Laboratory Reports

Superoxide radical generation and histopathological changes in hippocampal CA1 after ischaemia/reperfusion in gerbils

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Purpose: We investigated the relationship between the generation of superoxide radicals and histopathological changes on delayed neuronal death in the hippocampal CA1 subfield.

Methods: Seventy gerbils were randomly assigned to two groups, a sham group and an ischaemia/reperfusion (I/R) group. In the I/R group, transient forebrain ischaemia was induced by occluding the bilateral common carotid arteries for four minutes. The cerebrum was removed after reperfusion at intervals of one minute, six, twelve and twenty-four hr and at three, five and seven days. Each forebrain was cut into two portions including the hippocampus. The quantity of superoxide radicals was measured by using chemiluminescence, and histopathological changes in the hippocampal CA1 subfield were examined.

Results: In the I/R group, superoxide radicals increased on the 3rd and 5th days compared with the sham group (16.1 ± 3.4 vs 3.2 ± 1.0 on the third day ($P < 0.0001$); 10.9 ± 1.9 vs 3.3 ± 0.8 on the fifth day ($P < 0.0001$)). In the I/R group, the pyramidal cells were atrophic and pycnotic; vacuolation, and structural disruption of the radial striated zone were observed from the third through the seventh day. In the sham group, these changes were not observed. There were differences of degenerative ratios in the pyramidal cells between the two groups from the third to seventh days (5.6 ± 2.0 vs 80.9 ± 3.3 on the third day ($P < 0.05$); 6.9 ± 0.4 vs 93.6 ± 2.4 on the fifth day ($P < 0.05$); 6.2 ± 1.5 vs 95.0 ± 1.3 on the seventh day ($P < 0.05$)).

Conclusion: There is a correlation between the generation of superoxide radicals and histopathological changes of the pyramidal cells in the hippocampal CA1 subfield.

Objectif : Investiguer la relation entre la production des radicaux superoxydes et les changements histopathologiques sur le décès neuronal retardé dans le champ hippocampique CA1.

Méthodes : Soixante-dix gerbilles ont été aléatoirement réparties en 2 groupes, un groupe contrôle et un groupe ischémie/reperfusion (I/R). Dans le groupe I/R, une ischémie transitoire du prosencéphale était induite par l'occlusion bilatérale des carotides communes pour quatre minutes. Après reperfusion, le cerveau était retiré de l'animal après une minute de même qu'à six, douze et vingt-quatre heures ainsi qu'à trois, cinq et sept jours. Chaque prosencéphale était coupé en deux parties incluant l'hippocampe. La quantité de radicaux superoxydes était mesurée par chemiluminescence et les changements histopathologiques dans le champ hippocampique CA1 étaient observés.

Résultats : Dans le groupe I/R, les radicaux superoxydes ont augmenté aux jours 3 et 5 comparativement au groupe témoin ($16,1 \pm 3,4$ vs $3,2 \pm 1,0$ au jour 3 ($P < 0,0001$); $10,9 \pm 1,9$ vs $3,3 \pm 0,8$ au jour 5 ($P < 0,0001$)). Dans le groupe I/R, les cellules pyramidales étaient atrophiques et picnotiques; du 3e au 7e jour, on a observé de la vacuolisation et de la destruction structurale de la zone striée radiaire, et ces changements n'ont pas été retrouvés dans le groupe témoin. On a observé des différences dans le pourcentage dégénératif des cellules pyramidales entre les deux groupes à partir du jour 3 au jour 7 ($5,6 \pm 2,0$ vs $80,9 \pm 3,3$ au jour 3 ($P < 0,05$); $6,9 \pm 0,4$ vs $93,6 \pm 2,4$ au jour 5 ($P < 0,05$); $6,2 \pm 1,5$ vs $95,0 \pm 1,3$ au jour 7 ($P < 0,05$)).

Conclusion : Il y a une corrélation entre la production de radicaux superoxydes et les changements histopathologiques des cellules pyramidales du champ CA1 de l'hippocampe.

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SUPEROXIDE radicals may play an aetiological role in tissue damage after ischaemia/reperfusion.¹ The brain is particularly vulnerable. Superoxide radicals induce brain death²⁻⁷ by causing cell destruction due to peroxidation of the polyunsaturated fatty acid in the lipid of the cell membrane.⁸ Free radical scavengers alleviate brain damage after ischaemia/reperfusion.⁹⁻¹² No previous study however, has demonstrated the relationship between the generation of superoxide radicals and extent of brain damage in cases of delayed neuronal death after ischaemia/reperfusion.

In the present study we measured the amount of superoxide radicals generated from the hippocampal CA1 subfield after ischaemia/reperfusion using chemiluminescence.^{13,14}

Methods

The study was conducted according to the animal experimentation guidelines of Dokkyo University School of Medicine, which adhere to the National Institute of Health Animal Experimental Guidelines.

Experimental protocol

Seventy Mongolian gerbils weighing 75–95 g were divided into two groups.

Control group (sham group): Sham operation performed (n = 35).
Ischaemia/reperfusion group (I/R group): Ischaemia/reperfusion performed (n = 35).

After ether induction of anaesthesia in a glass cage, a rubber bag was placed over the head of each gerbil and nitrous oxide 50% in oxygen with halothane (end-tidal concentration: 1.0–1.5%) was administered throughout the experiment. The animals were then placed in the supine position and the common carotid arteries were exposed bilaterally. In the sham group, no further procedure was performed. In the I/R group, the common carotid arteries were occluded with miniature aneurysmal clips for four minutes. During this period we verified an absence of blood supply from the vertebral arteries with a microscope. Four minutes after occluding the bilateral carotid arteries, the clips were released and we confirmed visually spontaneous circulatory reperfusion with the microscope. The temperature of the tympanic membrane was monitored (Mon-a-therm Model 6510; Mallinckrodt), and maintained with a heating blanket at $37 \pm 0.2^\circ\text{C}$ during the experiment.

Animals' ventilation was shallow during the procedure but this improved after reperfusion. They were kept and fed in the cage at a room temperature of 26°C before decapitation.

At intervals of one minute, six, twelve and twenty-four hr, and at three, five and seven days five gerbils in each group were decapitated under ether anaesthesia and the forebrain was removed within two minutes. Each forebrain was cut into two portions including the hippocampus in the coronal direction: in one portion we measured the quantity of superoxide radicals using chemiluminescence, and in the another portion we examined histopathological changes in the hippocampal CA1 subfield.

Measurement of superoxide radicals

After sectioning the forebrain, one portion was immersed in liquid nitrogen for five minutes, and then sliced 18 μm tissue sections at -20°C using a cryostat. Superoxide radicals were measured using the chemiluminescence method developed by Nakano *et al.*¹³ and Hayashi *et al.*¹⁴ Next, the bottom of a transparent plastic Tissue-Tek box (Miles Inc., Indiana, USA) was covered with a piece of millipore filter (CA 250/0, Schuell Co., Dassel, Germany) that had been saturated with 0.1 ml 2-methyl-6-phenyl-3, 7-dihydroimidazol (1,2a) -prazin-3-one solution (CLA-phenyl, A5307, Tokyo Kasei Kogyo Co., Japan). Each tissue slice was placed directly on the filter paper and ASA 20,000 polaroid film was placed under the Tissue-Tek box. The film was exposed to the chemiluminescence emitted from the tissue for three minutes in a dark-room. The superoxide radicals in the brain tissue, which react with CLA-phenyl, emit chemiluminescence at 380 nm and expose the film. The light intensity and the exposed area on the film which correlated positively with the quantity of superoxide radicals were measured by a camera-microcomputer picture analysis system, Model TIAS software (ACI Co., Japan), and were expressed in integrated light absorption (ILA) values.

Histopathological changes

The second portion of the forebrain was fixed by solution, containing formaldehyde 4%, phosphate buffer solution and distilled water in a ratio of 1:2:7, and embedded in paraffin, then sliced into 5 μm sections in the coronal direction and stained with haematoxylin and eosin. Histopathological changes were examined using a light microscope (Olympus BH-2, Olympus, Japan). Morphological changes were detected in the pyramidal cells and structural changes in the cell layers of the CA1 subfield of the hippocampus. In order to compare the ratio of degenerative pyramidal cells between the two groups the total number and the degenerative number were counted for a uniform 1 mm length of the central area of hippocampal CA1

subfield. The degenerative ratios were determined according to the following formula: Degenerative ratio (%) = (number of degenerative pyramidal cells/total number of pyramidal cells per 1 mm of hippocampal CA1 subfield) $\times$ 100.

Atrophy, pycnosis, vacuolation and deformity of pyramidal cells, as well as disruption of the radial striated zone were taken to be degenerative cells.

Statistical analysis

Data are presented as mean $\pm$ SD. Data were analysed using repeated measures ANOVA with Bonferroni's correction as post hoc testing. Statistical significance was considered to be $P < 0.05$.

Results

Generation of superoxide radicals

The quantity of superoxide radicals expressed as ILA is shown in Table I. No differences were found in the two groups within 24 hr of the procedures. In the I/R group, superoxide radicals increased three and five days later compared with one minute after the procedure ($P < 0.0001$). In the sham groups, there were no differences at any time.

TABLE I Generation of superoxide radicals in the hippocampal CA1 subfield after ischaemia/reperfusion

	Sham group	I/R group
1 min	3.0 $\pm$ 1.0	3.8 $\pm$ 1.2
6 hr	3.5 $\pm$ 0.8	4.2 $\pm$ 0.7
12 hr	4.3 $\pm$ 0.5	5.3 $\pm$ 1.2
24 hr	3.2 $\pm$ 1.3	5.0 $\pm$ 1.8
3rd day	3.2 $\pm$ 1.0	16.1 $\pm$ 3.4*†
5th day	3.3 $\pm$ 0.8	10.9 $\pm$ 1.9*†
7th day	3.7 $\pm$ 1.0	5.6 $\pm$ 2.0

* $P < 0.05$ vs sham group

† $P < 0.05$ vs 1 min in I/R group

TABLE II Degenerative ratios of pyramidal cells after ischaemia/reperfusion in the hippocampal CA1

	Sham group	I/R group
1 min	5.9 $\pm$ 0.7	6.0 $\pm$ 1.3
6 hr	5.8 $\pm$ 1.3	5.9 $\pm$ 1.2
12 hr	5.6 $\pm$ 1.4	5.8 $\pm$ 1.0
24 hr	5.9 $\pm$ 1.6	7.4 $\pm$ 1.3
3rd day	5.6 $\pm$ 2.0	80.9 $\pm$ 3.3*†
5th day	6.9 $\pm$ 0.4	93.6 $\pm$ 2.4*†
7th day	6.2 $\pm$ 1.5	95.0 $\pm$ 1.3*†

* $P < 0.05$ vs sham group

† $P < 0.05$ vs 1 min in I/R group

Relationship between superoxide radicals and histopathological findings

Photographs of chemiluminescence and histopathology at one minute, 24 hr, three, five and seven days after reperfusion are shown in the Figure on pages 229 and 230. In the sham group, the normal pyramidal cells were well preserved and no disruption of the radial striated zone was detected during the study. In the I/R group, the pyramidal cells were atrophic and pycnotic. They also exhibited dense staining of the cell cytoplasm as well as vacuolation and structural disruption of the radial striated zone by the third day. It extended to almost all the pyramidal cells on the fifth and seventh days.

Degenerative ratios

The degenerative ratios of each group are shown in Table II. There were no differences between the two groups within 24 hr after the procedures. In the I/R group, the degenerative ratio increased three, five and seven days after reperfusion compared with one minute after reperfusion ($P < 0.0001$). In the sham group, no differences were found during the study.

Discussion

Transient ischaemia/reperfusion induces delayed neuronal death a few days after reperfusion. In a study using Mongolian gerbils, Kirino¹⁶ reported that destruction of the pyramidal cells in the hippocampal CA1 subfield occurs two days after transient ischaemia. The cerebellar Purkinje fibres, the pyramidal cells of the hippocampal CA1 subfield and the third and fifth layers of the cerebral cortex are selectively vulnerable to short periods of forebrain ischaemia.¹⁶ Extracellular increase of excitatory amino acids such as glutamate,¹⁷⁻¹⁹ accumulation of intracellular calcium,²⁰⁻²⁴ disturbance of protein synthesis,^{25,26} microvascular disturbance²⁷ or generation of superoxide radicals²⁻⁷ are responsible for delayed neuronal death after ischaemia/reperfusion. In particular, glutamate is responsible for ischaemia/reperfusion brain damage. It combines with a N-methyl-D-aspartate (NMDA) receptor in low pH and opens the NMDA gate to pass extracellular calcium into the cell. Accumulation of intracellular calcium induces neuronal damage because it generates superoxide radicals. Free radical scavengers such as superoxide dismutase prevent neuronal damage after ischaemia/reperfusion.⁹⁻¹² To date, however, no single study has compared the generation of superoxide radicals to histopathological cerebral changes after ischaemia/reperfusion.

We investigated the relationship between the quantity of superoxide radicals and histopathological changes in the hippocampal CA1 subfield after ischaemia/reperfusion. We used chemiluminescence with a cypridina

FIGURE (A) The sham group; Photographs of chemiluminescence (left) and histopathology (right) at 1 min (A-1), 24 h (A-2), the 3rd day (A-3), the 5th day (A-4) and the 7th day (A-5) after reperfusion. Clear chemiluminescence was not observed and the pyramidal cells were well preserved.

(B) The I/R group; Photographs of chemiluminescence (left) and histopathology (right) at 1 min (B-1), 24 h (B-2), the 3rd day (B-3), the 5th day (B-4) and the 7th day (B-5) after reperfusion. Clear chemiluminescence was not observed at 1 min, 24 h and the 7th day, but it did not appear on the 3rd and the 5th days. The pyramidal cells were well preserved at 1 min and 24 h, but the degenerative changes of the pyramidal cells were present on the 3rd, the 5th and the 7th days.

Figure continued on page 230

FIGURE Continued

luciferin analog (CLA) which emits 320 nm of light when it reacts with superoxide radicals.¹⁴ Although there are some methods for measuring free radicals, chemiluminescence with CLA is more specific for measuring superoxide radicals. In the present study, superoxide radicals increased from the third through the fifth day after reperfusion, and histopathological changes in the pyramidal cells were seen from the third to the seventh day after reperfusion. Coinciding with an increase of superoxide radicals and histopathological changes, superoxide radicals may contribute considerably to delayed neuronal death. It seems reasonable to assume that the delayed onset of neuronal damage may correlate well with delayed generation of superoxide radicals in the hippocampal CA1 subfield after ischaemia/reperfusion.

There are three hypotheses to explain the mechanism of superoxide radical generation after ischaemia/reperfusion: (1) an abnormal electron transport system of

mitochondria, (2) an abnormal hypoxanthine-xanthine oxidase system, and (3) activated neutrophils.^{7,28-30} When the electron transport system in the mitochondria is disturbed by ischaemia, excess leakage of electrons and reduction of oxygen result followed by the generation of superoxide radicals. Moreover, many more superoxide radicals appear after reoxygenation due to reperfusion.⁷ In the hypoxanthine-xanthine oxidase system, the depletion of the cell's ATP after ischaemia results in an elevated concentration of AMP, containing catabolised adenosine, inosine, and hypoxanthine. The elevated intracellular calcium caused by cerebral hypoxia activates a protease capable of converting xanthine dehydrogenase to oxidase. Superoxide radicals are then generated by oxidation of hypoxanthine under the presence of xanthine oxidase after ischaemia/reperfusion.^{31,32}

Neutrophils are another potential source of superoxide radicals. Neutrophils concentrate in one ischaemic

area after ischaemia/reperfusion and superoxide radicals are generated by a nicotinamide-adenine dinucleotide phosphate oxidase enzyme.^{28,32-36} Many investigators have reported that superoxide radicals are generated in the mitochondria immediately after ischaemia/reperfusion. However, it takes longer for the hypoxanthine-xanthine oxidase system and activated neutrophils to generate superoxide radicals.³¹⁻³⁶ Therefore, excessive generation of superoxide radicals on the third and fifth days after reperfusion in the present study may result from the hypoxanthine-xanthine oxidase system and from activated neutrophils. Ito *et al.*³⁷ reported amelioration of ischaemia/reperfusion cerebral damage by administering a xanthine oxidase inhibitor. Shiga *et al.*³⁸ reported that pretreatment of monoclonal antibody of neutrophils inhibited the brain oedema on the day following a transient ischaemia, probably due to exhaustion of circulatory neutrophils. These reports support our assumptions about the hypoxanthine-xanthine oxidase system and about activated neutrophils.

Hamada *et al.*³⁹ and Tagaya *et al.*⁴⁰ reported that administration of scavengers such as baialein and recombinant human superoxide dismutase prevented hippocampal neuronal death after ischaemia/reperfusion. In clinical practice, since the generation of superoxide radicals occurs from the third to fifth days after the incidence of ischaemia/reperfusion, it may be possible to prevent delayed neuronal death by administering free radical scavengers such as superoxide dismutase within three days.

We could not clarify the mechanism for delayed generation of superoxide radicals in this study. Additional studies are needed to examine why delayed generation of superoxide radicals occurs in the hippocampal CA1 subfield.

In conclusion, there was a correlation between the quantity of superoxide radicals and the histopathological changes of the pyramidal cells in the hippocampal CA1 subfield after ischaemia/reperfusion. These results suggest that superoxide radicals may play an important role in delayed neuronal death.

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