Protective Effects of Human Recombinant Superoxide Dismutase on Transient Ischemic Injury of CA1 Neurons in Gerbils

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Background and Purpose: It has been postulated that oxygen-derived free radicals are produced in significant quantities upon reperfusion of ischemic brain and that the free radicals play a pivotal role in triggering the ischemic neuronal damage causing delayed neuronal death. This study was undertaken to examine the effects of human recombinant superoxide dismutase on the delayed neuronal death of CA1 neurons and on the change in the expression of messenger ribonucleic acid for endogenous copper-zinc superoxide dismutase after transient ischemia.

Methods: Human recombinant superoxide dismutase (8 × 10⁵ units/kg) or apo-superoxide dismutase was administered intravenously 1 minute before bilateral carotid artery occlusion in gerbils divided among four experimental groups. Endogenous copper-zinc superoxide dismutase messenger ribonucleic acid was analyzed by in situ hybridization histochemistry using a sulfur-35-labeled oligonucleotide probe. Immunohistochemical localizations of administered human recombinant superoxide dismutase were investigated.

Results: All gerbils receiving apo-superoxide dismutase exhibited almost complete destruction of CA1 neurons 7 days after 5 minutes of ischemia. The gerbils treated with human recombinant superoxide dismutase showed mild lesions (p < 0.01). Discrete localizations were observed for endogenous copper-zinc superoxide dismutase messenger ribonucleic acid. Transient ischemia increased labeling throughout the hippocampus after 30 minutes and 24 hours of reperfusion. This increase was abolished by treatment with human recombinant superoxide dismutase. This phenomenon was confirmed by Northern blot analysis. The interneurons in CA3 and cells in the hilus were mainly stained against administered superoxide dismutase at 5 and 30 minutes, and these reactions had disappeared at 20 hours after the administration.

Conclusions: Our data demonstrate protective effects of human recombinant superoxide dismutase against ischemic neuronal damage and support the hypothesis that the generated free radicals induce a vicious cycle leading to delayed neuronal death. (Stroke 1992;23:75–81)
ous phenomenon. However, the precise mechanism involved during ischemia and recirculation remains to be clarified.

Recently, generated free radicals have been shown to cause an increased output of excitatory amino acids and to trigger the delayed loss of neurons following transient ischemia. Moreover, it has been reported that preischemic treatment with a derivatized SOD, pyran copolymer-conjugated SOD, shows clear protective effects against both the neuronal damage detected by immunohistochemistry after 5 minutes of ischemia and the delayed neuronal necrosis after 1 week of recovery.

The present study was undertaken to investigate the possible role of free radical formation in delayed neuronal death. We examined the effects of human recombinant copper-zinc SOD (h-rSOD), which has been shown to reduce the vasogenic edema developing after 3 hours of reperfusion and to afford significant cerebroprotection in gerbils undergoing 1 hour of bilateral carotid artery occlusion and reperfusion, on the delayed neuronal death of CA1 neurons and on the change in messenger ribonucleic acid (mRNA) expression for endogenous copper-zinc SOD (Cu-Zn SOD) after transient ischemia in gerbils. We also investigated the immunohistochemical localization of administered h-rSOD.

Materials and Methods

Five adult male Mongolian gerbils weighing 60–80 g were used in each experiment. The animals were lightly anesthetized with ether inhalation. Using an operating microscope, the right saphenous vein was cannulated with polyethylene catheter (PE-10) and the neck was dissected to allow simultaneous occlusion of both carotid arteries with aneurysm clips. Exactly 5 minutes after bilateral carotid artery occlusion, the aneurysm clips were removed and cerebral circulation was restored. To evaluate the protective effects of h-rSOD (Nippon Kayaku Co., Ltd., Tokyo, Japan), the gerbils were divided into the following groups: ischemia + apo-SOD (h-rSOD without the metal chelate), ischemia + h-rSOD, sham operation + apo-SOD, and sham operation + h-rSOD, where $8 \times 10^8$ units/kg h-rSOD, apo-SOD, or saline were administered intravenously via the saphenous vein 1 minute before bilateral carotid artery occlusion.

Endogenous Cu-Zn SOD mRNA was analyzed by in situ hybridization histochemical techniques according to the methods of Arentzen et al and Young et al. For hybridization, the 48-base-long sequence from the rat Cu-Zn SOD sequence (bases 465–512) was chosen. For the control experiments, in situ hybridization was carried out using a sense orientation probe or in the presence of a 100-fold excess of unlabeled probe. Probes were 3'-end-labeled with $\alpha$-32P-dATP (Oligonucleotide 3'-End Labeling System, New England Nuclear/Du Pont, Boston, Mass.) to a specific activity of $1.5 \times 10^9$ dpm/ug.

For in situ hybridization, the gerbils were decapitated 30 minutes, 3 hours, 6 hours, 24 hours, or 7 days after clip removal. The brains were quickly removed and frozen. Frozen 16-µm sections were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Sections were dried in warm air briefly and postfixed for 30 minutes in 4% paraformaldehyde in phosphate buffered saline. The sections were then rinsed twice in phosphate buffered saline followed by rinsing in 0.25% acetic anhydride in a 0.1 M triethanolamine HCl/0.9% NaCl solution. The sections were next rinsed in a series of ethanols (70%, 80%, 95%, and 100%) and in chloroform and ethanol (100% and 95%, respectively). Hybridization was performed by overnight incubation in a moist chamber kept at 42°C with the sections immersed in 100 µl of a mixture containing sulfur-35-labeled probes (1–5×10^6 dpm), 20 mM dithiothreitol, 50% deionized formamide, 4x sodium chloride–sodium citrate buffer (SSC), 0.48 µg/µl herring deoxyribonucleic acid (DNA), 1× Denhardt’s solution, and 10% dextran sulfate. Subsequently, the sections were washed in 1× SSC at 55°C for 15 minutes, followed by washing twice for 45 minutes each in the same solution at room temperature. The sections were then dehydrated in a graded alcohol series and dried. Slides were dipped in Kodak NTB-3 emulsion (Rochester, N.Y.) and stored at 4°C for 3 weeks. Autoradiograms were developed in Kodak D-19 developer and counterstained with cresyl violet.

For Northern analysis, the gerbils were decapitated 3 hours, 24 hours, or 3 days after clip removal. The brains were removed and frozen in liquid nitrogen and then stored at −80°C. Ribonucleic acid (RNA) was prepared from the brains by a guanidine thiocyanate method essentially as described by Chirgwin et al. Tissue (0.8–1.0 g) was homogenized in 15 ml of 4 M guanidine thiocyanate stock solution using a Polytron tissue homogenizer (Kinematica AG, Littau/Lucerne, Switzerland). The homogenate (7 ml) was layered onto 4 ml of 5.7 M CsCl, 10 mM ethylenediaminetetra-acetic acid (EDTA) in tubes for the Beckman SW 40 rotor (Fullerton, Calif.) and centrifuged at 32,000 g and 20°C for 20 hours. The pellet was dissolved in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS), extracted with an equal volume of chloroform: isooamyl alcohol (24:1), and precipitated with the addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The RNA was quantified spectrophotometrically, assuming that 40 µg RNA in 1 ml gives an absorbance of 1.0 at 260 nm. Integrity of the RNA preparations and consistent sample loading was verified by ethidium bromide staining of the transferred RNAs.

For electrophoresis, 15 µg total RNA was denatured and size-fractionated on a formaldehyde-agarose (1.2%) gel. After electrophoresis the RNA was blotted by capillary transfer onto nylon membranes (Gene Screen Plus, New England Nuclear). The membranes were prehybridized for 2 hours at 42°C in a buffer containing 50% deionized formamide, 10× Denhardt’s solution, 1 M NaCl, 0.5% SDS, 100
FIGURE 1. Representative photomicrographs of hippocampal CA1 region in gerbils 7 days after 5 minutes of bilateral carotid artery occlusion. A, B: treatment with apo-superoxide dismutase. Note marked damage to CA1 pyramidal cells. C, D: treatment with human recombinant superoxide dismutase. Most CA1 neurons are preserved. Cresyl violet stain; ×7 for A and C, ×35 for B and D.

μg/ml salmon sperm DNA, 25 mg/ml yeast transfer ribonucleic acid, and 100 μg/ml polyadenylic acid. Hybridization was carried out overnight at 42°C with the phosphorus-32-labeled oligonucleotide in the same buffer containing additional 10% dextran sulfate. The membranes were washed twice for 30 minutes each in 2× SSC, 0.1% SDS at room temperature and once for 10 minutes in 0.2× SSC, 0.1% SDS at 36°C. The membranes were subjected to autoradiography for several days at −80°C.

For the immunohistochemical studies of h-rSOD administered intravenously 1 minute before bilateral carotid artery occlusion, the gerbils were perfused with Zamboni's fixative under anesthesia with 50 mg/kg i.p. sodium pentobarbital 5 minutes, 30 minutes, 1 hour, 3 hours, or 20 hours after clip removal. Subsequently, the brains were removed and postfixed in the same fixative for 24 hours at 4°C. After immersion of the brains in 0.1 M phosphate buffer containing 30% sucrose, 16-μm sections were cut on a cryostat and subjected to immunohistochemistry. The sections were placed in a small vial and incubated overnight at 4°C with a 1:100 dilution of the primary antiserum. They were then stained according to the avidin-biotin-peroxidase complex method (Vecstatin ABC Kit, Burlingame, Calif.).

The antiserum employed was raised in New Zealand White rabbits to h-rSOD that was purified by adsorbing with rat Cu-Zn SOD (generous gift from Nippon Kayaku Co., Ltd.). To test the specificity of the immunohistochemical procedure, Western blot analysis and control experiments (omission of h-rSOD and use of the antiserum following its adsorption with excess h-rSOD) were performed.

Data are presented as mean±SD. Statistical analysis was performed by using Bonferroni's modified t test for examining differences in cell counts among the groups.

Results

The effect of h-rSOD on delayed neuronal death in the CA1 region was determined by cresyl violet staining 7 days after 5 minutes of ischemia. All gerbils that received apo-SOD 1 minute before carotid artery occlusion exhibited almost complete destruction of the CA1 region, and no sham-operated gerbil showed any lesions. On the other hand, all gerbils receiving preischemic h-rSOD treatment showed only mild lesions (Figure 1).

In the sham-operated groups neuronal cell density of the CA1 region was 44.0±3.0/200 μm. There was no difference between the sham operation + h-rSOD and sham operation + apo-SOD groups. In the ischemia + apo-SOD group, the number of CA1 pyramidal cells was markedly decreased (9.6±2.3/200 μm, p<0.01). Preischemic treatment with h-rSOD significantly (p<0.01) protected against pyramidal cell loss in the CA1 region (35.5±9.4/200 μm) (Figure 2).

In the hybridization experiments, discrete localizations were observed for endogenous Cu-Zn SOD mRNA, with a particularly dense band of radiolabeling in the hippocampus. No detectable hybridization was observed with a sense orientation probe. Negligible antisense labeling was observed in sections
FIGURE 2. Effect of human recombinant superoxide dismutase (SOD) on delayed neuronal death of hippocampal CA1 neurons in gerbils after 5 minutes of bilateral carotid artery occlusion. APO, apo-SOD. *p<0.01 different from treatment with APO.

Northern blot analysis confirmed the elevation of brain mRNA coding for endogenous Cu-Zn SOD 3 and 24 hours after ischemia. The amount of mRNA decreased to the control level 3 days after ischemia. This elevation of brain mRNA coding for endogenous Cu-Zn SOD was abolished by treatment with h-rSOD (Figure 4).

With respect to the immunohistochemical studies of administered h-rSOD, marked topographical differences in intensities of the immunoreactions were apparent. Cells in the CA3 and subiculum regions, which may be interneurons morphologically, and cells in the hilus were mainly stained 5 and 30 minutes after the administration of h-rSOD. Moreover, the perivascular and periventricular interstitial regions were stained. The immunohistochemical reaction for h-rSOD decreased between 1 and 3 hours and had completely disappeared at 20 hours after the administration (Figure 5). In control experiments using gerbils given neither h-rSOD nor apo-SOD (omission of the administration of h-rSOD and use of coincubated with a 100-fold excess of unlabeled antisense probe (data not shown). Labeling was increased virtually throughout the hippocampus following ischemia, from 30 minutes to 6 hours after clip removal. Twenty-four hours after the insult increased labeling could still be observed in the CA1 pyramidal neurons, but in the other regions labeling was decreased to the control level. Little labeling occurred in the CA1 region with cell loss 7 days after ischemia. This increase was abolished by treatment with h-rSOD (Figure 3).

FIGURE 3. Representative photomicrographs showing effect of human recombinant superoxide dismutase (h-rSOD) on endogenous Cu-Zn SOD hybridization in gerbil hippocampus following ischemia. A, B: apo-SOD treatment. C, D: h-rSOD treatment. A, C: sham operation. Panels illustrate representative sections obtained 3 hours (B, D) after 5 minutes of ischemia. Note increased labeling in dentate granule cell layer (arrowheads); this increase was abolished by h-rSOD treatment (B, D).
the antiserum following its adsorption with excess h-rSOD), no tissue sections showed immunostaining.

**Discussion**

The phenomenon of selective vulnerability of the hippocampus following transient cerebral ischemia has long received considerable attention. In gerbils, forebrain ischemia for 5 minutes leads to ischemic cell death (delayed neuronal death) that is restricted to the CA1 pyramidal neurons of the hippocampus. In recent years, two metabolic events (viz., free radical formation and excitatory amino acid release) have been proposed to explain this phenomenon. Formation of oxygen-derived free radicals has been demonstrated and proposed as an important step in the sequence of events that link cerebral blood flow reduction to neuronal death, both during the acute ischemic attack and when blood and oxygen eventually return to the brain upon reperfusion. Pyran copolymer-conjugated SOD has been reported to reduce ischemic neuronal damage in vivo. Another line of research has demonstrated that increased release of the excitatory amino acids aspartate and glutamate has neurotoxic effects and that excitatory amino acid receptor antagonists offer protection against ischemic brain damage in vivo. Recently, Pellegrini-Giampietro et al. have hypothesized that these two metabolic events are related and cooperate and that the generated free radicals cause a vicious cycle. In our present study, h-rSOD showed clear protective effects against delayed neuronal death after 1 week of recovery. Our observations may support the hypothesis of Pellegrini-Giampietro et al.

The very high dose of h-rSOD that we used was the dose required to reduce the vasogenic edema developing after 3 hours of reperfusion and to afford significant cerebroprotection in gerbils undergoing 1 hour of bilateral carotid artery occlusion and reperfusion. Tagaya et al. performed dose–response studies and showed the necessity of nearly that amount of h-rSOD to protect against early ischemic brain damage and delayed neuronal death in gerbils. Apo-SOD was used to negate the protein effect for the control experiment. However, we cannot deny the possibility that apo-SOD picks up copper. We performed immunohistochemistry to investigate localization of the administered h-rSOD. Cells in CA3 and the subiculum region were mainly stained; these cells are morphologically considered to be interneurons. Benveniste et al. have reported that ischemia-induced damage to pyramidal cells in CA1 is dependent on glutamate release from the intact innervation of CA3. γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter of the mammalian central nervous system, and interneurons include GABAergic neurons. Agents associated with the GABAergic system have been shown to provide protection against delayed neuronal death. Protection of the CA3 GABAergic system might be responsible for the protective effects of administered h-rSOD. However, interneurons themselves are rather resistant. Further studies are needed to establish the exact mechanisms and sites of the protective effects afforded by h-rSOD.

Gerbils are considered to have two forms of intracellular SODs because rats have two forms. One form is Cu-Zn SOD and the other is manganese (Mn) SOD. Immunolocalization of both Cu-Zn SOD and Mn SOD in various rat tissues has been investigated using the indirect immunoenzyme method, but there is little information on the morphological localization of both SODs in the hippocampus. Akai et al. have shown by the localization of Mn SOD that CA1 pyramidal cells were weakly immunostained, whereas CA3 pyramidal cells were strongly reactive. These authors emphasized that the difference in densities of Mn SOD immunostaining might relate to the vulnerability of CA1. The cellular localization of Cu-Zn SOD mRNA has been determined in the human hippocampus by in situ hybridization. This localization is almost the same as our results in gerbils. Endogenous Cu-Zn SOD mRNA expression showed a transient increase, being strongly induced in all major hippocampal neuronal populations. Notably, the most prolonged expression of Cu-Zn SOD mRNA was observed in vulnerable CA1 neurons.

If the generated oxygen-derived free radicals cause an increased output of excitatory amino acids and trigger a series of events that explain the delayed neuronal death following transient ischemia, why does an increase in Cu-Zn SOD mRNA fail to protect against neuronal death (Figure 4)? There are three possible answers to this question. First, the prolonged increase in Cu-Zn SOD mRNA does not necessarily indicate increased synthesis of Cu-Zn SOD protein. It could reflect the disturbed translation of Cu-Zn SOD mRNA. The same phenomenon is observed with 70-kd heat shock protein. Second, the increase in Cu-Zn SOD after 30 or more minutes of recirculation cannot reduce delayed neuronal death because superoxide radicals are generated
during ischemia and immediately upon recirculation. Third, too much cellular Cu-Zn SOD activity might itself contribute to the neurodegenerative process. This hypothesis is motivated by patients with Down’s Syndrome who have increased Cu-Zn SOD activity in various tissues including the brain. This question remains to be answered, however. It would be worthwhile investigating other enzymatic steps, such as those involving catalase and glutathione peroxidase. Moreover, quantitative analysis of Cu-Zn SOD protein is in progress for evaluating the level of transcription and translation of the Cu-Zn SOD gene.

References

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