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Effect of ischemic preconditioning on the expression of c-myb in the CA1 region of the gerbil hippocampus after ischemia/reperfusion injury

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ABSTRACT

Objective(s): In the present study, we investigated the effect of ischemic preconditioning (IPC) on c-myb immunoreactivity as well as neuronal damage/death after a subsequent lethal transient ischemia in gerbils.
Materials and Methods: IPC was subjected to a 2 min sublethal ischemia and a lethal transient ischemia was given 5 min transient ischemia. The animals in all of the groups were given recovery times of 1 day, 2 days and 5 days and we examined change in c-myb immunoreactivity as well as neuronal damage/death in the hippocampus induced by a lethal transient ischemia.
***Results*:** A lethal transient ischemia induced a significant loss of cells in the stratum pyramidale (SP) of the hippocampal CA1 region at 5 days post-ischemia, and this insult showed that c-myb immunoreactivity in cells of the SP of the CA1 region was significantly decreased at 2 days post-ischemia and disappeared at 5 days post-ischemia. However, IPC effectively prevented the neuronal loss in the SP and showed that c-myb immunoreactivity was constitutively maintained in the SP after a lethal transient ischemia.
Conclusion: Our results show that a lethal transient ischemia significantly decreased c-myb immunoreactivity in the SP of the CA1 region and that IPC well preserved c-myb immunoreactivity in the SP of the CA1 region. We suggest that the maintenance of c-myb might be related with IPC-mediated neuroprotection after a lethal ischemic insult.

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Introduction

Transient global cerebral ischemia causes irreversible neuronal damage in specific brain regions such as the hippocampus (1). The Mongolian gerbil is a good animal model to investigate the molecular mechanism of selective neuronal death following transient global cerebral ischemia (2, 3) because gerbils lack the posterior communicating arteries in the Willis' circle (4-6). Thus, five min or more periods of transient ischemia usually kill pyramidal neurons in the CA1 region of the hippocampus 4 or 5 days after transient ischemic insult (7).

In the brain, ischemic preconditioning (IPC) as a strategy to reduce ischemia-reperfusion injury has been firmly established (8). Kitagawa *et al* (9) firstly introduced the concept of ischemic tolerance in the brain, thereafter, further studies using IPC have been demonstrated in animal models of global and focal cerebral ischemia (10-13). Also, we recently reported that IPC (2 min period of transient ischemia), which was applied 1 day before 5 min of ischemia, kept about 95% of pyramidal neurons in the hippocampal CA1 region from transient cerebral

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ischemic insult (14, 15), although mechanisms of IPC-mediated neuroprotection are not fully understood yet.

A pleiotropic transcription factor c-myb, which has been most extensively studied among *myb* gene family, is known to play pivotal roles in regulating multiple cellular and tissue processes including cellular proliferation, differentiation, growth, metabolism, and apoptosis (16, 17). The basal level of c-myb protein is relatively low in the mouse hippocampus (18) and c-myb is constitutively expressed in many different neuron types in the adult rat brain (19). Although it has been suggested that c-myb might play a role in normal physiology of the brain, until now, a few studies regarding the expression and change of c-myb in some pathological states have been reported. Shin *et al* (20) first investigated change in c-myb immunoreactivity in the brain of a transgenic mouse expressing a human copper/zinc superoxide dismutase (Cu/Zn SOD) mutation. In addition, Jeon *et al* (18) showed the delayed and protracted induction of c-myb in the hippocampus of the mouse with kainic acid-induced seizure and suggested that the induction of c-myb might be associated with neuronal loss and/or astrogliosis in this model. Furthermore, we previously observed that ischemia-induced change in c-myb was associated with the delayed neuronal death in ischemic gerbil hippocampus (21). On the basis of these findings, we assume that c-myb plays crucial roles in neuronal damage/death following cerebral ischemic insults.

To the best of our knowledge, anyway, the expression pattern of c-myb protein in IPC-induced hippocampus following transient cerebral ischemia has not been studied. Thus, this study was performed to investigate effects of IPC on cellular localization and changes of c-myb in IPC-induced hippocampus, which is an important structure for studies regarding neuronal damage following a subsequent transient cerebral ischemia in the gerbil, which is a good animal for studies on mechanisms of neuronal damage/death induced by transient cerebral ischemia (22, 23).

Materials and Methods

Experimental animals

As we described previously (24), 140 of Mongolian gerbils (total number = 140, body weight 65-75 g, 6 months of age) were obtained from the Experimental Animal Center, Kangwon National University, Chunchon, South Korea. The animals were housed in a conventional state under adequate temperature (23ºC) and humidity (60%). All the experimental protocols were approved (approval no. KW-130424-1) by the Institutional Animal Care and Use Committee (IACUC) at Kangwon University and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011).

Experimental groups and ischemic surgery

As we previously described (24), gerbils were divided into four groups (n = 14 at each point in time in each group): (1) sham-operated-group, which was exposed bilateral common carotid arteries and no ischemia was given (sham-operation); (2) ischemia-operated-group, which was given 5 min of transient cerebral ischemia; (3) IPC plus sham-operated-group (IPC+sham-operated-group), which was subjected to a 2-min sublethal ischemia prior to sham-operation and given no ischemia; and (4) IPC+ischemia-operated-group, which was subjected to a 2 min sublethal ischemia and given 5 min transient ischemia. The IPC paradigm has been proven to be very effective at protecting neurons from ischemic insult in this animal model (25). The animals in all of the groups were given recovery times of 1 day, 2 days and 5 days, because pyramidal neurons in the hippocampal CA1 region do not die until 3 days and begin to die 4 days after ischemia/reperfusion (25).

Transient cerebral ischemia was developed according to our method (26). In brief, the experimental animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Ischemia was induced by occluding the arteries with non-traumatic aneurysm clips (Yasargil FE 723K, Aesculap, Tuttlingen, Germany). After 2 or 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The body (rectal) temperature under free-regulating or normothermic (37 ± 0.5ºC) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and maintained using a thermometric blanket before, during and after the surgery until the animals completely recovered from anesthesia. Thereafter, animals were kept on the thermal incubator (temperature, 23 °C; humidity, 60%) (Mirae Medical Industry, Seoul, South Korea) to maintain the body temperature of animals until the animals were sacrificed.

Cresyl violet (CV) and Fluoro-Jade B (F-J B) histofluorescence staining

As we previously described (24), briefly, the gerbils were deeply anesthetized with pentobarbital sodium (30 mg/kg, IP) (JW pharmaceutical, Seoul, Korea) and perfused through the left ventricle with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). The brain tissues were embedded in tissue-freezing medium and serially sectioned into 30 μm coronal sections in a cryostat (Leica, Wetzlar, Germany).

To investigate neuronal damage in the hippocampus after ischemia-reperfusion, CV and F-J B histofluorescence staining were performed as we described previously (27). In brief, for CV staining, the sections were stained with 1.0% (w/v) cresyl violet acetate (Sigma–Aldrich, St. Louis, MO, USA), dehydrated and mounted with Canada balsam (Kanto chemical, Tokyo, Japan). For F-J B histofluorescence, the sections were immersed in a 0.0004% F-J B (Histochem, Jefferson, AR, USA) staining solution. After washing, the sections were examined using an epifluorescent microscope (Carl Zeiss, Göttingen, Germany) with blue (450-490 nm) excitation light and a barrier filter.

Immunohistochemistry for neuronal nuclei (NeuN) and c-myb

Immunohistochemical staining were carried out according to our method (27). Briefly, the sections were blocked with 10% normal goat serum in 0.05 M PBS followed by staining with primary mouse anti-NeuN (a neuron-specific soluble nuclear antigen) (diluted 1:1,000, Chemicon International, Temecula, CA, USA) and rabbit anti-c-myb (diluted 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were next incubated with the secondary antibodies (Vector Laboratories Inc., Burlingame, CA, USA) and were developed using Vectastain ABC (Vector Laboratories Inc.). And they were visualized with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer. In order to establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of primary antibody. The negative control resulted in the absence of immunoreactivity in any structures.

Western blot analysis

Change in c-myb protein level in the ischemic CA1 region was done according to our method (24). In brief, hippocampal CA1 regions (n = 7 at each point in time) dissected and homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, USA). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Then, each aliquots were loaded onto a 12.5% polyacryamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY). The membranes were incubated with rabbit anti-c-myb antiserum (1:2,000, Santa Cruz Biotechnology), peroxidase-conjugated goat anti-rabbit IgG (Sigma–Aldrich) and an ECL kit (Pierce Chemical).

Data analysis

Numbers of NeuN-immunoreactive and F-J B-positive cells were counted as we described previously (24). Briefly, the brain sections were selected according to anatomical landmarks corresponding to AP from −1.4 to −2.2 mm of gerbil brain atlas, and the cells were counted in a 200×200 μm square at the center of the CA1 region including the stratum pyramidale. Cell counts were obtained by averaging the total cell numbers from each animal per group. A ratio of the averaging the total cell numbers was calibrated as % of the sham-operated group (NeuN-immunoreactive cells) or ischemia-operated group (F-J B-positive cells).

Change in c-myb protein level in the CA1 region was analyzed according to our method (27). briefly, the result of western blot analysis was scanned, and the quantification of the analysis was done using Scion Image software (Scion Corp., Frederick, MD), which was used to analyze relative optical density (ROD): A ratio of the ROD was calibrated as % of the sham-operated group.

Statistical analysis

All data are presented as mean ± S.E.M. A multiple-sample comparison was applied to test the differences between groups and days. The differences between groups in same day were assessed by using one-way ANOVA and a Tukey's *post hoc* test. For analysis of time-dependent differences in between the groups, two-way ANOVA were used with the Bonferroni *post hoc*. Statistical significance was considered at $P<0.05$.

Results

CV-positive (CV+) cells

CV+ cells were distributed in all of the subregions of the hippocampus in the sham-operated-group, and the CV+ cells in the stratum pyramidale were pyramidal in shape (Figures 1A and 1B). In the ischemia-operated-group, the morphology of CV+ cells in the stratum pyramidale was not changed at 2 days post-ischemia (Figures 1E and 1F). However, at 5 days post-ischemia, CV+ cells in the stratum pyramidale was significantly decreased in the CA1 region, not the CA2/3 region, compared with those of the sham-operated-group (Figures 1I and 1J), and the CV+ cells of the stratum pyramidale were shrunken and contained dark and polygonal nuclei (Figure 1J).

In the IPC+sham-operated-group, the distribution pattern of CV+ cells in the hippocampus was similar to that in the sham-operated-group (Figures 1C and 1D). In the IPC+ischemia-operated-group, the distribution pattern of CV+ cells in the stratum pyramidale of the CA1 region was also similar to that in the IPC+sham-operated-group (Figures 1G, 1H, 1K and 1L).

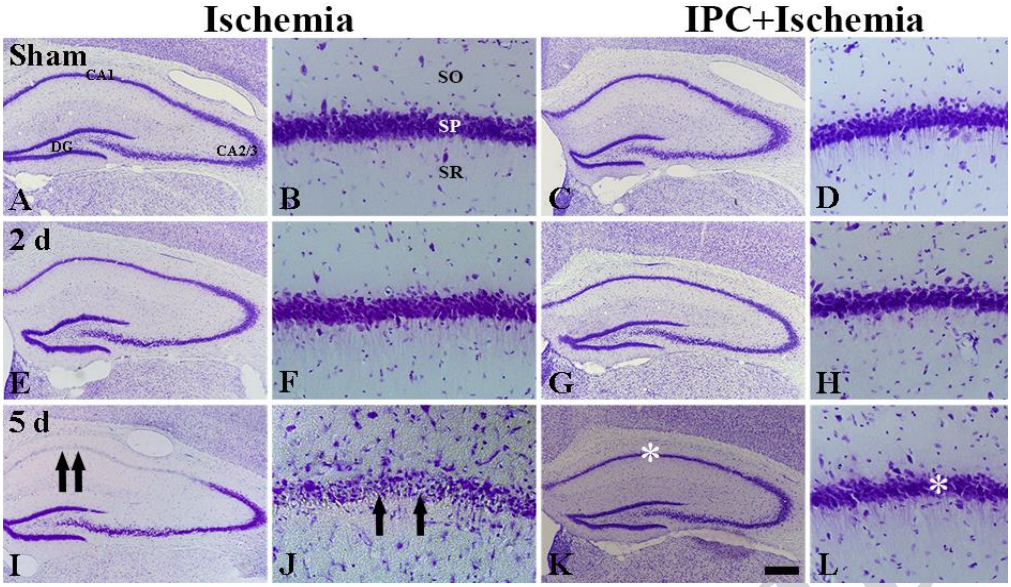

Figure 1 Cresyl violet (CV) staining in the hippocampus of the ischemia-operated- (left two columns) and IPC+ischemia-operated- (right two columns) groups at 2 (E – H) and 5 days (I – L) after ischemia-reperfusion. CV^+ cells in the stratum pyramidale (SP, arrows) of the CA1 region are damaged at 5 days post-ischemia in the ischemia-operated-group; however, CV^+ cells (asterisks) in the IPC+ischemia-operated-group are similar to those in the sham-operated-group. SO, stratum oriens; SR, stratum radiatum. Scale bar = 800 μm (A, C, E, G, I and K), 50 μm (B, D, F, H, J and L)

NeuN⁺ and F-J B⁺ cells

In the sham-operated-group, NeuN+ cells were easily detected in the stratum pyramidale of the CA1 region (Table 1, Figure 2A), and no F-J B+ neurons were found in the stratum pyramidale of the CA1 region (Table 1, Figure 2B). In the ischemia-operated-group, we did not find significant change in numbers of NeuN+ and F-J B+ cells in the stratum pyramidale neurons at 2 days post-ischemia (Table 1, Figures 2E and 2F); however, 5 days after ischemia-reperfusion, a significant loss of NeuN+ cells and a significant increase of F-J B+ cells was observed in the stratum pyramidale of the CA1 region (Table 1, Figures 2I and 2J): at this point in time, the mean percentage of NeuN+ cells in the stratum pyramidale was 14.9% of that in the ischemia-sham-operated-group (Table 1).

In the IPC+sham-operated-group, distribution patterns of NeuN+ and F-J B+ cells in the CA1 region were similar to those in the ischemia-sham-operated-group (Table 1, Figures 2C and 2D). In the IPC+ischemia-operated-group, distribution patterns of NeuN+ and F-J B+ cells in the stratum pyramidale were not significantly changed at 2 days post-ischemia compared with that in the IPC+sham-operated-group (Table 1, Figures 2G and 2H). At 5 days post-ischemia, many NeuN+ cells were found in the stratum pyramidale of the CA1 region (Table 1, Figure 2K): 94.5% of CA1 pyramidal neurons were stained with NeuN compared to that in the IPC+sham-operated-group (Table 1). In addition, at this point in time, a few F-J B+ cells were detected in the stratum pyramidale (Table 1, Figure 2L): the mean percentage of the F-J B+ cells in the stratum pyramidale was 10.1% of the ischemia-operated-group (Table 1).

Table 1. Change in the mean number of cells in the stratum pyramidale of the hippocampal CA1 region in the ischemia-operated- and IPC+ischemia-operated-groups

Time after I-R	Group			
	Ischemia		IPC+Ischemia	
	$NeuN^+$	F-J B^+	$NeuN^+$	F-J B^+
Sham	100.0±2.88	0	100.8±3.21	0
1 d	101.7±3.39	0	99.8±3.87	0
2 d	100.6±3.01	0	102.7±3.65	0
5 d	14.9±4.24*	100±2.33*	94.5±3.16*#	

The mean number of $NeuN^+$ and F-J B^+ cells is counted in a 200×200 μm square of the stratum pyramidale of the CA1 region after ischemia-reperfusion (I-R). A ratio of the cell number was calibrated as % of the sham-operated-group ($NeuN^+$ cells) or ischemia-operated-group (F-J B^+ cells) designated as 100 %. (n = 7 per group; $^*P < 0.05$, significantly different from the corresponding sham-group, $^{\#}P < 0.05$, significantly different from the respective pre-time point group)

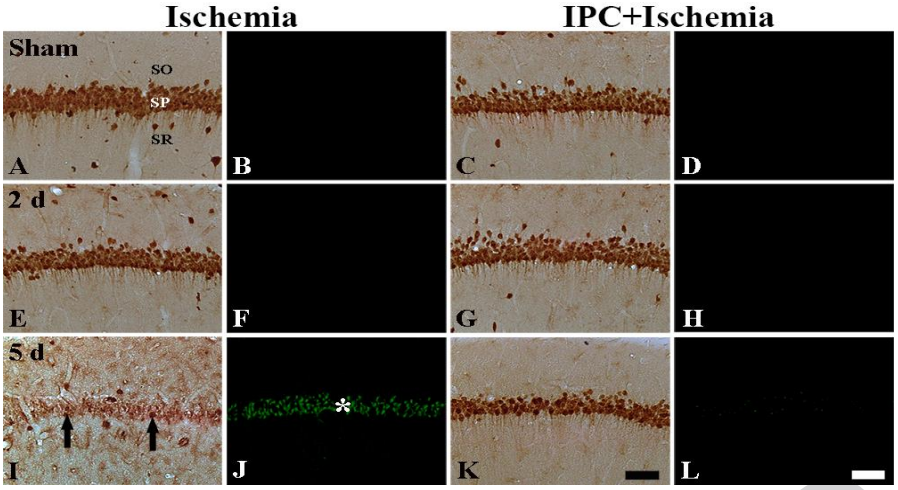

Figure 2. NeuN immunohistochemistry (first and third longitudinal columns) and F-J B histofluorescence staining (second and fourth longitudinal columns) in the CA1 region of the ischemia-operated (left two columns) and IPC+ischemia-operated- (right two columns) groups at 2 (E – H) and 5 days (I – L) after ischemia-reperfusion. In the ischemia-operated-group, a few $NeuN^+$ (arrows) and many F-J B^+ (asterisk) cells are detected in the stratum pyramidale (SP) at 5 days post-ischemia; in the IPC+ischemia-operated-group, NeuN and F-J B staining is similar to that in the sham-operated-group. SO, stratum oriens; SR, stratum radiatum. Scale bar = 50 μm

c-myb immunoreactivity

CA1 region: Strong c-myb immunoreactivity was detected in the stratum pyramidale of the CA1 region (Figure 3A). In the ischemia-operated-group, c-myb immunoreactivity in the stratum pyramidale 1 day after ischemia-reperfusion was not changed (Figure 3C); however, c-myb immunoreactivity in the stratum pyramidale was apparently decreased 2 days after ischemia-reperfusion (Figure 3E). Five days after ischemia-reperfusion, c-myb immunoreactivity in the stratum pyramidale was hardly found of the CA1 region, whereas, strong c-myb immunoreactivity was newly expressed in many cells in the strata oriens and radiatum (Figure 3G).

In the IPC+sham-operated-group, c-myb immunoreactivity in the CA1 region was similar to that in the sham-operated group (Figure 3B). In the IPC+ischemia-operated-group, c-myb immunoreactivity in the stratum pyramidale was not significantly changed until 5 days after ischemia-reperfusion (Figures 3D, 3F and 3H); however, c-myb immunoreactivity was shown in cells in the strata oriens and radiatum 2 and 5 days after ischemia-reperfusion (Figures 3F and 3H).

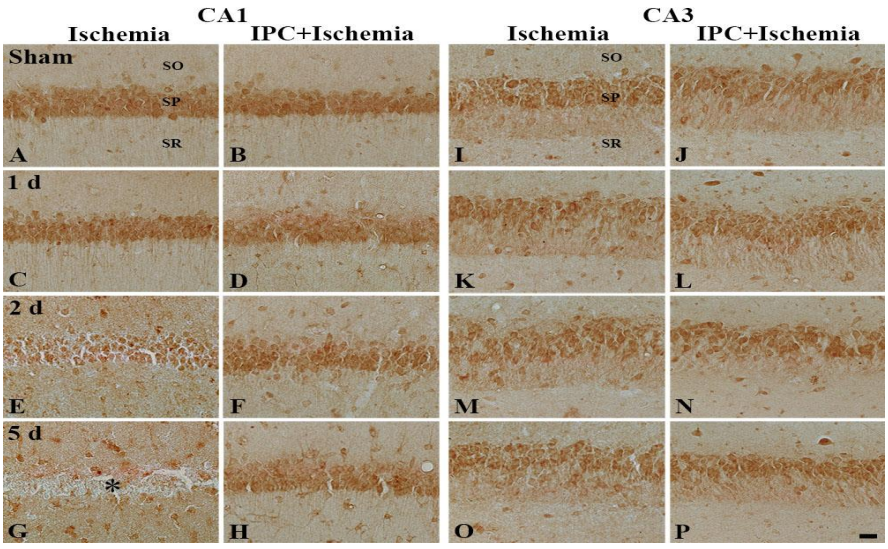

Figure 3. Immunohistochemical staining for c-myb in the CA1 (left two columns) and CA3 (right two columns) regions of the ischemia-operated- (first and third columns) and IPC+ischemia-operated- (second and fourth columns) groups at 1 (C, D), 2 (E, F) and 5 days (G, H) after ischemia-reperfusion. c-myb immunoreactivity in the stratum pyramidale (SP, asterisk) of the CA1 region is barely detected at 5 days post-ischemia; in the IPC+sham- and operated-groups, c-myb immunoreactivity is similar to that in the sham-operated-group. c-myb immunoreactivity in the CA3 region is not changed in all of the experimental groups. SO, stratum oriens; SR, stratum radiatum. Scale bar = 50 μm

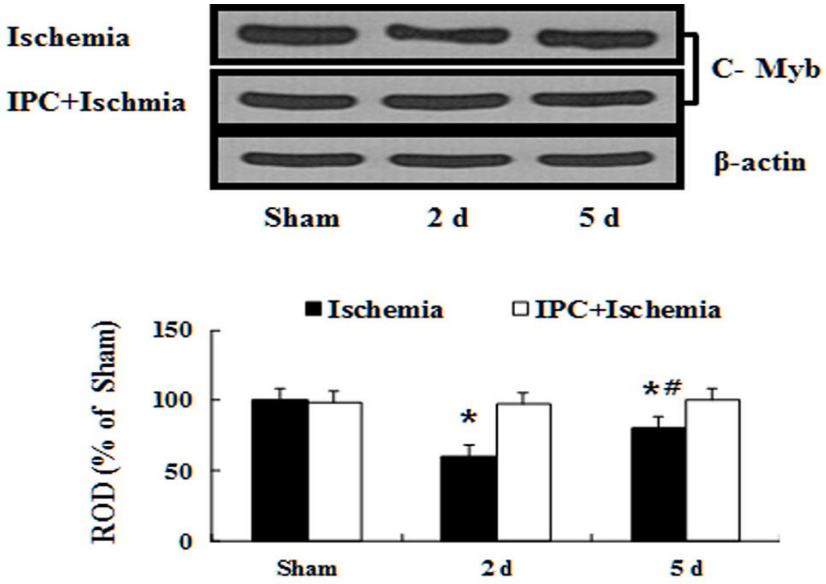

Figure 4. Western blot analysis of c-myb protein in the CA1 region derived from the ischemia-operated and IPC+ischemia-operated-groups. Relative optical density (ROD) as % values of immunoblot band is represented (n = 7 per group; $^{*}P$<0.05, significantly different from the sham-operated-group, $^{\#}P$ < 0.05, significantly different from the pre-time point group). The bars indicate the means ± SEM

CA2/3 region: In the CA2/3 region of the sham-operated-group, c-myb immunoreactivity was strongly detected in the stratum pyramidale (Figure 3I), and c-myb immunoreactivity in the stratum pyramidale was not significantly changed after ischemia-reperfusion (Figures 3K, 3M and 3O). In all of the IPC+sham- and ischemia-operated-groups, c-myb immunoreactivity in the stratum pyramidale was not significantly changed compared with the sham-operated-group (Figures 3J, 3L, 3N and 3P).

Protein levels of c-myb

The change pattern of c-myb protein levels in the hippocampal CA1 after ischemia-reperfusion was generally similar to the immunohistochemical change (Figure 4). Two days after ischemic insult, c-myb protein level significantly decreased, and, 5 days after ischemic insult, c-myb protein level increased CA1 (Figure 4).

In the IPC+ischemia-operated-group as well as in the IPC+sham-operated-group, c-myb protein levels were similar to that in the sham-operated group (Figure 4).

Discussion

In the CNS, neurons in the hippocampal CA1 region are selectively damaged even after a brief ischemic insult. This topographical heterogeneity is known as “selective vulnerability of the brain” (1). Especially, the pyramidal neurons in the CA1 region do not die immediately after transient cerebral ischemia and die over several days; this unique process is termed “delayed neuronal death”. However, pyramidal neurons in the hippocampal CA3 region are much less vulnerable to ischemic insults (28). In this study, we found the delayed neuronal death in the CA1 region at 5 days post-ischemia using CV histochemistry, NeuN immunohistochemistry and F-J B histofluorescence.

IPC, which can be developed by a brief transient ischemia, prevents against ischemic injury following a subsequent longer or lethal transient ischemic insult (29). The first description of IPC in the brain was demonstrated by Kitagawa *et al* (30) in a gerbil model, and similar findings have been reported in rats (31, 32) and mice (33). In the present study, we found that the CA1 pyramidal cells did not die in the IPC-induced hippocampus after transient cerebral ischemia. The neuroprotection was assessed by CV histochemistry, NeuN immunohistochemistry and F-J B fluorescence staining; the last of which is very sensitive to neuronal injury (34). Based on those and our studies, remarkable protections induced by IPC are able to be an attractive target for a potential therapeutic development; however, exact molecular mechanisms regarding IPC-mediated neuroprotection have not been fully understood yet (12).

Although c-myb is associated with diverse biologic processes, such as cellular proliferation, differentiation, growth and apoptosis (16, 17), its exact role in the brain after ischemia-reperfusion has not been elucidated. It was reported that death of cortical and sympathetic neurons in response to DNA damage or NGF withdrawal might be related with a rapid induction of c-myb (35). We previously reported that c-myb immunoreactivity was significantly increased in the stratum pyramidale of the gerbil hippocampal CA1 region 12 hr after ischemia-reperfusion, began to be decreased 2 days after ischemia-reperfusion and was

hardly observed at 5 days post-ischemia (21). In the present study, c-myb immunoreactivity in the CA1 pyramidal neurons was significantly decreased at 2 days post-ischemia and disappeared at 5 days post-ischemia, although c-myb immunoreactivity was not changed 1 day after ischemic insult. On the basis of those and our present studies, the change of c-myb expression in the pyramidal neurons in the CA1 region after ischemic insult may be associated with neuronal death.

On the other hand, in the late 1990's, some experiments showed that human lymphoid and myeloid tumors over-expressed c-myb (36) and that removing c-myb mRNA in cycling hematopoietic cells finally induced cell death (37) and suggested that c-myb is required for the prevention of cell death. Furthermore, Liu *et al* (38) reported that the suppression of c-myb using antisense oligonucleotide and siRNA constructs provided a substantial protection against the death of cultured cortical neurons elicited by either NGF withdrawal or DNA damage and suggested that c-myb might be associated with neuronal death in cortical neurons. In our present study, IPC effectively protected pyramidal neurons of the CA1 region following a lethal ischemic insult and maintained c-myb immunoreactivity in the neurons. Therefore, we insist that c-myb could be another substantial candidate to protect neurons form ischemic insults.

Conclusion

Our present study demonstrates that IPC significantly prevented neuronal death in the hippocampal CA1 region following a lethal transient ischemia in a gerbil model of transient cerebral ischemia and that IPC well preserved c-myb immunoreactivity in pyramidal neurons of the CA1 region. These findings suggest that consistently maintained level of c-myb in CA1 pyramidal neurons following IPC may participate in endogenous protective response to a subsequent ischemic injury.

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Conflict of interest

The authors have no financial conflict of interest.

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