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Modulation of Ischemic Neuronal Death by Non-lethal Ischemia and Ubiquitin Reappearance in Gerbils

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Abstract Tolerance to lethal ischemia can be induced in gerbils by prior subjection to non-lethal ischemia. The sequential changes of ubiquitin immunoreactivity in the hippocampi of gerbils subjected to non-lethal ischemia followed by lethal ischemia were investigated. The double-ischemic group was subjected to non-lethal ischemia followed by lethal ischeima, the single-ischemic group was subjected to lethal ischemia, only, the sham-ischemic qroup was subjected to sham operation followed by lethal ischemia, and the control group underwent no operation and were not subjected to ischemia. The densities of the CA (Cornu ammonis) 1 neurons were assessed by hematoxylin-eosin staining. In the double-ischemic group, the extent of the neuronal damage was less than that in the single-ischemic and sham ischemic groups (both $P < 0.02$) and half that in the control group ($P < 0.002$). Ubiquitin immunoreactivity (UIR) disappeared once after lethal ischemia in all the ischemic groups. In the single and sham ischemic groups, UIR never reappeared, whereas in the double-ischemic group, it recovered partially during the early postischemic period and almost completely in the surviving cells eventually. Non-lethal ischemia reduces the ischemic damage caused by subsequent lethal ischemia in the CA1 neurons of gerbils, and ubiquitination is related to the damage reduction.

Key Words:Ischemia, Ischemic tolerance, Non-lethal ischemia, Ubiquitin, Gerbil

Introduction

Neurons in the hippocampal CA (Cormu ammonis) 1 sector, cerebellar cortex, dorsolateral striatum, and third and fifth layers of the cerebral cortex have been observed to be selectively vulnerable to ischemic injury[1]. Kirino reported that delayed neuronal death (DND) occurred in the selectively vulnerable hippocampal CA1 sector of gerbils subjected to ischemia[2]. Elevation of extracellular glutamate levels[3], disturbance of protein synthesis[4,5], and free radical formation[6] have been proposed as causes of this DND, which could be avoided under mild hypothermic condition in animal models[7,8]. Magnusson et al. reported that ubiquitin immunoreactivity (UIR) was lost permanently in the CA1 neurons of gerbils after transient ischemia under normothermic conditions[9]. Yamashita et al. demonstrated that under hypothermic conditins, the UIR loss in the CA1 sector of gerbils after ischemia was transient, UIR recovered and the CA1 neurons were preserved[8]. Ubiquitin, one of the heat-shock proteins, is present in the normal state and is essential for normal cellular function[10,11,12]. It was found that DND also could be avoided by prior induction of non-lethal ischemia, which induced tolerance to lethal ischemia[13,14]. Kitagawa et al. reported that subjecting gerbils to double 2-min periods of

ischemia before 5-min ischemia induced a greater protective effect against DND than a single 2-min ischemic period[13]. Kirino et al. reported that heat-shock protein 70 (hsp 70), a stress-protein, appeared in the CA1 neurons of gerbils in which tolerance to ischemia had been induced[14]. However, the hsp 70 staining pattern did not correlate consistently with the induced tolerance pattern, and whether hsp 70 was indispensable for the tolerance could not be determined in this model.

In this study, we investigated the modulation of ischemic neuronal death and sequential changes of hippocampal UIR in the double ischemic gerbil model subjected to nonlethal ischemia with 2-min occlusion followed by 5-min occlusion of both common carotid arteries.

Materials and Methods

Male Mongolian gerbils (Seiwa Experimental Animals) weighing 60-90 g were used in this study. Their bilateral common carotid arteries were exposed though a midline cervical incision under halothane anesthesia and occluded with aneurysm clips 1 min after discontinuation of halothane administration as follows. In the double-ischemic group, the first period of ischemia lasted 2 min and, 2 days later, the carotid arteries were reexposed and occluded for 5 min. The sham-ischemic group was subjected to sham operation, during which the carotid arteries were exposed but not occluded initially, then, 2 days later, they were occluded for 5 min. The control group comprised untreated gerbils. In the single-ischemic group, the carotid arteries were occluded once only for 5 min. During the operation, the rectal (RT) and scalp (RT) temperatures were regulated with a heat lamp and maintained at 36.5-38.5 and 36-38°C respectively and after recovery they were monitored and regulated for 10 min.

For the histopathological study of the tissues, the brain was removed from each gerbil 7 days after the final experimental procedure as follows. The gerbils were perfused and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) transcardiacally at a pressure of 120 cm H_2O. After brain had been removed, the regions containing the dorsal hippocampi, 1.4 and 1.9 mm caudal to the bregma, were excised, placed in the perfusion solution at 4°C overnight, dehydrated with a graded ethanol series, soaked in xylene, and embedded in paraffin. Coronal sections (6 μm thick) of the dorsal hippocampus were cut, deparaffinized and stained with hematoxylin-eosin. The number of undamaged CA1 neurons in the bilateral hippocampal CA1 sectors in one section from each gerbil was counted as similar neuronal damage occurred throughout the rostrocaudal extent of the dorsal hippocampus. The density of intact neurons per 1 mm linear length of the stratum pyramidale was calculated, and the average value of the left and right hippocampi was used for each animal. The neuronal densities in the CA1 pyramidal cell layers of 5 intact, untreated gerbils used as normal controls also were evaluated. The data obtained for each group were expressed as means ± standard errors, and statistical analysis of these was performed using one-way analysis of variance (ANOVA) and Bonferroni's correction was used for the post-hoc test; differences at $P<0.05$ were considered to be significant.

For the immunohistochemical study of the tissues, the brains were removed from 6 gerbils each at 6 h, 24 h, and 48 h and 7 days after the final period of ischemia and coronal sections were prepared, as described above for the histopathological study. One section from each gerbil was examined for UIR using a monoclonal antibody against ubiquitin (Chemicom International Inc., USA) at a dilution of 1:100 as follows. The sections were deparaffinized, rinsed with phosphate-buffered saline, pretreated with 0.3% hydrogen peroxide, incubated with the primary antibody solution for 12 h at 4°C followed by incubation with biotinylated anti-immunoglobulin in phosphate-buffered saline with normal serum, carrier protein and a preservative (Nichirei, Tokyo, Japan) for 30 min at room temperature and with horseradish peroxidase-conjugated streptavidin in phosphate-buffered saline with carrier protein and preservative (Nichirei, Tokyo, Japan) for a further 30 min at room temperature. The sections were washed and the horseradish peroxidase binding sites were stained with 0.02% diaminobenzidine tetrahydrochloride in 0.05M Tris buffer containing 0.005% H_2O_2. The UIRs in various hippocampal regions were classified into the following 4 grades; 0, no neurons positive; 1, only a small number of neurons positive

(0-30%); 2, a large number of neurons positive (30-70%); 3, most or all neurons positive (70-100%).

Results

The mean intact neuronal density in the CA1 pyramidal cell layer in the control group (n=5) was 254±4/mm (mean±standard error), in the double-ischemic group (n=6), it was 113±17/mm, and in the single- (n=6) and sham-ischemic (n=6) groups, the values were 11±3 and 12±2/mm, respectively. The mean neuronal density in the double-ischemic group was significantly lower than that in the control group ($p<0.002$) and significantly higher than that in the groups subjected to single-ischemia and sham operation followed by ischemia ($p<0.02$, Fig. 1).

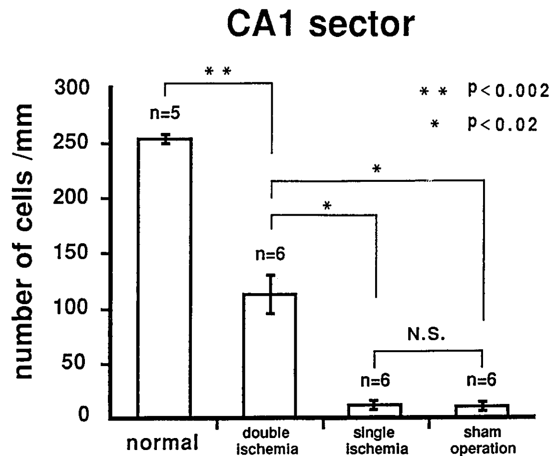

Fig. 1: Mean neuronal densities in the CA1 sectors 7 days after the final ischemic insult. In the double-ischemic group (n=6), the neurons in the CA1 sectors were preserved. Statistically significant differences from the normal group are indicated by a double asterisk ($p<0.002$), and from the double-ischemic group by a single asterisk ($p<0.02$).

In the control group, UIR was observed in the cell nuclei, cytoplasm and dendrites in most of the dorsal hippocampal neurons (Fig. 3A). In the single ischemic group, no UIR was observed in any hippocampal region 6 h after the final ischemic insult. In the CA1 sector, no UIR was observed at any time after the ischemic insult (Fig. 2A). The UIR of this group was grade 0 in all but one gerbil in which it was grade 1. In all the CA3 sector and dentate gyrus neurons, UIR was observed 24 h, 48 h and 1 week after the insult (Fig. 2B). In the double-ischemic group, no UIR was observed in the CA1 sector neurons 6 h after the final ischemic insult (Fig. 3B), but it recovered partially 24 h and 48 h after the insult. The postischemic UIR grades were 1 in 2 gerbils after 24 h, and 2 in one and 1 in 3 after 48 h (Fig. 2A). One week after the final ischemic insult, the UIR had recovered in the surviving CA1 pyramidal neurons (Fig. 3C). In the CA3 sector neurons, some UIR was observed 6 h after the final insult with grade 1 staining in 3 gerbils and grade 2 staining in one. The UIR recovered in all the CA3 sector and dentate gyrus neurons 24 h, 48 h and 1 week after the final ischemic insult. No marked differences between the UIRs of the right and left sides of the hippocampus were observed in any group. Sections incubated with nonimmune immunoglobulin G showed no significant staining.

Discussion

Induced tolerance to various types of stress in living cells and organisms has been reported and the effect of hyperthermic stress has been the most intensively studied. Prior

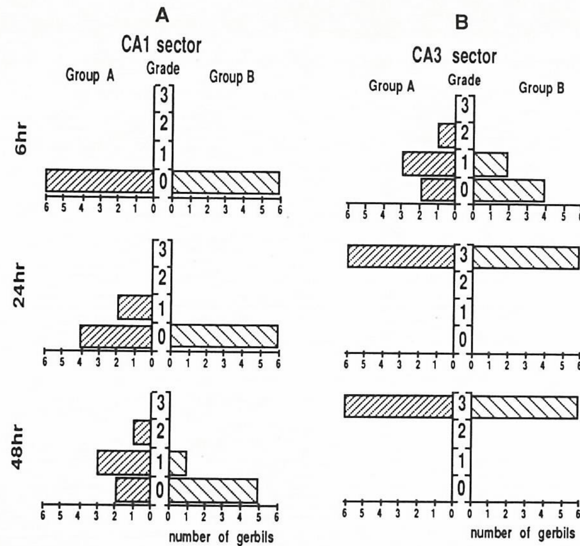

Fig. 2: Ubiquitin immunoreactivity (UIR) grades in the CA1 and CA3 sectors 6, 24 and 48 h after the final ischemic insult. Groups A and B are the double- and single-ischemic groups respectively. The number of gerbils is indicated on the abscissa and the UIR grade on ordinate.

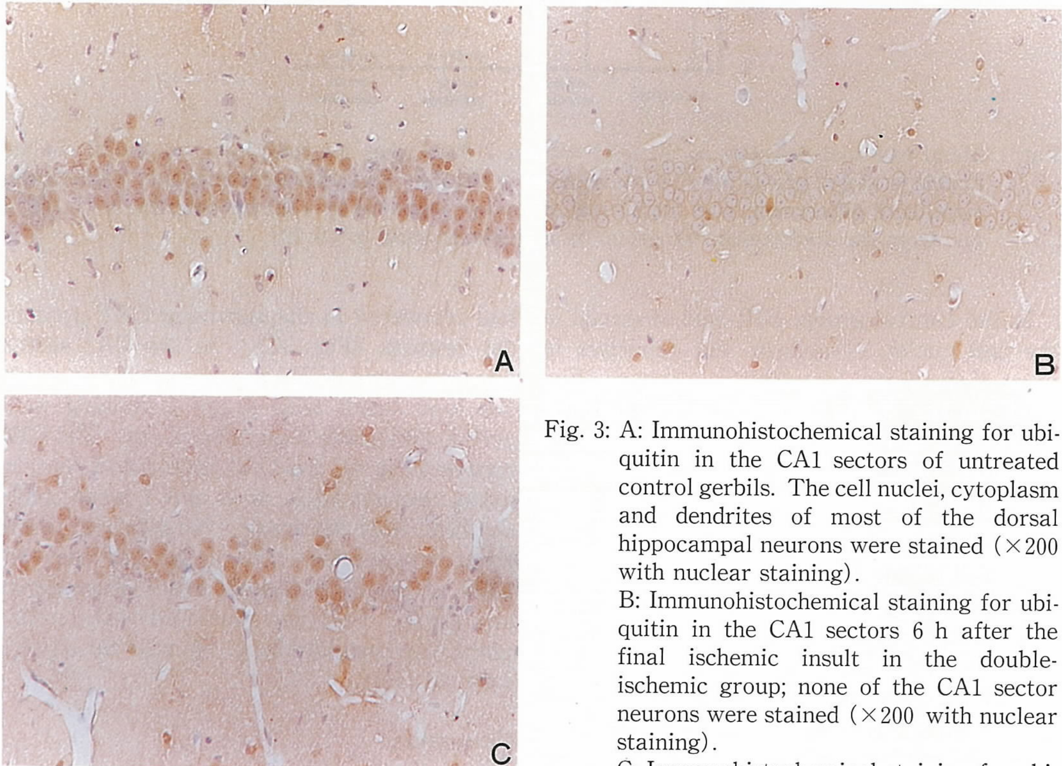

Fig. 3: A: Immunohistochemical staining for ubiquitin in the CA1 sectors of untreated control gerbils. The cell nuclei, cytoplasm and dendrites of most of the dorsal hippocampal neurons were stained (×200 with nuclear staining).
B: Immunohistochemical staining for ubiquitin in the CA1 sectors 6 h after the final ischemic insult in the double-ischemic group; none of the CA1 sector neurons were stained (×200 with nuclear staining).
C: Immunohistochemical staining for ubiquitin 7 days after the final ischemic insult in the double-ischemic group; staining recovered in the surviving CA1 sector neurons (×200 with nuclear staining).

exposure of HeLa cells to sublethal hyperthermia induced heat tolerance with production of heat-shock proteins[16], which exhibited a high degree of conservation from bacterial to mammalian cells and were synthesized in response to heat-shock[17]. As fibroblasts pretreated with antibodies against hsp 70 in vitro did not survive after brief incubation at 45°C, hsp 70 was thought to be necessary for the survival of these cells when subjected to heat-stress[18]. Prior exposure to hyperthermia protected gerbils against ischemic stress and this protection correlated well with the appearance of heat-shock proteins[19]. Therefore, these proteins appear to play a role in enhancing survival during and after stress.

Brief bilateral carotid artery occlusion in gerbils has proven to be a useful model for studying the effects of ischemia on neuronal death. Five-minute ischemia in this model constantly causes DND in the gerbil hippocampal CA1 sector within 7 days after ischemia, whereas 2-min ischemia does not. Thus, 5- and 2-min ischemia are defined as lethal and non-lethal, respectively.

Prior subjection to ischemia for 2 min prevents the hippocampal DND caused by 5-min ischemia and in study, such non-lethal ischemia reduced the severity of such DND induced by 5-min occlusion in gerbils. Selective changes of gene expression and protein synthesis alterations are considered to be involved in the mechanism responsible for this prevention. Induction of the messenger RNA encoding the 70-kDa heat-shock protein and of the c-fos product in the gerbil hippocampus from 15 min to 72 h after transient forebrain ischemia has been observed[20] and the expression of the c-fos product, found mainly in the hippocampal neurons, was found to be resistant to ischemic injury[21].

Ubiquitin is one of the low molecular weight heat-shock proteins present in all eukaryotic cells under normal conditions[11]. It regulates the cell cycle in the cell nucleus, binds to the short-lived (high turnover rate) and denatured proteins produced in response to various forms of injury (hypoxia, ischemia or heat exposure) and the ubiquitinated proteins are broken down by proteases in an ATP-dependent manner[11,12].

Magnusson et al. demonstrated the UIR disappeared from the CA1 pyramidal neurons after 5-min ischemia and did not reappear under normothermic conditions[9], and the results of our study agree with theirs. Kitagawa et al. reported that ischemia for 2 min did not change the UIR in the CA1 pyramidal neurons after reperfusion for 3 days under normothermic conditions[13]. In our study, 2-min non-lethal ischemia resulted in no immunohistochemical lesions in the gerbil hippocampal CA1 sector after reperfusion for 7 days. In the double-ischemic group subjected to prior non-lethal ischemia, UIR disappeared just after the lethal ischemic insult, reappeared partially during the early postischemic period and was demonstrated in the surviving cells in the CA1 sector 7 days after the lethal ischemia. This result is similar to that of Yamashita et al., who observed that under mild hypothermic conditions, UIR disappeared from the CA1 neurons 6 h after 5-min ischemia and reappeared after 24-48 h[8].

Loss of UIR may be related to suppression of the stress response and permanent loss of ubiquitin has been proposed to be one of the mechanisms responsible for DND. In our study, prior exposure to non-lethal ischemia led to the reappearance of UIR in the CA1 neurons after lethal ischemia and early recovery of ubiquitin synthesis was demonstrated in all the surviving cell. Therefore, ubiquitin would appear to be indispensable for neuronal restoration after transient ischemia. The reappearance of UIR is believed to induce gene expression and protein synthesis changes that may play roles in protecting neurons against lethal ischemia. Our results suggest that prior exposure to non-lethal ischemia produces "ischemic tolerance"[13,14] to lethal ischemia through ubiquitin synthesis.

The reappearance of UIR may be important to prevent DND, but at least 2 problems, need to be solved to confirm this. One is, what is the optimum interval between 2- and 5-min ischemia for the induction of tolerance. An interval of more than 1 day between non-lethal and lethal ischemia has been reported to be needed for the induction of tolerance[13]. When the interval was less than 1 day, the neuronal damage increased[15]. The second problem is that the recovery rate of

UIR 24-28 h after the second ischemic insult did not correlate well with the number of CA1 sector neurons preserved after 7 days. The UIR in the preserved neurons was restored eventually, but it has not been demonstrated clearly whether synthesis of normal proteins or just that of stress proteins (for example, hsp 70) recovered. Further studies are needed to resolve these problems.

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