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Down-regulation of Ras/Akt Signaling and Hippocampal Neuronal Death following Transient Global Ischemia-Reperfusion in Rat Brain

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Abstract Apoptosis is one of the components of delayed neuronal death in the hippocampal CA1 following transient cerebral ischemia. However, the precise signal transduction for inducing this type of death is not fully determined. Recent research has shown that oncogenic Ras protein plays a role in regulation of cell survival and death. We hypothesized that apoptosis in delayed neuronal death is regulated with Ras and its signaling cascades. We examined the temporal profile of Ras and its downstream cascade following transient forebrain ischemia (15 min) in rat brain. Neuronal death was inconsistently observed in the CA1 of the hippocampus (HE staining) 2 days after ischemia, whereas most neurons in the CA1 showed marked damage 3 days after ischemia. These damaged neurons exhibited positive TdT dUTP nick end labeling staining. α-Fodrin breakdown product in the hippocampus increased time dependently, 2 days and 3 days after reperfusion (by 14 and 25 times for 145-,150-kDa, and by 30-40% for 120-kDa, respectively). The amounts of Ras and phospho-Akt were significantly decreased by approximately 30-50%. The results suggest that delayed neuronal death in the hippocampal CA1 following transient forebrain ischemia is characterized by an apoptotic nature and associated with an inhibition of the Ras-Akt cascade.

Key words: Ras, Akt, ischemia-reperfusion, hippocampus, apoptosis

Introduction

It has been shown that the CA1 pyramidal neurons in the hippocampus are selectively vulnerable to transient ischemia, and they undergo delayed neuronal death.[1-3] Apoptosis is one of the components of this delayed neuronal death.[4-6] Accumulated data showed that excitotoxicity[7,8] and mitochondrial dysfunction might be the mechanisms for this type of neuronal death.[5,9,10] However, the precise signal transduction for inducing this type of death is not fully determined. Elucidation of this signal transduction would provide robust therapeutic approach because this type of cell death develops with time delay, usually 2 to 3 days, suggesting that there is a therapeutic time window.

Recently, it has been shown that GTPase-regulated oncogenic Ras protein has a role in regulation of cell survival and death.[11] Furthermore, Ras has been shown to paradoxi cally induce pro- and anti-apoptotic signaling.[12] Ras protein superfamily comprise a large number of molecules, selective activation of H-Ras by Ras-GRF has recently been reported in *in vitro* study, suggesting the potential participation of each Ras homologue in different signaling pathways.[13] PI3-kinase is a critical effector of Ras function in various

conditions such as growth factor stimulation,[14] and appears to provide a universal survival signal downstream of Ras. PI3-kinase is also strongly linked to pro-survival signaling, because it has an ability to activate the serine/threonine kinase Akt, also known as protein kinase B (PKB).[15] It has also been reported in *in vitro* cell lines that activation of PI3-kinase or Akt abrogates apoptosis by blocking the release of cytochrome *c*.[16] However, the changes of Ras signaling cascades in relation to the ischemic cell damage in the brain following ischemia-reperfusion have not been systematically determined *in vivo*.

In the present study, we examined the temporal profiles of H-Ras in relation to Akt and its activated form phospho-Akt (p-Akt) in the rat hippocampus 2-3 days after ischemia-reperfusion when hippocampal CA1 neurons may develop apoptotic cell death. We also measured fragments of α-fodrin, which is one of the major constituents of the membrane skeleton, as an indicator of ischemic neuronal damage in the hippocampus.

Materials and methods

Animals and experimental groups

The study protocol was approved by the Committee of the Ethics for Animal Experiments of Yamaguchi University School of Medicine. Thirty-six male Wistar rats weighing 260-280 g were randomly assigned to one of the following three groups: sham control group that received only surgical preparation but not subjected to ischemia (n=12); ischemia-reperfusion 2-day group (IR-2D group) that were subjected to 15 min of forebrain ischemia followed by two days of reperfusion (n=12); and ischemia-reperfusion 3-day group (IR-3D group) that were subjected to 15 min of forebrain ischemia followed by three days of reperfusion (n=12).

Forebrain ischemia

With the animal under isoflurane (1.5 %) anesthesia with 70% nitrous oxide and artificial ventilation (F_IO_2 0.3) through the tracheal tube, both carotid arteries were isolated carefully for temporal occlusion, while the pericranial temperature was maintained at 37 ± 0.2° C. Forebrain ischemia was induced by the method of Smith et al.[17] with a slight modification as we described previously.[18)19] The blood pressure was first decreased with intravenous administration of phentolamine mesilate (0.05 mg) and both carotid arteries were occluded with vascular clips. Thereafter, mean arterial blood pressure (MAP) was maintained at approximately 50 mm Hg during ischemia by withdrawing or infusing blood through the catheter placed in the right atrium through the external jugular vein. In this model, cerebral blood flow in the forebrain including hippocampus has been reported to decrease to near-zero values.[20]

After ischemia of 15 min, vascular clips were removed and recirculation was verified with the color of circulating blood and good pulsation of both carotid arteries. In the control group, all procedures were the same as described above (including carotid artery exposure) but cerebral ischemia was not induced. Then, the catheters were removed and wounds were sutured. Anesthesia was discontinued and the tracheal tube was removed after spontaneous respiration recovered. During the following 2-3 days, the rats were kept in the chamber maintained at 25° C and freely fed.

Histopathological evaluation

Eighteen rats in total (six in sham control group, six in IR-2D group and six in IR-3D group) were evaluated for histopathological findings.

At two or three days after 15 min ischemia or three days after sham treatment, the animals were re-anesthetized, and trans-cardiac perfusion and fixation were performed with 200 ml of 10% phosphate-buffered formalin. The 6 μm thick cross-sections corresponding to the stereotaxic section 3.8 mm caudal to bregma containing both dorsal hippocampi, were stained with hematoxylin-eosin (HE) and TdT dUTP nick end labeling (TUNEL) staining. The latter was performed to detect double-strand breaks in genomic DNA using a kit (Apoptosis in situ Detection Kit, WAKO Jun-yaku, Tokyo, Japan) according to the manufacturer's specifications with slight modifications. In TUNEL staining, we counted the number of neurons showing the nucleus stained dark brown with or without

chromatin condensation.

Immunoblotting

In eighteen rats (six in each group), immunoblotting analysis was performed. In the sham control group, three rats were killed 2 days after sham treatment and the other three rats were killed 3 days after sham treatment under anesthesia. Rats in the IR-2D and IR-3D groups were killed 2 days and 3 days after ischemia, respectively. Immediately before the rats were killed, the brains were frozen *in situ* according to the method of Pontén et al.[21]

After decapitation of the rats the brain was chiseled out and stored at -40℃. Thereafter the hippocampus was isolated at -20℃ and stored again at -40℃ for later analysis. The frozen hippocampus was homogenized, and sodium dodecyl sulfate-acrylamide gel electrophoresis and immunoblotting were performed according to the method previously reported.[17][18] The antigen-antibody complex was visualized by the enhanced chemiluminescence western blotting detection kit. The bands of immunoblotting were quantified using an image analyzer (Densitograph AE-6905C, Atto, Tokyo, Japan). Protein concentration was measured according to the method of Lowry et al.[22] using bovine serum albumin as a standard. All antibodies were purchased from Santa Cruz (Santa Cruz, CA), except that α-fodrin antibody from Biomol (Plymouth Meeting, PA) and p-Akt antibody from Cell Signaling (Danvers, MA). The molecular weight standard was purchased from Bio-Rad (Richmond, CA), and the enhanced chemiluminescence western blotting detection kit from Amersham International (Buckinghamshire, United Kingdom).

Statistical analysis

Data are presented as mean±SD. One-way analysis of variance followed by Fisher's post hoc test was used to detect significant difference. A value of $p< 0.05$ is considered statistically significant.

Results

The physiological variables before ischemia (or sham treatment) are shown in Table 1. There were no significant differences in any physiological variables among the groups. Figure 1 shows representative microphotographs of hippocampal CA1 stained by HE or TUNEL. The CA1 neurons in the control group appeared normal (Fig. 1, A-1), whereas the neurons in the IR-2D group were partially damaged, some neurons exhibiting cell shrinkage and clamping of nuclear chromatin (Fig. 1, B-1). At 3 days after ischemia-reperfusion, almost all neurons in the hippocampal CA1 were damaged, normal neurons being

Table 1 Physiological variables in sham-operated (control) and ischemia-reperfusion (IR) groups

Series for Westernblotting

	Hb (g/dl)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	mBP (mmHg)	Glucose (mg/dl)
Control	12.8±0.7	7.35±0.02	42±1	118±8	151±14	120±9
IR-2D	12.0±0.5	7.39±0.03	39±4	122±14	150±16	119±15
IR-3D	12.8±0.5	7.36±0.03	41±3	129±8	149±8	115±11

Series for Histopathology

	Hb (g/dl)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	mBP (mmHg)	Glucose (mg/dl)
Control	13.4±0.6	7.40±0.02	40±1	118±8	170±16	117±7
IR-2D	13.4±0.9	7.37±0.04	42±2	118±6	149±9	119±5
IR-3D	13.4±1.1	7.37±0.02	39±1	126±13	149±14	119±18

Data are mean±SD (n=6 per group). Upper and lower tabulated data are for the series of experiment for Westernblotting and Histopathological evaluation, respectively.

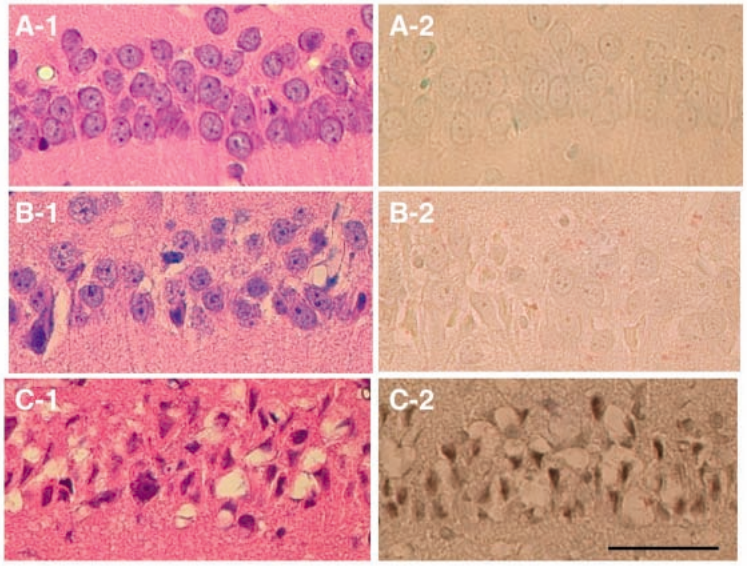

Fig. 1 Coronal section of rat hippocampus, sham controls (A-1, A-2), 2 days (B-1, B-2) and 3 days (C-1, C-2) after ischemia-reperfusion. In sham control, regularly arranged normal neurons stained by HE (A-1) with no TUNEL positive cells (A-2). Neurons were partially damaged (B-1) with some TUNEL positive cells (B-2) 2 days after ischemia-reperfusion. Neurons were shrunk or lost (C-1) with marked TUNEL positive staining (C-2) 3 days after ischemia-reperfusion.
Scale bar = 50 μm

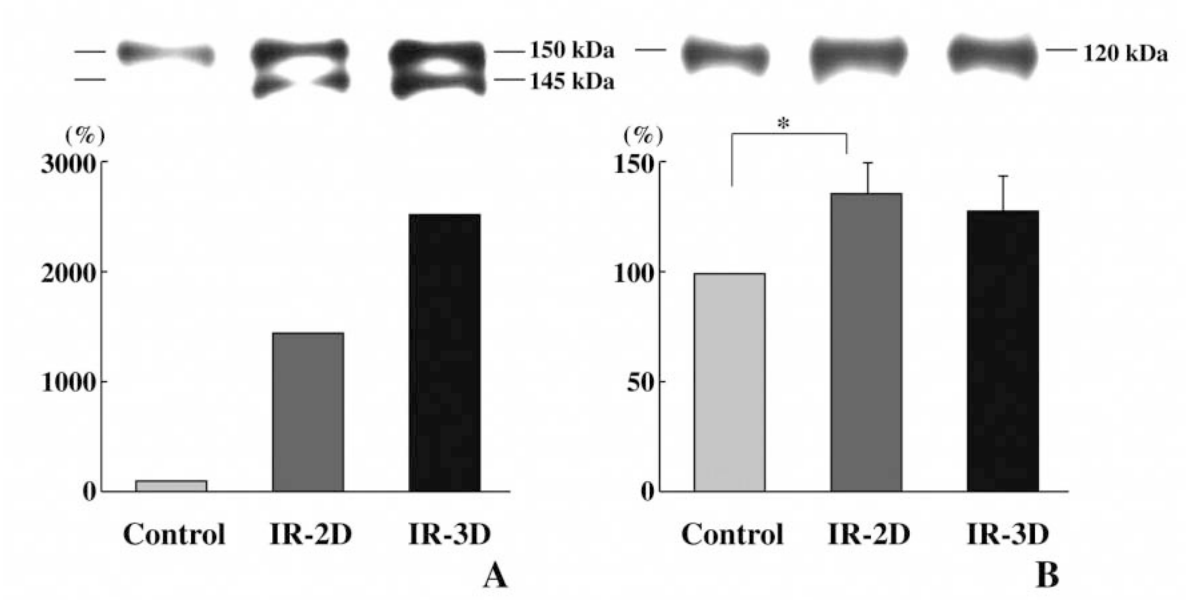

Fig. 2 The amounts of α-fodrin BDPs (145-,150-kDa, and 120-kDa). The bands of immunoblotting are shown in upper panels with the results of their analysis in lower panels. For sham control, the blotting band is the one obtained 3 days after sham operation and the bar represents the average value of samples obtained 2 days and 3 days after sham operation. Evidently, the IR-2D and IR-3D group have higher levels of BDPs (145-,150-kDa) of α -fodrin than sham control group (A).120-kDa BDPs also increased significantly after ischemia-reperfusion (B).
*Significantly different from the control group ($P < 0.05$).

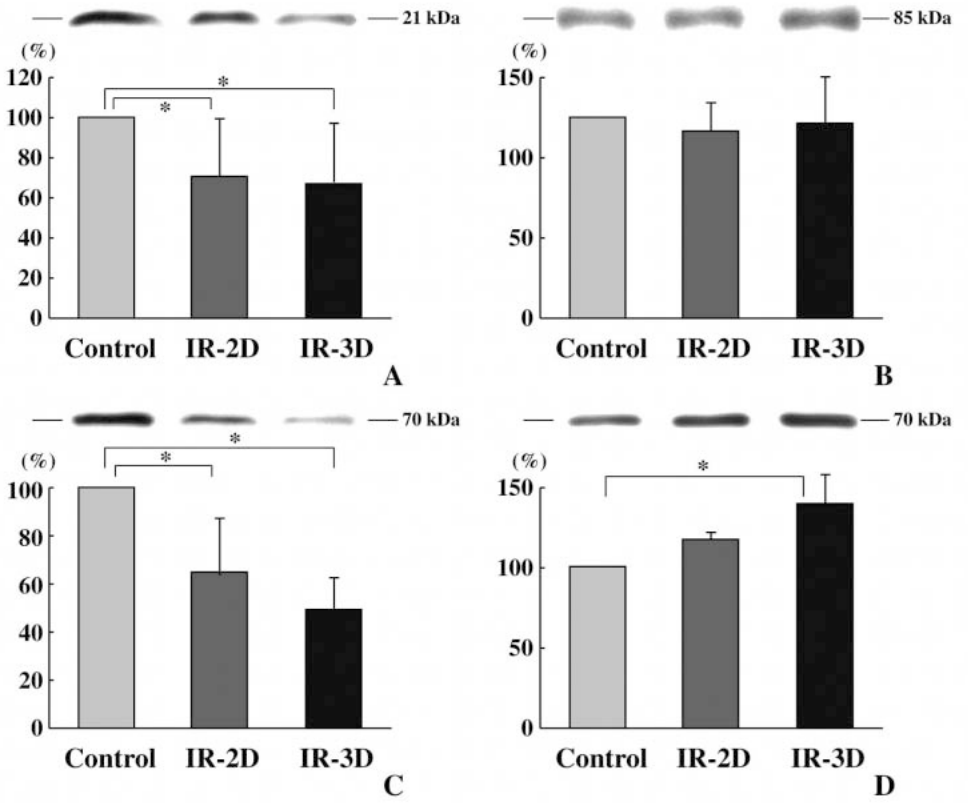

Fig. 3 The amounts of H-Ras, PI-3 kinase, p-Akt and Akt1.
The bands of immunoblotting are shown in upper panels with the results of their analysis in lower panels. For sham control, the blotting band is the one obtained 3 days after sham operation and the bar represents the average value of samples obtained 2 days and 3 days after sham operation. The level of H-Ras has significantly decreased in the IR-2D and IR-3D compared to sham control group (A), but no significant difference was shown in PI-3 kinase (p85a) among the three groups (B). The amount of p-Akt (C) was decreased time-dependently, while Akt1 (D) was increased 3 days after ischemia-reperfusion.
*Significantly different from the control group ($P < 0.05$).

scarcely observed (Fig. 1, C-1). There were no TUNEL positive cells in the control group (Fig. 1, A-2). TUNEL positive cells started to be observed in the IR-2D group (Fig. 1, B-2) and became prominent in the IR-3D group (Fig. 1, C-2). The numbers of normal neurons/TUNEL positive neurons in the hippocampal CA1 (1mm) in the control, IR-2D and IR-3D groups were 148 ± 8/0±0, 117± 32/10±11, and 0±0/116±13, respectively.

The results of the immunoblot analysis are shown in Figure 2 and Figure 3. The breakdown-products (BDPs) of α-fodrin were significantly increased time-dependently after reperfusion, the levels of BDPs of α-fodrin (145-, 150-kDa fragment) being increased almost 25 folds in the IR-3D group as compared to the control (Fig. 2A). The 120-kDa fragment of BDPs of α-fodrin were increased at 2 days after ischemia-reperfusion (Fig. 2B).

The results of the H-Ras signaling cascades are shown in Figure 3. The level of H-Ras was significantly decreased in the IR-2D and IR-3D groups compared to the control group (Fig. 3A). There was no significant difference in PI-3 kinase (p85α) among the three groups (Fig. 3B). The amount of p-Akt (Ser473) was significantly decreased time-dependently

after ischemia-reperfusion, reaching to 50% of the control 3 days after ischemia-reperfusion (Fig. 3C), while Akt (Akt1) was increased in the IR-3D group (Fig. 3D).

Discussion

It has been shown that the CA1 pyramidal neurons in the hippocampus are selectively vulnerable to transient ischemia,[1-3] and they undergo apoptotic cell death.[4-6] The present study reconfirmed this, and the CA1 pyramidal neurons were selectively and markedly damaged, starting 2 days after and being maturated 3 days after 15 min of forebrain ischemia. Most of the CA1 pyramidal neurons were damaged 3 days after ischemia and revealed TUNEL positive (Fig.1), the staining being used widely to detect DNA fragments *in situ* as a marker of apoptosis.[23] The delayed neuronal death with apoptotic nature observed in the present study is in accordance with the previous reports,[4-6] despite of some variations in the severity of ischemia (duration and systemic hypotension). In considering the decrease in the total number of neurons in IR-3D group, approximately 20% of neurons were lost, being undefined whether those were necrotic or apoptotic type.

In the present study we analyzed α-fodrin breakdown as a marker of cytoskeletal damage. The α-fodrin (240-kDa) is proteolyzed into several fragments, called breakdown products (BDPs).[19] Our results showed that α-fodrin BDPs (145-, 150-kDa and 120- kDa) increased at 2 days and 3 days after ischemia-reperfusion, indicating that biochemical neuronal derangement has been occurring at 2 days after ischemia despite the inconsistent neuronal damage observed by light microscope. The disproportional increase (relative values) in α-fodrin BDPs (145-, 150-kDa) compared to the histological damage at 2 days after ischemia-reperfusion may be due in part to very low baseline values of α-fodrin BDPs and to the limited sensitivity of histological evaluation with light microscopy.

In the present study, we focused on the signal transduction pathway possibly related to apoptosis, namely Ras and its signal cascades. One important signaling cascade modulating apoptotic cell death is Ras-PI3K-Akt/PKB system. Ras interacts directly with the catalytic subunit of PI-3 kinase in a GTP-dependent manner through the Ras effector site. Ras can probably regulate PI-3 kinase, providing a point of divergence in signaling pathways downstream of Ras.[14] Survivin, one of the inhibitors of apoptosis proteins (IAPs), is up-regulated by H-Ras, which is mediated by PI-3 kinase activity.[24] The present study showed that H-Ras in CA1 pyramidal neurons in the hippocampus decreased significantly 2 and 3 days after ischemia-reperfusion in association with decreased activity of Akt (p-Akt). This could be related to the development of apoptotic cell death observed in the hippocampal CA1. Activation of Akt/protein kinase B has been reported to prevent apoptosis in several cell types.[25-27] Critical to the understanding of the regulation of Akt in the cells was the findings that Akt kinase activity is induced following PI-3 kinase activation in various growth factor receptor-mediated signaling cascades.[28-30]

Yano et al.[31] examined whether induction of ischemic tolerance resulting from a sub-lethal ischemic insult requires Akt activation and they showed that Akt activation was induced by a sub-lethal ischemic insult in gerbil hippocampus and contributes to neuroprotective effect (ischemic tolerance) in CA1 pyramidal neurons. In their study, after lethal ischemia, phosphorylation of Akt-Ser-473 showed a marked decrease in gerbils. Changes in Akt-Ser-473 phosphorylation were correlated with changes in Akt activities, as measured by an in vitro kinase assay. Sub-lethal ischemia gradually and persistently stimulated phosphorylation of Akt-Ser-473 in the hippocampal CA1 region after reperfusion and protected against the neuronal damage after subsequent otherwise lethal ischemia. Our results that p-Akt has decreased in association with decrease of Ras 2 and 3 days after ischemia-reperfusion are compatible with the decrease in p-Akt after lethal ischemia in the study by Yano et al.[31] In the present study, PI-3 kinase did not show any significant change and Akt (Akt1) increased, which revealed dissociation with a decrease in Akt activity (p-Akt). We have no clear explanation for these findings. This remains to be clarified in the future. Also, in

the present study, limited cascades of multitude downstream signaling pathway of Ras were examined, which needs to be evaluated together with examining whether modification of Ras attenuates neuronal damage induced by ischemia.

In summary, we showed that the CA1 pyramidal neurons were selectively damaged 3 days after 15 min of forebrain ischemia and that this delayed neuronal death were characterized by an apoptotic nature and associated with an inhibition of the Ras-Akt pathway. The present study suggests that down-regulation of the Ras-Akt pathway may be one of the mechanisms for delayed neuronal death in the hippocampal CA1 neurons induced by transient ischemia-reperfusion. Further studies are necessary to examine the effect of modification of Ras signaling cascades on ischemic neuronal damage.

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