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'Ischemic tolerance' phenomenon found in the brain

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We investigated the possibility that neuronal cells given a mild ischemic treatment sufficient to perturb the cellular metabolism acquired tolerance to a subsequent, and what would be lethal, ischemic stress in vivo. Cerebral ischemia was produced in the gerbils by occlusion of both common carotids for 5 min, which consistently resulted in delayed neuronal death in the CA1 region of the hippocampus. Minor 2-min ischemia in this model depletes high-energy phosphate compounds and perturbs the protein synthesis, but never causes neuronal necrosis, and therefore was chosen as mild ischemic treatment. Single 2-min ischemia 1 day or 2 days before 5 min ischemia exhibited only partial protective effects against delayed neuronal death. However, two 2-min ischemic treatments at 1 day intervals 2 days before 5 min ischemia exhibited drastically complete protection against neuronal death. The duration and intervals of ischemic treatment, enough to perturb cellular metabolism and cause protein synthesis, were needed respectively, because neither 1-min ischemia nor 2-min ischemia received twice at short intervals exhibited protective effects. This 'ischemic tolerance' phenomenon induced by ischemic stress — which is unquestionably important — and frequent stress in clinical medicine, is intriguing and may open a new approach to investigate the pathophysiology of ischemic neuronal damage.

INTRODUCTION

The molecular mechanism of the selective vulnerability of the nerve cells in the hippocampus to global cerebral ischemia¹³ has long received considerable attention. Several pathophysiological mechanisms such as an increased excitatory input^{7,27}, intracellular calcium overload^{5,26} and free radical formation¹⁶ have been proposed for this curious phenomenon. However, recent several reports have demonstrated selective gene expression and protein synthesis after cerebral ischemia^{11,13,23}, and it is conceivable that neuronal cells like other types of cells^{6,18–20,25} respond and resist the suffering detrimental stress. In this study, we tried to determine if tolerative property of neurons to ischemic stress is induced by prior milder ischemic treatment using an in vivo gerbil model of cerebral ischemia. Occlusion of both common carotids of the gerbils easily produces global forebrain ischemia and 5-min ischemia of this model leads to a subsequent ischemic cell death and delayed neuronal cell necrosis, which is restricted to the CA1 pyramidal neurons of the hippocampus following 7-day recirculation¹⁴. Minor 2-min ischemia of this model transiently perturbs cellular metabolism^{21,22}, but neuronal necrosis never occurs⁹. One-min ischemia is not enough to deplete high-energy

phosphate compounds²¹ and to perturb the protein metabolism²². We used 1-min and 2-min ischemia as a mild ischemic treatment before detrimental 5-min ischemia.

MATERIALS AND METHODS

A total of 61 adult Mongolian gerbils (*Meriones unguiculatus*) of both sexes, weighing 60–80 g, were used in the present study. They were kept at constant temperature (about 25 °C) in an air-conditioned room for at least 10 days prior to study under a 12-h light/dark cycle. Each gerbil was lightly anesthetized with ether inhalation and both common carotid arteries were exposed and bilateral cerebral ischemia was produced by occlusion of these arteries with miniature aneurysmal clips. Body temperature was maintained at 36.5–37 °C using warming blankets. All animals were divided into a sham-operated group (group A; *n* = 5), a 5-min ischemic control group (group B; *n* = 10), and mild ischemic treatment groups before 5-min ischemia (group C–G; *n* = 46) as shown in Table I. Mild ischemic treatment groups were further divided into the following 5 groups by the number of times and the degree of ischemic treatment, and intervals between ischemic treatments and a subsequent 5-min ischemia: a single 2-min ischemic treatment 1 day or 2 days before 5-min ischemia (group C; *n* = 9 or group D; *n* = 10, respectively), where animals had received single 2-min ischemia a day (group C) or 2 days (group D) before 5-min ischemia. Two 2-min ischemic treatments at 1 day interval 2 days before 5-min ischemia (Group E; *n* = 11), where animals had received 2-min ischemia twice at 1-day interval, and then 5-min ischemia 2 days after the last 2-min ischemic treatment. Two 2-min ischemic treatments at 12-h intervals before 5-min ischemia (group

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TABLE I

Incidence of each histological grading and neuronal density in sham-operated (A) and cerebral ischemia group (B-G)

Cerebral ischemia groups are divided into the following 6 groups. B group ($n = 10$), where animals had only 5-min ischemia; C group ($n = 9$), where animals had received single 2-min ischemia 1 day before 5-min ischemia; D group ($n = 10$), where animals had received single 2-min ischemia 2 days before 5-min ischemia; E group ($n = 11$), where animals had received 2-min ischemia twice at 1 day interval 2 days before 5-min ischemia; F group ($n = 6$), where animals had received 2-min ischemia twice at 12-h intervals before 5-min ischemia; G group ($n = 10$), where animals had received 1-min ischemia twice at 1 day interval 2 days before 5-min ischemia. Animals were decapitated 7 days after 5-min ischemia and histological findings were graded from 0 (no cell necrosis) to III (almost complete cell necrosis). Statistically significant differences from the ischemic control (group B) and two 2-min ischemia at 1-day interval (2 days before) group (group E) were indicated by asterisks and swords, respectively: $*P < 0.01$, $^{\dagger}P < 0.05$ and $^{++}P < 0.01$ (Wilcoxon's ranked sum test for unpaired samples). For details of histological gradings, see text.

Groups	Histological gradings				Neuronal density (mean \pm S.E.M.)
	0	I	II	III	
A: Sham-operated	5/5	0	0	0	193.6 \pm 3.1*
B: Ischemic control	0	0	1/10	9/10	12.9 \pm 2.2
C: One 2-min ischemia (1 day before)	2/9	3/9	3/9	1/9	107.9 \pm 22.2* ^{††}
D: One 2-min ischemia (2 days before)	3/10	3/10	3/10	1/10	129.1 \pm 21.1* [†]
E: Two 2-min ischemia at 1-day interval (2 days before)	9/11	2/11	0	0	188.5 \pm 3.6*
F: Two 2-min ischemia at 12-h intervals before	0	0	3/6	3/6	37.8 \pm 11.1 ^{††}
G: Two 1-min ischemia at 1 day interval (2 days before)	0	0	1/10	9/10	17.6 \pm 4.3 ^{††}

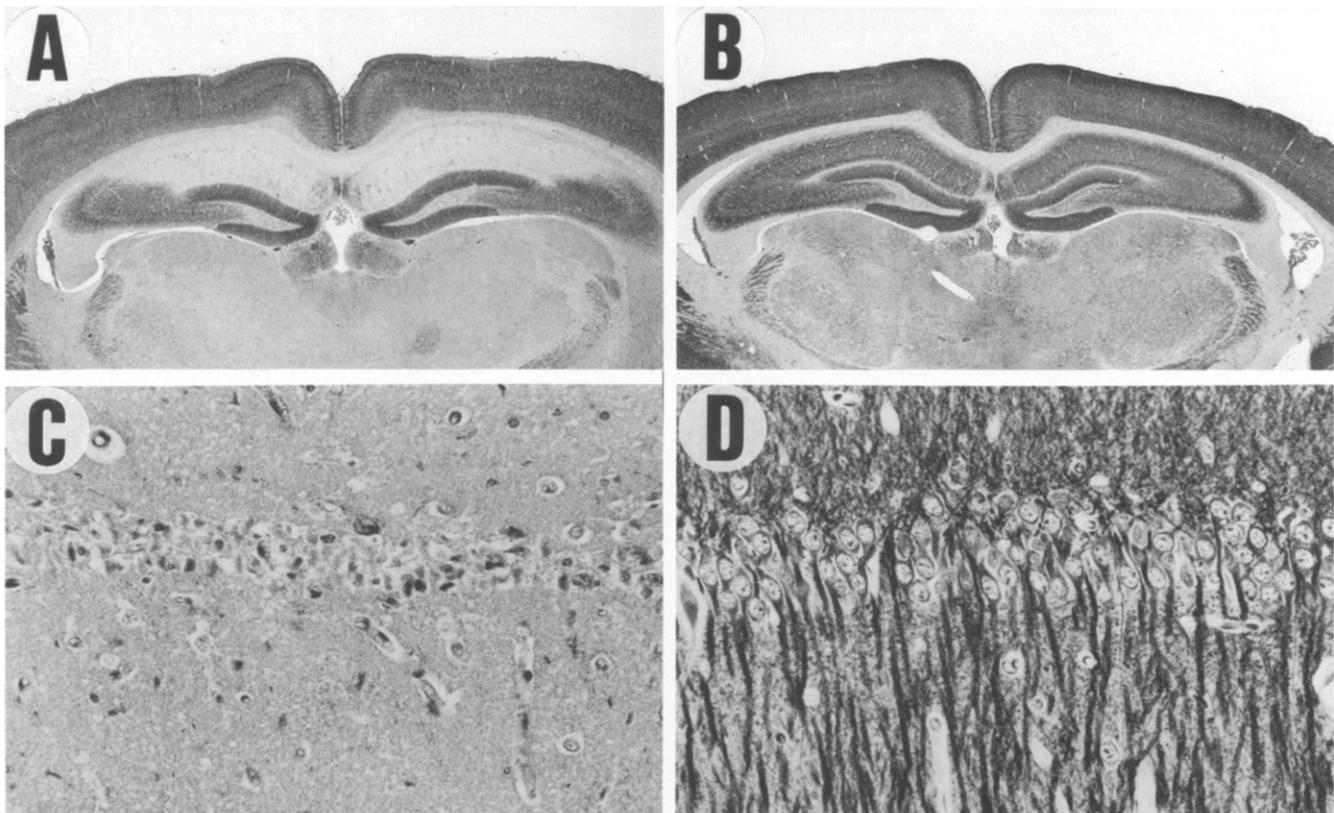


Fig. 1. Photomicrographs of immunohistochemical reaction for MAP2 taken from A, an ischemic control subject and B, a subject with 5-min ischemia that had received 2-min ischemia twice. C and D show higher magnification in the hippocampal CA1 area of A and B, respectively. All are shown 7 days after 5-min forebrain ischemia. Note clear loss of neuronal staining for MAP2 in the CA1 region of the gerbil with 5-min ischemia (A and C), while in the animal that had received 2-min ischemia twice at 1 day interval 2 days before 5-min ischemia (B and D) neuronal staining for MAP2 was completely preserved as in the sham-operated animals. The original photographs for A and B were taken at $\times 4$ magnification and those for C and D at $\times 100$ magnification.

F; $n = 6$), where animals had received 2-min ischemia twice at 12-h intervals before 5-min ischemia. Two 1-min ischemic treatments at 1 day interval 2 days before 5-min ischemia (group G; $n = 10$), where animals had received ischemic treatments before 5-min ischemia with the same schedule concerning the numbers of times and intervals as group E, but the length of ischemic treatments was not 2 min but 1 min. For histological examination in the ischemic control and mild ischemic treatment groups, both common carotid arteries were exposed under light ether anesthesia and occluded by miniature aneurysmal clips for 5 min. Seven days after clip removal the animals were again anesthetized, decapitated and the brains of these animals were promptly removed, divided 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 4–5 h, each tissue block was dehydrated in ethanol and embedded in paraffin. The 5 μ m thick cross-sections corresponding to the stereotaxic section 1.4–1.6 mm caudal to bregma containing both dorsal hippocampi, were stained with hematoxylin–eosin (HE) and the immunohistochemical reaction for microtubule-associated protein 2 (MAP2). The peroxidase–antiperoxidase method was employed for the immunostaining as described previously¹⁵. In the sham-operated group, both common carotid arteries were exposed but no carotid occlusion took place. MAP2 has been proved to be localized in neuronal soma and dendrites and the immunoreaction for MAP2 clearly visualized the surviving neurons in our previous study¹⁵. With respect to the neuronal destruction of the CA1 region 7 days after 5-min ischemia, the brain sections were examined by an investigator who was blinded to the detail of the experiment and the following 4 gradings were established based on the histological findings with HE staining and immunostaining for MAP2 (ref. 16): grade 0, no cell necrosis; grade I, scattered single cell necrosis or small cell group necrosis; grade II, scattered cell group necrosis; grade III, almost complete cell necrosis in the CA1 area. The neuronal density, i.e. the number of the surviving pyramidal neurons per 1 mm length of the medial CA1 region was also counted under a light microscopy at $\times 320$ magnification. Differences in mean cell densities were analyzed using Wilcoxon's ranked sum test for unpaired samples, with $P < 0.05$ being required for statistical significance.

RESULTS

The results are summarized in Table I. All 10 ischemic control animals (group B) showed almost complete (grade III, $n = 9$) or moderate (grade II, $n = 1$) necrotic lesions in the CA1 area of hippocampus 7 days following recirculation as previously reported by other investigators^{10,24} and us¹⁶ (Fig. 1A and C). All animals of the non-ischemic sham-operated group (group A) exhibited no cellular necrosis (grade 0) in whole brain area, including the hippocampus. More than half of the animals, which had received single 2-min ischemia 1 day before (group C) or 2 days before (group D) 5-min ischemia, showed no (grade 0) or mild (grade I) necrotic lesions, and these single 2-min ischemic treatment groups exhibited an insufficient but clear protective effect on ischemic neuronal death. More surprisingly, all 11 animals, which had received 2-min ischemia twice at 1-day interval 2 days before 5-min ischemia (group E), showed no (grade 0) or mild (grade I), necrotic lesions in 9 or 2 animals, respectively, and this ischemic treatment exhibited almost a complete neuroprotective effect against

delayed neuronal death (Fig. 1B and D). All 6 animals, which had received 2-min ischemia twice at 12-h intervals before 5-min ischemia (group F), showed moderate (grade II) or severe (grade III) necrosis, and exhibited lesser neuroprotective effects than those with single 2-min ischemia 1 day before 5-min ischemia. All animals, which had received 1-min ischemia twice at 1-day interval 2 days before 5-min ischemia (group G) showed an almost complete necrotic change (grade III) and this group exhibited no protective effect (Table I).

DISCUSSION

In the present study, the gerbil model of bilateral cerebral ischemia was chosen as a suitable model for detecting 'ischemic tolerance' phenomenon. Because, in this model, we can obtain both non-lethal ischemic stress and mild but lethal ischemic stress in a simple, reproducible way^{12,29} just by changing duration of bilateral carotid occlusion. In our previous studies, bilateral⁹ or unilateral¹⁵ ischemia for up to 2 min never showed any ischemic lesions even with sensitive immunohistochemical methods, whereas faint ischemic lesions were detected in the subiculum-CA1 area of the hippocampus following 3 min bilateral ischemia. Bilateral ischemia for up to 2 min was therefore selected as a non-lethal, reversible ischemic stress and bilateral ischemia for 5 min, which is well known to cause delayed neuronal death in the vulnerable CA1 area of the hippocampus¹⁴, as a mild but lethal ischemic stress. Our results clearly show that 2 repetitive brief periods of ischemia alter the neuronal cells of the hippocampi so that they are quite resistant to subsequent detrimental ischemic stress. Single mild ischemia exhibited significant but insufficient protective effect. More than 1-day interval between non-lethal and lethal ischemia were needed for appearance of neuroprotective effects because animals of group F only showed lesser protective effects than those of group C, D and E. Ischemic stress, enough to perturb the energy metabolism of neuronal cells, was essentially required for induction of tolerance because 2 1-min ischemic treatments showed no protective effects. From these observations, it is conceivable that mild ischemic stress showed neuroprotective action by causing the changes of gene expression and alteration of protein synthesis. In fact, several reports^{11,13,23} demonstrated selective gene expression and protein synthesis at 3–72 h following transient forebrain ischemia, though none showed the 'ischemic tolerance' phenomenon.

It is fascinating to speculate on the biological basis underlying the observed 'ischemic tolerance' phenomenon. There are several examples in other areas of biology about acquisition of tolerance to stress with a treatment

of mild stress. It is reported that synthesis of a small group of highly conserved proteins in response to elevated temperature and other agents that induce stress is a universal feature of prokaryotic and eukaryotic cells^{1,28}, and these proteins play a role in enhancing survival during and after stress^{2,17}. Another example is acquisition to 100% oxygen toxicity in rats by previous 85% oxygen stress and this effect is thought to be caused by augmentation of enzyme activity of superoxide dismutase^{4,8}. From these reports, it is probable that the acquisition of tolerance to various kinds of exogenous stresses is essential for survival of individual organisms and cells, and cells primarily have endogenous protective mechanisms against damaging stress. In particular in neuronal cells which have a longer life span, several strategies for survival should be operative. Our present results strongly suggest that neurons acquire an endoge-

nously tolerative property to ischemia through the alteration of gene expression and protein synthesis. This 'ischemic tolerance' phenomenon should be found also in other types of cells including myocardial cells, in which ischemic damage is an important clinical problem. An investigation at the level of cultured cells, i.e. neurons, myocardial cells and endothelial cells, also seems to be worthwhile and promising. Future studies would clarify more precisely the adaptation of gene expression in neurons which acquire tolerance to ischemia.

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