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Expression of Bcl-2 Protein in the Gerbil Hippocampus Following Transient Forebrain Ischemia and Its Modification by Ischemic Preconditioning

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=국문초록=

모래쥐 해마에서 일과성 전뇌 허혈과 허혈 전처치에 의한 Bcl-2 단백의 발현

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목 적 : 신경 세포 손상 과정에는 괴사(necrosis)와 고사(apoptosis)가 모두 관여한다고 알려져 있으나, 허혈 전처치에 의한 허혈 내성 발현 과정에서 고사의 역할에 대해서는 잘 알려져 있지 않다. 허혈 전처치에 의한 허혈 내성 현상과 고사의 연관성을 알아보기 위하여 본 연구를 시행하였다.

실험재료 및 방법 : 모래쥐를 사용한 일과성 전뇌 허혈(transient global ischemia) 모델에서 허혈 전처치에 의한 고사 조절 단백질의 발현을 살펴보았다. 겉보기 수술군과 허혈 전처치를 가하지 않은 단순 허혈 조작군 및 허혈 전처치 후 허혈 조작을 가한 군으로 실험 대상군을 나누었고, 각각 1, 3, 7일 후 해마(hippocampus)의 CA1 영역에서 Bcl-2 단백질의 발현을 면역화학적 방법으로 살펴보았다.

결 과 : 허혈 전처치군에서 단순 허혈군에 비해 뚜렷한 신경세포 보호가 관찰되었고, 허혈 전처치 후 허혈 내성이 유발된 군에서 고사 억제 물질로 알려진 Bcl-2 단백질의 발현이 CA1 영역에서 1, 3일째 나타났다.

결 론 : 본 연구에서 고사 억제 단백질인 Bcl-2의 발현이 허혈 전처치에 의한 허혈 내성의 발현 기전에 관여되며, 그 작용기전은 아직 명확하지 않지만 허혈 내성의 발현에도 고사가 중요한 역할을 할 가능성이 높음을 확인 할 수 있었다.

중심 단어 : Ischemic preconditioning · Iischemic tolerance · Apoptosis · Bcl-2.

Introduction

It is well known that ischemic preconditioning induces tolerance and protects brain from subsequent ischemia[1)2)]. However, the mechanisms by which preconditioning prevents lethal cell injury have not been clearly elucidated yet.

Recently, it is becoming evident that both apoptosis and necrosis, two distinctly different types of

cell death, are linked to ischemic cell death[3]. Necrosis is characterized by rapid cell swelling, plasma membrane breakdown, and a concomitant inflammatory response. In contrast, apoptosis is identified by enzymatic internucleosomal degradation of chromatin in the absence of membrane rupture and inflammatory response. Internucleosomal cleavage of the genomic DNA, which is considered a hallmark of apoptosis, occurs in ischemic brain following focal and global ischemia[4][5]. Unlike necrosis, apoptosis is controlled by specific genetic programs, which can be activated by a wide range of physiological and pathological events. Among the apoptosis regulating molecules studied in neuronal cell death and survival, the Bcl-2 family has attracted the most attention. The best characterized of these are coded for by the antiapoptotic gene *bcl-2*.

One group has recently shown that ischemic preconditioning reduces ischemic injury by decreasing apoptosis in the heart[6]. Although ischemic tolerance in brain is different from the heart, it is possible that much longer time frame required for induction of tolerance in brain and its persistence over days is more compatible with new protein synthesis, which confer apoptosis, as a mechanism. To test the hypothesis that ischemic preconditioning reduces irreversible cerebral ischemic injury in part by decreasing apoptosis, changes in apoptosis-specific protein bcl-2 were studied in gerbil model of transient global ischemia with and without ischemic preconditioning, in which delayed neuronal death cell death occurred in the hippocampal CA1 region.

Material and Methods

1. Gerbil global ischemia model

Adult male Mongolian gerbils(*Meriones unguiculatus*) weighing 50 to 80g were kept at constant room temperature with a 12-hour light/dark cycle for at least 7 days before surgery, and had free access to food and water before and after surgery. Under ether anesthesia, common carotid arteries were exposed bilaterally through a midline incision in the neck and were occluded by using miniature aneurysmal clips. At the prescheduled time, the clips were released and patency of the arteries was ascertained before closure of skin incision. For the second occlusion of the common carotid arteries, the skin incision was opened again under ether anesthesia and miniature aneurys-mal clips were applied. The rectal temperature was kept at 37.0±0.5℃ by means of a heating pad. Animals that showed seizure or severe respiratory failure were not included in this study.

Gerbils were assigned to the following three experimental groups : (1) the sham operated control group, (2) the non-preconditioning group with 5-minute ischemia, (3) the ischemic-preconditioning group with a pretreatment with 2-minute ischemia 2 days before 5-minute ischemia. The reperfusion periods of 1 day, 3 days, or 7 days were investigated for ischemic treatment groups. Six gerbils were used for each group.

2. Tissue histology

At a prescheduled time, each gerbil was anesthetized with pentobarbital(50ml/kg) and the brain was fixed by transcardial perfusion with heparinized phosphate buffered saline(PBS ; pH7.5), followed by periodate lysine paraformaldehyde(PLP). The brain was removed and placed in PLP solution for 24 hours at 4℃. A standard block, corresponding to coronal section interaural 5.7－6.7mm, bregma －3.3 to －2.3 mm, was obtained using a rodent brain matrix. Blocks were embedded in wax. The adjacent 5μm-thick section was mounted on a poly-L-lysine treated slide and air-dried on the 37℃ heat plate at overnight. They were stained with H&E. Ischemic neuronal damage was determined by counting the number of surviving pyramidal neurons in the entire CA1 subfield of the hippocampus. Neurons with a sharply delineated nucleus and a clearly distinguishable nucleolus without eosinophilic cytoplasms were counted. The average of right and left neuronal densities was calculated for each gerbil as described by Kirino, *et al*[7]. The light microscopic identification of apoptosis depended upon the recognition of rounded or oval shaped apoptotic bodies, typically intensely dark

purple-blue masses.

3. Immunohistochemistry

Immunostaining was performed by using the avidin-biotin-alkaline phospatase complex(ABC) method. All sections were incubated in 10% normal goat serum(NGS) in PBS for 1 hour at room temperature and were then incubated in antibodies against Bcl-2(Ab-2)(rabbit polyclonal IgG, Oncogene Research Products, CA, USA) diluted 1 : 25 in PBS containing 3% NGS for 10－12 h at 4℃. The following immunohistochemical procedures were carried out at room temperature. After being washed in PBS for 30 minutes(3×10min), sections were incubated in biotinylated goat anti-goat and anti-rabbit IgG(Vector Laboratories, Burlingame, CA, USA) in PBS for 2 hours and then washed in PBS for 30 minutes(3×10min). They were then incubated for 2 hours in ABC solution in PBS. After being washed in two changes of PBS and three changes of 0.05M Tris-HCL buffer(TB, pH7.4) for 50 minutes(5×10min). They were then incubated in 0.02% Fast red substrate in 100mM Tris-HCL-buffer(pH 8.5) containing 0.05% levamisole and washed in three changes of PBS and counterstained by Mayer's Hematoxylin. For light microscopical observation, the immunostained sections were washed in three changes of PBS, mounted in glycerol.

4. Statistical analysis

All data were subjected to statistical analysis using Bonferroni unpaired test using SPSS 9.0 for windows. A two-tailed probability value of <0.05 was considered significant.

Results

1. Histology

The results are summarized in Table 1. In general, a decrease in neuronal density could be observed in the hippocampal CA1 region both in single ischemia (SI) and preconditioned ischemia group(PI) compared with sham-operated group at any of the time point studied. However, PI group exhibited significant protective effects in the CA1 of the hippocampus compared with SI group at 3 and 7 days($p<0.05$).

Table 1. Time course of neuronal density after transient global ischemia with and without preconditioning

	Reperfusion time		
	1 day	3 days	7 days
Neuronal density in the CA1 region(cells/mm)			
Sham	–	–	217±9
SI group	198±30	105±38[a]	25±12[a]
PI group	195±8	161±34[a]	127±54[a]

Values are mean±SD(n=6). SI and PI groups indicates the group without preconditioning and the group with preconditioning, respectively, before transient global ischemia for 5 minutes. [a]$p<0.05$ indicates the difference from the sham controls(one-factor analysis of variance followed by th Bonferroni multiple comparison test).

2. Immunohistochemistry

It is known that adult brain usually shows no immunohistochemically detectable expression of Bcl-2. In our present study, Bcl-2 immunoreactivity in hippocampal CA1 region was not significantly detected in sham operated or in SI group at any of the time points studied. However, analysis of PI group revealed weak, but detectable Bcl-2 immunoreactivity 1 day after reperfusion(Fig. 1a). The immunoreactivity was slightly accentuated at 3 days(Fig 1b), but disappeared at 7 days.

Discussion

Recently, activation of the apoptotic process has been described as a key phenomenon for delayed neuronal death, based on the studies showing the detection of DNA fragmentation in the CA1 hippocampal neurons following global ischemia and neural protection offered by protein synthesis inhibition[8,9].

Among the known regulators of apoptosis, the bcl-2 protooncogene is best defined. Bcl-2 protein stands out for its ability to suppress cell death induced by wide variety of insults and stimuli, including the nervous system. Recent studies show that expression of bcl-2 oncoprotein in the CA1 area following brief ischemia is closely related to the tolerance to ischemia-

induced neuronal death and the bcl-2 gene is induced in neurons that survive cerebral ischemia[10][11].

Ischemic tolerance phenomenon found in brain is quite intriguing in view of the induction of tolerance to ischemia in neuronal cells. The molecular mechanism contributing to the 'ischemic tolerance' is fascinating and synthesis of heat shock proteins(HSPs) is one of the most probable candidates[12][13]. Recently, Piot, *et al*[8] found that ischemic preconditioning reduced irreversible ischemic injury in part by decreasing apoptosis in rat hearts, based on the specific DNA fragmentation and infarct size assessment. Accordingly, hypothesizing that ischemic tolerance in brain reduces irreversible ischemic injury in part by preventing neuronal apoptosis, we studied time-course expression of bcl-2 in gerbil hippocampus following transient forebrain ischemia, with or without ischemic preconditioning.

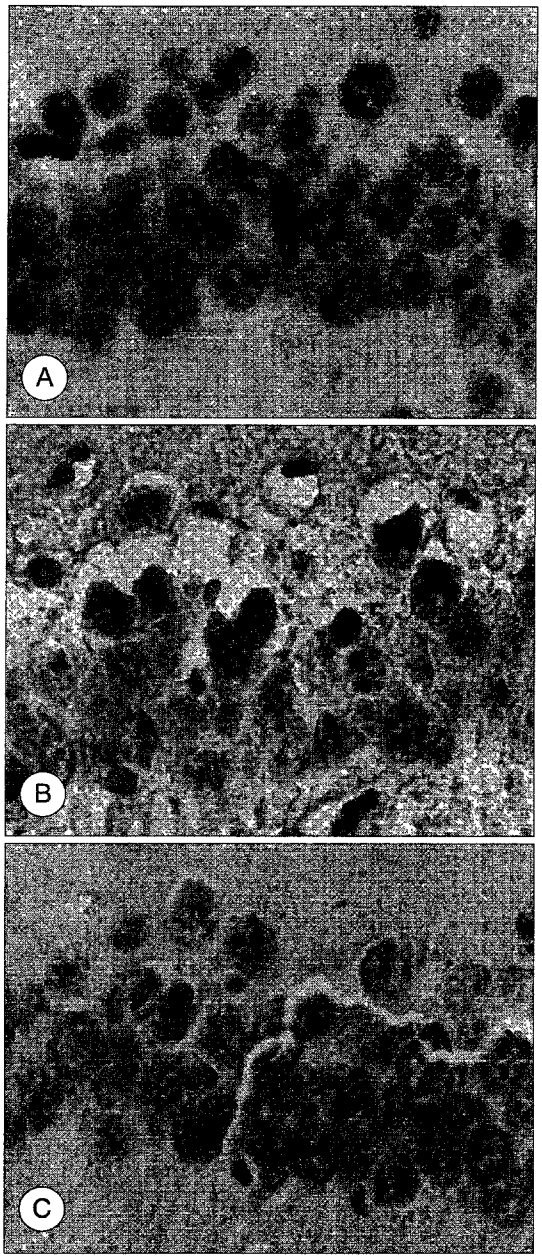

Fig. 1. Light micrographs of the pyramidal neurons in CA1 sector of hippocampus from the gerbil in PI group illustrating Bcl-2 immunoreactivity. Figures are taken in order of 1, 3 and 7 days after reperfusion(A, B, C), respectively. Magnifications : A-C ×200.

The major finding of present study is that the expression of antiapoptotic Bcl-2 oncoprotein was observed in CA1 neurons 1, 3 days after ischemia in PI group. Though many explanations for mechanism contributing to the 'ischemic tolerance' may exist, the data reported here suggest that one potential contributors could be the expression of Bcl-2. Expression of Bcl-2 may promote survival of ischemic neurons in the setting of ischemic tolerance. It is well known that adult brain usually shows no immunohistochemically detectable expression of Bcl-2 protein. Previous immunohistochemistry studies revealed that Bcl-2 immunoreactivity were detected at a high level in CA3 pyramidal neurons at 24 or 72 hours following ischemia. But while expression of Bcl-2 mRNA was induced in CA1, Bcl-2 protein was not expressed[14]. The ability of Bcl-2 protein to inhibit neuronal apoptosis is well documented, but the mechanism for this protection is unclear. Kane, *et al*[11] showed that Bcl-2 expression may prevent necrotic neuronal death as well as apoptosis. This observation suggests that, instead of inhibiting a cellular death program directly, Bcl-2 may modulate a generalized cellular process, such as generation of free radicals, that may contribute to either apoptotic or necrotic death. Recently, it has been reported that the expression of another protein Bcl-x also inhibits apoptosis in the similar manner as Bcl-2. In addition, Bcl-x and Bcl-2 regulate apoptotic process independently[15].

Therefore, it is suggested that another Bcl-2 family, such as Bcl-x, may play a role as an inhibitory factor in the machanism of delayed neuronal death. Although apoptosis and necrosis are considered to be distinct mechanisms of cell death, data from our present study and previous studies for Bcl-2 suggest the possibility of pathways of delayed neuronal cell death may be interrelated.

In summary, in neuronal cells which acquire ischemic tolerance, antiapoptotic Bcl-2 protein play a neuroprotective role. These results support that ischemic preconditioning reduces neuronal death in part by decreasing apoptosis.

Conclusions

It has been demonstrated that ischemic preconditioning prevents delayed neuronal death following transient ischemia. Although both apoptosis and necrosis have been shown to contribute to neuronal cell death, the ability of ischemic preconditioning to prevent apoptosis remains unknown. To test the hypothesis that ischemic preconditioning reduces irreversible cerebral ischemic injury in part by decreasing apoptosis, changes in apoptosis-specific protein bcl-2 were studied in gerbil model of transient global ischemia with and without ischemic preconditioning. The time-course expression of bcl-2 were examined immunohistochemically in the hippocampal CA1 region at 1, 3 and 7 days after ischemia. The expression of antiapoptotic bcl-2 oncoprotein was observed in CA1 neurons 3 days after ischemia in PI group. This study demonstrated that antiapoptotic Bcl-2 protein play a neuroprotective role in neuronal cells which acquire ischemic tolerance. Although the detailed relationship between the function of bcl-2 protein and ischemic tolerance is still unclear, our results provide a new evidence which indicates that ischemic preconditioning reduces neuronal death in part by decreasing apoptosis.

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