Effect of Acquired Tolerance to Ischemia on Focal Infarction in Gerbils

Takatoshi SORIMACHI, Shigekazu TAKEUCHI, Hiroshi ABE,
and Ryuichi TANAKA

Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata

Abstract

The protective effect of prior ischemia on focal cerebral infarction produced by permanent middle cerebral artery (MCA) occlusion was studied in a gerbil model. Forebrain ischemia for 2 and 5 minutes at an interval of 48 hours was used as the prior ischemic insult. Forty-eight hours after the second forebrain ischemia, the MCA was occluded. Progression of ischemic change caused by the MCA occlusion was delayed in the early ischemic period in animals with prior ischemia, as compared to animals without prior ischemia. The mean infarct volume 24 hours after the MCA occlusion was the same in animals with and without prior ischemia. Ischemic tolerance was acquired in this model, but had no effect on the extent of infarction caused by permanent ischemia.

Key words: ischemic tolerance, focal cerebral infarction, microtubule-associated protein 2, heat shock protein 70, gerbil

Introduction

Cells and organisms can acquire tolerance to heat and other physiological stresses.^{15,29)} Non-lethal mild ischemia, if produced 2 days before lethal ischemic stress, provides protection against neuronal damage.^{8,9,11)} Occlusion of both common carotid arteries (CCAs) in gerbils for 5 minutes produces global forebrain ischemia and leads to delayed neuronal death, which is restricted to the CA1 pyramidal neurons of the hippocampus 7 days after recirculation.⁷⁾ In contrast, ischemia for 2 minutes transiently perturbs cellular metabolism, but neuronal death never occurs. Hippocampal CA1 neurons subjected to non-lethal ischemia for 2 minutes can survive ischemia for 5 minutes, which is normally lethal.^{9,11)} Transient focal ischemia reduces neuronal damage caused by succeeding transient forebrain ischemia in rats.⁵⁾ Most previous studies have focused on the effect of preceding ischemia on succeeding transient ischemia, so the effect of preceding ischemia on subsequent infarction caused by permanent ischemia is unclear.

Morphological damage in the early period of

cerebral ischemia was difficult to detect with conventional light microscopic examination until the recent development of immunohistochemical methods for identifying microtubule-associated protein 2 (MAP2), which is one of the most sensitive marker proteins for early ischemic damage.^{3,10)} MAP2 is present in large amounts in the dendrites, where it is associated with the dendritic microtubules and postsynaptic dendrites.¹³⁾ MAP2 is degraded by proteinases immediately after cerebral ischemia, because of changes in the intracellular environment such as increased concentration of calcium ions.^{6,14,17,23,25)} Progressive laminar loss of staining for MAP2 in the cerebral cortex of gerbils occurs.¹⁰⁾ This clear discrimination of the damaged area is useful for the evaluation of the effects of pathophysiological factors and drugs on cerebral ischemia.^{3,10)} Various studies have demonstrated an increase in heat shock protein 70 (HSP70) synthesis in the brain following mild ischemia,^{5,8,20,28)} which suggests a correlation between HSP70 and ischemic tolerance.

The present study investigated the ischemic tolerance phenomenon in the cerebral cortex using a gerbil model of focal infarction induced by middle

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Author's present address: T. Sorimachi, M.D., Department of Neurology, University of Tennessee, Memphis, Tenn., U.S.A.

cerebral artery (MCA) occlusion and immunohistochemical staining for MAP2 and HSP70.

Materials and Methods

I. Animal model

Adult Mongolian gerbils of both sexes, weighing 60–80 g, were used in this study. Each gerbil was lightly anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Both CCAs were exposed through a midline cervical skin incision and anesthesia was then discontinued. The arteries were occluded with small aneurysm clips for 2 minutes. Forty-eight hours later, the CCAs were again occluded for 5 minutes in the same way. In sham-operated animals, both CCAs were exposed but not occluded. Forty-eight hours after the second CCA occlusion or sham operation, the left MCA was occluded. The left MCA was exposed by the method for rats²⁶⁾ with slight modifications.^{1,31)} The animal was anesthetized with halothane and a small craniectomy was made on the posterosuperior aspect of the orbit. The proximal portion of the MCA was then found on the surface of the olfactory tract. Electrocoagulation was used for the MCA occlusion at the lateral border of the olfactory tract. Animals with over 15 minutes of operation time and with massive bleeding during surgery were excluded. Eighty animals underwent MCA occlusion + prior sham operation and 72 underwent MCA occlusion + double ischemic pretreatment. Following the MCA occlusion, anesthesia was discontinued. The rectal temperature was maintained at 36–37°C using a heating lamp during these procedures.

II. MAP2 immunohistochemistry and histological grading

The animals were again anesthetized with ether inhalation and decapitated at 30 minutes, 1 hour, and 2 hours after MCA occlusion (animals with prior sham operation: $n = 22, 26, 32$; animals with prior ischemia: $n = 20, 23, 29$, respectively) for immunohistochemical investigation of MAP2 in the cerebral cortex. Their brains were removed immediately after decapitation, cut into coronal sections (about 5 mm in thickness), and immersion-fixed in a solution consisting of ethanol-glacial acetic acid (19:1) at 4°C. After fixation for 5 hours, each tissue block was dehydrated in ethanol and embedded in paraffin. The 5 μ m-thick cross sections corresponding to approximately 1.5 mm caudal to the bregma according to the stereotactic atlas¹⁶⁾ were stained by the immunohistochemical method for MAP2 using the peroxidase-antiperoxidase method

for light microscopy.¹⁰⁾ Briefly, each deparaffinized tissue section (5 μ m) was incubated with 10% non-immunized goat serum for 6 hours, with anti-MAP2 mouse serum (MAB364; Chemicon, Temecula, Cal., U.S.A.) (1:200) for 1 hour, with secondary antiserum (1:20) for 30 minutes, and with peroxidase-antiperoxidase complex (1:100) for 30 minutes (Vectastatin ABC-HRP; Vector Laboratories, Burlingame, Cal., U.S.A.). The peroxidase reaction was accomplished by incubation in the presence of 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Control sections were incubated with the absorbed primary antiserum. The most severely damaged region in the cerebral cortex was expressed using the following 5 grades based on the histological findings with immunostaining for MAP2 (Fig. 1): grade 0, all layers of the cerebral cortex stained with MAP2; grade 1, loss of immunohistochemical reaction in layers 3 and 4 of the cerebral cortex; grade 2, loss of reaction in layers 3, 4, and 6; grade 3, loss of reaction in layers 3–6; and grade 4, loss of reaction in layers 1–6. Statistical comparisons for histological grades were made by Ridit analysis.

III. Measurement of infarct volume

To estimate infarct volume 24 hours after MCA occlusion, 21 animals were reanesthetized with ether inhalation, and treated by transcatheter perfusion with 2% 2,3,5-triphenyl tetrazolium chloride (TTC). Their brains were removed and fixed with 10% formalin for 24 hours.²²⁾ The infarct area, which was not stained with TTC,²²⁾ was estimated by image analysis of five coronal sections (1.5 mm thickness) corresponding to the stereotactic sections,¹⁶⁾ –2.5, –1, 0.5, 2, and 3.5 mm caudal to the bregma. The infarct volume in the cerebral cortex and caudate putamen was then determined by summing the unstained areas for each of the five sections, multiplied by the section thickness, and expressed as a percentage of the contralateral hemispheric volume. The percentage hemispheric infarct in animals with prior ischemia ($n = 10$) was compared with that in animals with sham operation ($n = 11$) using the Mann-Whitney test.

IV. HSP70 immunohistochemistry

The immunohistochemical technique for detection of HSP70 was described previously.⁸⁾ Forty-eight hours after the second CCA occlusion, four animals were anesthetized with ether inhalation and treated by transcatheter perfusion with 100 mM sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in buffer. The brains were removed and placed in the same fixative overnight at

Fig. 1 Photomicrographs showing histological grades of immunostaining for microtubule-associated protein 2 (MAP2) in the cerebral cortex after middle cerebral artery occlusion. **A:** grade 0. All layers of the cerebral cortex were stained with MAP2. **B:** grade 1. Loss of immunohistochemical reaction in layers 3 and 4 of the cerebral cortex. **C:** grade 2. Loss of reaction in layers 3, 4, and 6. **D:** grade 3. Loss of reaction in layers 3-6. **E:** grade 4. Loss of reaction in layers 1-6. $\times 100$.

4°C, then transferred to 10 mM phosphate-buffered saline (PBS). Coronal sections (50 μ m) cut with a vibratome were reacted immunohistochemically using mouse monoclonal antibody C 92 to HSP70 (RPN 1197; Amersham, Little Chalfont, Buckinghamshire, U.K.). Biotinylated sheep anti-mouse im-

munoglobulin G was used as the second antibody. Sections were incubated with streptavidin "bridge" 1:200 in PBS for 1 hour, followed by biotinylated horseradish peroxidase 1:400 in PBS for 1 hour. The peroxidase was detected with diaminobenzidine. The sections were mounted on gelatine-coated slides

for light microscopic evaluation. Control sections were also examined in the experiment without primary antibody to exclude cross reactivity with anti-mouse secondary antibody and endogenous immunoglobulin.

Results

I. MAP2 immunohistochemistry and histological grades

The effects of prior mild ischemia on neuronal changes caused by the MCA occlusion were assessed by histological grades for MAP2 staining in the cerebral cortex. In all animals, the right cerebral cortex contralateral to the MCA occlusion showed grade 0. The laminar loss of immunoreaction for MAP2 was clearly visualized in the left cerebral cortex of all gerbils, occurring first in layers 3 and 4 (grade 1), second in layer 6 (grade 2), third in layer 5 (grade 3), and fourth in layers 1 and 2 (grade 4) (Table 1). Because the laminar loss occurred heterogeneously in each hemisphere, the most severely damaged region was used for evaluation. Thirty minutes after MCA occlusion, 15 of 22 animals with prior sham operation showed grade 2 in the left cerebral cortex. In contrast, nine of 20 animals with prior ischemia showed grade 1, and only one of 20 animals demonstrated grade 3. One hour after MCA occlusion, 18 of 26 animals with prior sham operation showed grade 3. Eighteen of 23 animals with prior ischemia showed grade 2. Two hours after MCA occlusion, 31 of 32 animals with prior sham operation showed grade 3 or 4, whereas 13 of 29 animals with prior ischemia showed grade 2. The animals with prior ischemia exhibited significantly less damage to neuronal structures than the animals undergoing prior sham operation at 30 minutes, 1 hour, and 2 hours after MCA occlusion (all $p < 0.01$).

II. Infarct volume

Twenty-four hours of the MCA occlusion resulted in a well-recognized infarct in the MCA territory of each animal. The lesion extended over 6 mm in a ros-

tral-caudal direction and included frontal, parietal, and occipital cortices and the lateral caudate putamen. There was a clear uniformity in the distribution of the infarct which was similar in both groups. Comparison of infarct volume showed the mean volume of infarction in the animals with prior ischemia was not significantly different from that in the animals with prior sham operation in both the cerebral cortex ($24.7 \pm 3.8\%$, $n = 10$; $26.0 \pm 5.3\%$, $n = 11$) and caudate putamen ($6.5 \pm 1.5\%$, $n = 10$; $7.3 \pm 1.6\%$, $n = 11$), respectively.

III. HSP70 immunohistochemistry

Five minutes ischemia 48 hours after 2 minutes ischemia induced the expression of HSP70 in the cerebral cortex. Staining for HSP70 was intense in the layers 3 and 4 but faint in the other layers (Fig. 2). Elsewhere in the brain, HSP70 was detected intensely in the hippocampus, and faintly in the habenula and lateral caudate putamen. Staining for HSP70 was negative in the control animals.

Discussion

The present study showed that mild ischemia before permanent MCA occlusion delays the progression of neuronal damage in the cerebral cortex, indicating that ischemic tolerance was acquired in this model after mild ischemia. Since the infarct volume 24 hours after MCA occlusion was the same with and without prior ischemia, the ischemic tolerance was so mild that the final infarct volume was not reduced.

We investigated the effect of preceding forebrain ischemia on focal infarction in gerbils. The reasons why we used gerbils in this experiment are that infarct area produced by MCA occlusion is more reproducible and that complete forebrain ischemia is more easily achieved in gerbils than in rats.^{1,31} We employed bilateral CCA occlusion as preceding ischemia, because no development of collateral circulation supplying the MCA territory can be expected. In comparison with single brief ischemia,

Table 1 Histological grade of neuronal damage caused by permanent middle cerebral artery (MCA) occlusion

Grade	30 min after MCA occlusion		1 hr after MCA occlusion		2 hrs after MCA occlusion	
	Sham operation (n = 22)	Ischemia (n = 20)	Sham operation (n = 26)	Ischemia (n = 23)	Sham operation (n = 32)	Ischemia (n = 29)
1	2	9	0	0	0	0
2	15	10	2	18	1	13
3	5	1	18	3	10	10
4	0	0	6	2	21	6

Fig. 2 Photomicrographs showing immunohistochemical staining for heat shock protein 70 (HSP70) 48 hours after the second common carotid artery occlusion. HSP70 staining was intense in layers 3 and 4 and faint in the other layers of the cerebral cortex in animals undergoing ischemia (upper), but no staining for this particular protein was observed in sham-operated animals (lower). $\times 100$.

double brief ischemia exhibits stronger protection against delayed neuronal death caused by succeeding lethal ischemia.¹¹⁾ The gerbils pretreated with 5 minutes ischemia 48 hours after 2 minutes ischemia were exposed to MCA occlusion 48 hours later. In this time interval, HSP70 staining was observed in the cerebral cortex at the time of the MCA occlusion, and no ischemic change was detected in the cerebral cortex contralateral to the MCA occlusion even with the sensitive immunohistochemical staining for MAP2.

Several mechanisms for ischemic tolerance have been proposed. Ischemic stress causing perturbation of energy metabolism in neuronal cells is required for induction of tolerance. Such ischemic stress also induces expression of HSP70,^{2,5,8,20)} suggesting that HSP70 is important in ischemic toler-

ance. The change in HSP70 staining with time does not correlate well with changes in induced tolerance.⁸⁾ Spreading depression induces ischemic tolerance in the cerebral cortex of rats where HSP70 messenger ribonucleic acid is not expressed.¹²⁾ In our study, expression of HSP70 was intense in layers 3 and 4 of the cerebral cortex but only faint in the other layers. Loss of immunoreaction for MAP2 was delayed in animals with prior ischemia compared to animals without prior ischemia, but the laminar loss of reaction occurred in the same order. Therefore, tolerance was acquired equally in all layers of the cerebral cortex, implying that the expression of HSP70, induced notably in layers 3 and 4, does not correlate well with ischemic tolerance. This suggests that other factors are involved in the ischemic tolerance phenomenon. Another possible mechanism of induced tolerance is that sublethal stress, such as brief ischemia, may facilitate recovery of normal protein synthesis patterns.^{4,19)} Neurons without induced tolerance undergo marked loss of dendritic microtubules after transient ischemia.³⁰⁾ In contrast, neurons with induced tolerance show similar loss of content of dendritic microtubules for 6 hours of the post-ischemic phase, but this returns to near the control level within 24 hours.¹⁹⁾ The reason may be the recovery of free polyribosomes not attached to endoplasmic reticulum, which synthesize proteins for intracellular use.²⁷⁾ Continuous supply of cytoskeletal proteins is essential for regeneration of damaged axons.²¹⁾ Ischemia-induced elevation in intracellular calcium ion concentration and subsequent activation of calcium-dependent phosphatases and proteases lead to dephosphorylation and proteolytic degradation of MAP2.^{6,14,17,23,25)} Delay of MAP2 degradation observed in this study suggested changes in this cascade induced by preceding ischemia.³⁾ These degradation processes might provide feedback to MAP2 expression after transient ischemia by regulation of the MAP2 splicing system.^{18,24)} It can be speculated that changes in the processing system of MAP2 induced by preceding ischemia might cause the functional recovery of microtubules after transient ischemia.

Sublethal ischemic pretreatment delayed the progression of ischemic changes caused by MCA occlusion in the early ischemic period, but did not reduce the final infarct volume. Mild forebrain ischemia used as a prior ischemic insult (5 minutes CCA occlusion 48 hours after 2 minutes CCA occlusion) induced expression of HSP70, especially in layers 3 and 4 of the cerebral cortex, but this did not correlate with the degree of ischemic tolerance which was found in all layers of the cerebral cortex. These findings appear to suggest that another factor might

participate in the ischemic tolerance phenomenon.

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Address reprint requests to: T. Sorimachi, M.D., Department of Neurosurgery, Brain Research Institute, Niigata University, 1-757 Asahi-machi, Niigata 951, Japan.

Commentary

Many experimental reports about the ischemic tolerance phenomenon have been published recently. Almost all of the experimental methods consisted of transient ischemia after preloading transient ischemia. Based on the results of former reports, the authors studied the changes of infarcted area by using permanent MCA occlusion after double transient ischemia. This experimental method is thought to be superior because of the similarity to clinical features. The second advantage of this report is to show the grade of neuronal damage by using MAP2 expression. The results of MAP2 staining clearly showed the delay of progression of ischemic changes after MCA occlusion. This is the interesting point of this report. The appropriate preloading ischemia may achieve reduction of the final infarct volume. To elucidate the ischemic tolerance phenomenon, nerve growth factors such as bFGF and TGF, and nitric oxide, etc. should be examined in the future.

Koki KADOTA, M.D.
Department of Neurosurgery
Faculty of Medicine
Kagoshima University
Kagoshima, Japan

The authors found that prior double forebrain ischemia delayed the progression of ischemic neuronal damage in the cerebral cortex caused by subsequent permanent focal brain ischemia, but did not reduce the final infarct volume. In the pioneer study of Kitagawa et al., 2 minutes of forebrain ischemia was

selected as a non-lethal ischemic stress and 5 minutes of forebrain ischemia as a lethal ischemic stress for hippocampal CA1 neurons (see ref. 11 of this article). However, what duration of forebrain ischemia is non-lethal and sufficient to perturb cellular protein synthesis in neurons of the cerebral cortex is unknown. In the present study, the pretreatment may have been too mild to induce ischemic tolerance in cortical neurons after permanent focal brain ischemia. Most previous studies have stressed the important role of heat shock protein in ischemic tolerance phenomenon, but its definite role in the mechanism is still unknown. Glazier et al. pointed out that prior cortical lesions may exert a protective effect on adjacent subcortical tissue against the subsequent episodes of forebrain ischemia, possible via diffusion of local factors (ref. 5). We have the impression that determination of the mechanism of the ischemic tolerance phenomenon requires study of the roles of non-neuronal cells, such as microglia and astrocytes, which produce many types of neurotrophic factors. Although the results of the present study are challenging and their scientific interpretation difficult, it is our hope that they will inspire further research on the ischemic tolerance phenomenon.

Takahito MIYAZAWA, M.D.
and Katsuji SHIMA, M.D.
Department of Neurosurgery
National Defense Medical College
Tokorozawa, Saitama, Japan.

Dr. Sorimachi and his colleagues have shown in relatively few animals that mild ischemia before middle cerebral artery occlusion delays the progression of neural damage in the cerebral cortex, but did not alter the ultimate size of the final infarct volume. The notion that ischemic stress induces tolerance in the cortex is intriguing, but elusive and is likely to have little practical value. This study suggests that the previously postulated biochemical mechanisms are either incorrect or minimally important. Solid, basic research, such as this work, will continue to be necessary to improve our understanding of the ischemic mechanisms and how to modify them.

Sydney J. PEERLESS, M.D., F.R.C.S.C.
Mercy Neuroscience Institute
Florida, U.S.A.