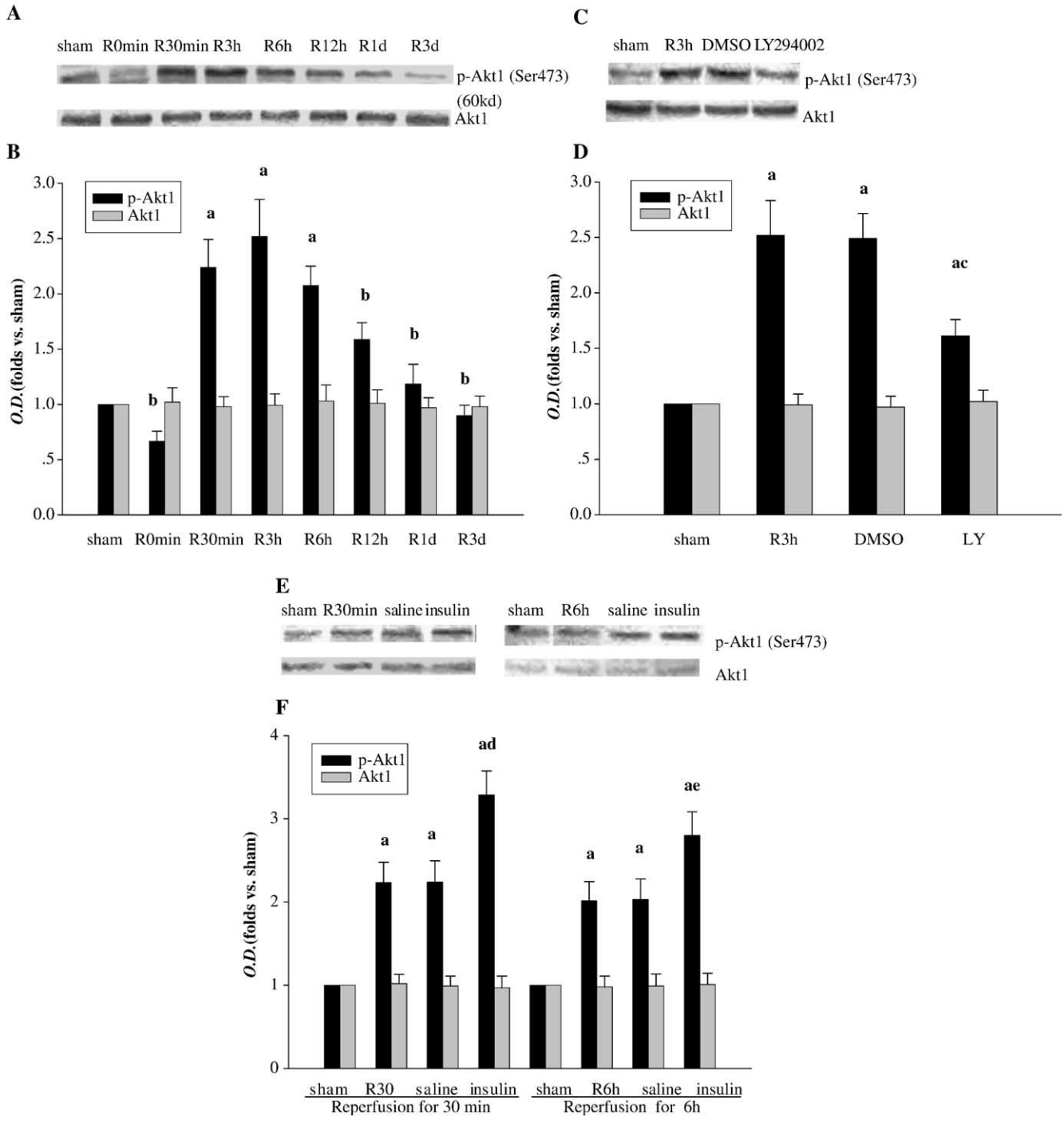

Fig. 2. Time course of I/R-induced alteration of Akt1. (A, B) p-Akt1 and Akt1 expression was examined by immunoblotting analysis from hippocampus after I/R. Bands corresponding to Akt1 and p-Akt1 were scanned and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD derived from four independent animals ($n = 4$). [a]$P < 0.05$ vs. sham, [b]$P < 0.05$ vs. R 3 h. Effects of LY204002 on p-Akt1 at R 3 h and insulin on Akt1 activation and expression at 30 min and 6 h in hippocampus after I/R. (C, D) Akt1 activation was suppressed by LY294002 at 3 h of reperfusion compared with vehicle DMSO. (E, F) Akt1 activation was strengthened by insulin at 30 min and 6 h of reperfusion compared with the rat treated with saline. Data were expressed as mean ± SD derived from four independent animals in each experiment group. [a]$P < 0.05$ vs. sham, [c]$P < 0.05$ vs. R 3 h + DMSO, [d]$P < 0.05$ vs. R 30 min + saline, [e]$P < 0.05$ vs. R 6 h + saline.

30 min and 3 days of reperfusion that we previously reported [9]. As shown in Figs. 3A and B, insulin obviously inhibited JNK1/2 activation at 30 min and 3 days following 15 min of ischemia. On the contrary, as shown in Figs. 3C and D, LY294002 significantly strengthened JNK1/2 activation at 30 min and 3 days after 15 min of ischemia. The protein levels of JNK1/2 were unaffected by insulin or LY294002.

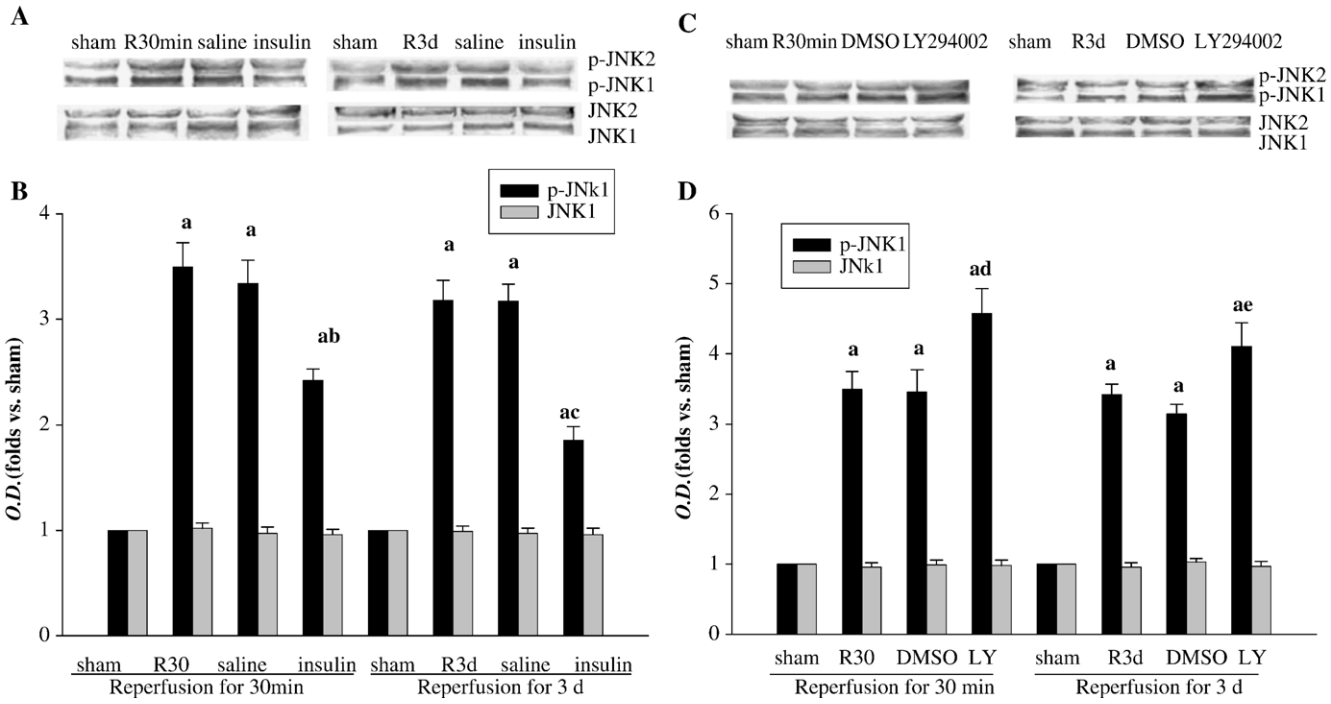

Fig. 3. Effects of insulin and LY294002 on JNK1/2 activation and expression at 30 min 3 days in hippocampus after I/R. (A, B) JNK1/2 activation was attenuated by insulin at 30 min and 3 days after 15 min of ischemia compared with the rat subjected with vehicle control. (C, D) JNK1/ 2 activation was strengthened by LY294002 at 30 min and 3 days of reperfusion compared with vehicle DMSO. The products reveal the approximate size 46 kDa (JNK1α, JNK2α) and 54 kDa (JNK1β, JNK2β). Semiquantitative representative of JNK1 activation level on Western blots. Data were expressed as mean ± SD derived from four independent animals in each experiment group. $^{a}P < 0.05$ vs. sham, $^{b}P < 0.05$ vs. R 30 min + saline, $^{c}P < 0.05$ vs. R 3 days + saline, $^{d}P < 0.05$ vs. R 30 min + DMSO, $^{e}P < 0.05$ vs. R 3 days + DMSO.

3.4. Effects of insulin on phosphorylation of c-Jun, Bcl-2, and activation of caspase-3 following cerebral ischemia

To address whether insulin inhibited the activation of c-Jun, a nuclear substrate of JNK, and LY294002 reversed the effect during reperfusion. Because we have previously examined that c-Jun was activated after reperfusion and reached its activation peak at 3 h of reperfusion [10], we firstly explored the effects of insulin on c-Jun activation at 3 h of reperfusion. As shown in Figs. 4A and B, treatment with insulin prevented the increased c-Jun activation at 3 h of reperfusion. On the contrary, LY294002 significantly strengthened c-Jun activation at 3 h of reperfusion. The increased protein expression of c-Jun was unaffected by insulin or LY294002.

We have previously examined that the phosphorylated Bcl-2 (Ser-87), a non-nuclear substrate of JNK, reached its peak at 3 h of reperfusion [10]. We also examined levels of Bcl-2 phosphorylation during reperfusion after the treatment with insulin. The study was carried out by Western immunoblotting analysis with anti-phospho-Bcl-2–Ser-87 antibody or conventional anti-Bcl-2 antibody. As shown in Figs. 4C and D, intraventricular administration of insulin 20 min before ischemia prevented the increased Bcl-2–Ser-87 phosphorylation observed at 3 h after 15 min ischemia. On the contrary, intraventricular administration of LY294002 20 min before ischemia strengthened the Bcl-2–Ser-87 phosphorylation observed at 3 h after 15 min ischemia. Level of total Bcl-2 protein was unaffected by insulin or LY294002.

To examine the effect of insulin on caspase-3 activation, the study was carried out by Western immunoblotting. Caspase-3 had only one activated peak at 3 days of reperfusion in our previous studies [30]. As shown in Figs. 4E and F, using immunoblotting assay, we confirmed that pretreatment with insulin rescued the ischemia-induced increase in caspase-3 activation observed at 3 days of reperfusion. However, pretreatment with LY294002 increased the caspase-3 activation observed at 3 days of reperfusion.

4. Discussion

Preclinical data from animal models indicate that insulin may reduce damage in both global and focal ischemia. Two kinds of mechanisms may be involved: one in which insulin interacts directly with brain tissue and one in which insulin acts indirectly by reducing peripheral blood glucose levels. Animal data indicate that the direct mechanism is mediated by insulin-like growth factor-1 receptors. The direct effect appears to predominate in global ischemia [1]. Insulin mediates all of its effects by initially binding and activating its specific cell-surface receptor. Conformational changes induced by insulin binding lead to activation of intrinsic

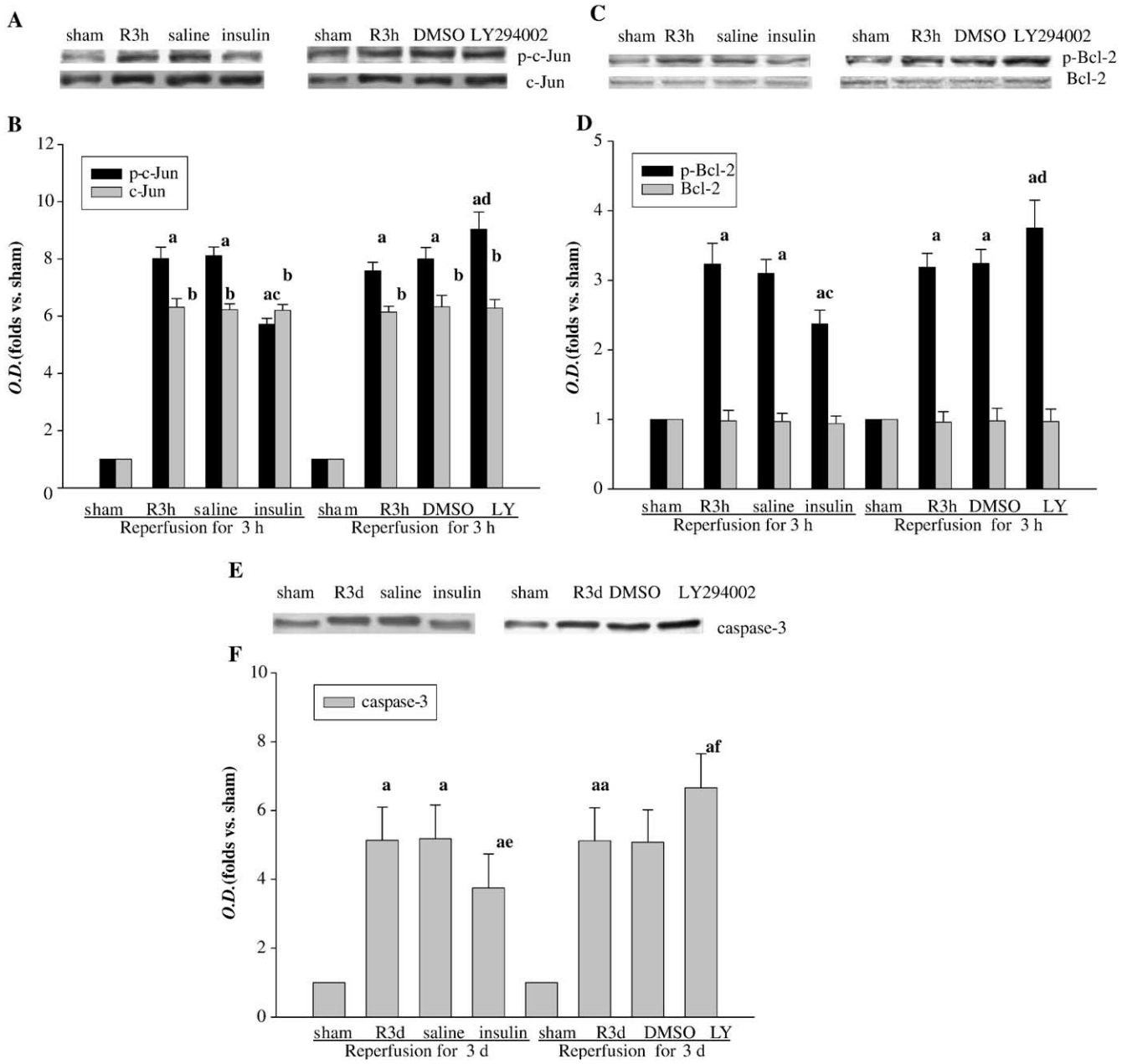

Fig. 4. Effects of insulin and LY294002 on c-Jun, Bcl-2 phosphorylation, and expression at 3 h and effects of insulin and LY294002 on caspase-3 activation at 3 days in hippocampus after I/R. (A–D) c-Jun (p-c-Jun, Ser63) and Bcl-2 (p-Bcl-2, Ser87) phosphorylation was attenuated by insulin at 3 h after 15 min of ischemia compared with the rat subjected with vehicle control. c-Jun (p-c-Jun, Ser63) and Bcl-2 (p-Bcl-2, Ser87) phosphorylation was strengthened by LY294002 at 3 h of reperfusion compared with vehicle DMSO.(E, F) Caspase-3 activation was attenuated by insulin at 3 days after 15 min of ischemia compared with the rat subjected with vehicle control. And caspase-3 activation was strengthened by LY294002 at 3 days of reperfusion compared with vehicle DMSO. Bands corresponding to p-c-Jun, c-Jun, p-Bcl-2, Bcl-2, and caspase-3 were scanned and the intensities were represented as folds vs. sham control. Data were expressed as mean ± SD derived from four independent animals in each experiment group. $^{a,b}P < 0.05$ vs. sham, $^{c}P < 0.05$ vs. R 3 h + saline, $^{d}P < 0.05$ vs. R 3 h + DMSO, $^{e}P < 0.05$ vs. R 3 days + saline, $^{f}P < 0.05$ vs. R 3 days + DMSO.

receptor tyrosine kinase. In the current study, we investigated the neuroprotective action of insulin on ischemic injury possibly by activating PI3K/Akt pathway and blocking the activation of JNK pathway in hippocampus following global ischemia.

Our current results showed that the decreased Akt1 activation was caused by the lack of ATP and subsequently resulted in neuronal damage at the end of 15 min of ischemia [6], which were consistent with previous reports. Then, the increased Akt1 activation participated in endogenous neuroprotection following brain ischemia as shown before [7] and in the current study. The mechanisms that Akt rescues cells from apoptosis have been extensively studied. For example, Akt phosphorylates Bad in vitro and vivo, blocking the Bad-induced death of primary neurons in a site-specific manner [5]. But here, we presented another

neuroprotective pathway that phosphorylation of Akt could negatively regulate JNK signaling, which might have a more important meaning in cerebral ischemia.

In order to elucidate the possible mechanism of Akt1 activation, we injected directly PI3K inhibitor LY294002 and PI3K agonist insulin into the brain ventricles, respectively. In our study, we initially examined that LY294002 decreased Akt1 activation in cerebral ischemia in rat, which was consistent with previous reports that it negatively affected on Akt activation in MCAO mice [19]. Our results suggested that Akt1 was activated through PI3K pathway. It was reported that IGF-1 could block the decreased Akt phosphorylation after brain ischemia by intraventricular administration [7]. Our results clearly showed that insulin could effectively activate Akt1 via PI3K pathway during reperfusion.

What's more, it had been reported that insulin could suppress the JNK signaling pathway through activation of PI3K-Akt pathway in HepG2, whereas PI3K inhibitor LY294002 reversed this effect [2]. Our present findings indicated that insulin could weaken JNK1/2 activation at 30 min and 3 days of reperfusion; however, LY294002 could strengthen JNK1/2 activation at the two activation peaks. The findings also suggested that insulin could down-regulate JNK pathway via PI3K/Akt pathways. Together with previous evidences, our observation suggested that there might be a cross-talk between Akt1 and JNK1/2 through the activation of PI3K/Akt signaling pathways in cerebral ischemia in rat. Taken together, there was a close relationship between strengthened Akt1 activation and suppressed JNK1/2 activation, which was mediated by insulin.

Recently, several mechanisms of the cross-talk between Akt and JNK pathways have been reported. One group showed that the phosphorylated Akt1 (Ser-473) could phosphorylate MLK3 on serine 674, which inhibited MLK3-mediated JNK activation [2]. Other report indicated that Akt phosphorylates serine 71 of Rac1 and inhibited Rac1-GTP binding which resulted in the activation of JNK [16]. Moreover, Akt decreased JNK signaling by phosphorylating ASK1 on serine 83 [13]. It also has been well accepted that Akt1 binding to JIP1 decreases JIP1's ability to enhance JNK activity by interfering with JIP1-mediated assembly of an active JNK signaling complex in neurons undergoing excitotoxic apoptosis [14]. Consequently, these studies suggest that the Akt pathway represents a crucial point of anti-apoptotic signaling by downregulating JNK signaling.

JNK was a serine/threonine protein kinase that phosphorylates c-Jun [12]. Further study showed that a c-Jun mutant cannot be phosphorylated on serine 63, which inhibited programmed neuronal cell death in vitro [25]. Also, our previous results indicated that brain ischemia promoted both the expression and phosphorylation on serine 63 of c-Jun, which peaked at 3 h in the vulnerable hippocampus [10]. In this study, insulin significantly diminished the increase of p-c-Jun at 3 h after ischemia. However, the increased expression of c-Jun was not affected by insulin.

A current study also concluded that JNK was responsible for paclitaxel-induced Bcl-2 phosphorylation in Jurkat cells overexpressing Bcl-2 [28]. It was likely that the role of Bcl-2 phosphorylation in promoting or inhibiting apoptosis might depend on the phosphorylation site(s) involved and the cellular context where this event took place, as well as on its duration. In this study, our results demonstrated that insulin significantly diminished the increase of p-Bcl-2 at Ser87 at 3 h after ischemia. The results suggested that JNK could phosphorylate Bcl-2 during reperfusion and the phosphorylation might inactivate Bcl-2, thus promoting apoptosis.

Caspase-3 was a key step in the execution process of apoptosis, and the inhibition of its activation could block apoptotic cell death. Increased caspase-3-like proteased activity in the hippocampal CA1 region that might be responsible for the delayed neuronal cell death after cerebral ischemia had been recently reported [15]. Our studies proved that insulin could decrease the activation of caspase-3, which might take effect at the final step of the apoptotic cascade.

In conclusion, our present results provided a new clue in an in vivo ischemic model. Utilizing insulin in both of these clinical situations could be evaluated in clinical trials that attempt to reduce ischemic brain damage because insulin has a long and safe history of human use in diabetes treatment. During reperfusion, insulin could activate Akt1 via PI3K pathway. Moreover, insulin might play a neuroprotective role against ischemic insults via inhibition of the JNK pathway, involving the death effector of caspase-3. Additionally, there appeared a cross-talk between PI3K/Akt and JNK pathways that led to protecting neuron in brain ischemia. Finally, the mechanisms of interaction between Akt and JNK in ischemic brain injury needed to be further elucidated.

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