The Protective Effect of L-Threo-3,4-dihydroxyphenylserine on Ischemic Hippocampal Neuronal Death in Gerbils

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**Background and Purpose** L-Threo-3,4-dihydroxyphenylserine (DOPS) is reported to increase the nerve growth factor (NGF) synthesis in cultured mouse L-M fibroblast and astroglial cells, and this effect is not blocked by treatment with decarboxylase inhibitor. NGF is suggested to play an important role in neuronal survival and regeneration under pathological conditions. We evaluated the possible protective effect of DOPS against hippocampal CA1 cell death after transient forebrain ischemia in gerbils.

**Methods** Male mongolian gerbils were treated with DOPS (30, 100, or 300 mg/kg IP) plus benserazide (10 mg/kg IP) (n=28) or vehicle (n=7) before 3.5 minutes of forebrain ischemia. For histopathological study, the animals were decapitated 7 days after recirculation, and neuronal density of the hippocampal CA1 area was counted after cresyl violet staining. For immunohistochemical study, another group of gerbils (n=34) was recovered for 1, 3, and 8 hours and 1, 2, and 7 days, when they were decapitated. The brain sections were stained against NGF, NGF receptor, and HSP70 using the avidin-biotin-peroxidase method.

**Results** Preservation of the hippocampal CA1 cells was found in the brains treated with 300 mg/kg DOPS plus benserazide (neuronal density, 125±24 cells per millimeter) compared with the vehicle-treated ones (49±11 cells per millimeter) (P<.01). The immunoreactive NGF was greatly reduced from 1 hour after recirculation in the vehicle group, but it was much less reduced in the 300-mg/kg-DOPS-plus-benserazide group as compared with the vehicle group. The immunoreactivity for NGF receptor was gradually induced from 1 hour after recirculation with the peak at 1 day in the vehicle group, but it was only slightly induced at 8 hours in the 300-mg/kg-DOPS-plus-benserazide group. HSP70 immunoreactivity was also induced from 3 hours with the peak at 1 day in the vehicle group. However, in the 300-mg/kg-DOPS-plus-benserazide group, the induction of HSP70 was found from 8 hours and was much less intensive.

**Conclusions** Treatment with DOPS is protective to the ischemic hippocampal CA1 cells, and the NGF-receptor system may play a role in this protective effect of DOPS. (Stroke. 1994;25:1425-1432.)

**Key Words** cerebral ischemia, transient hippocampus neuroprotection gerbils

The norepinephrine precursor L-Threo-3,4-dihydroxyphenylserine (DOPS) can penetrate the blood-brain barrier and induce a central effect in both animals and humans; it is used clinically to treat the patients of Parkinson's disease. It can be directly converted into L-norepinephrine by L-aromatic amino acid decarboxylase in the brain and kidney. Therefore, DOPS may act as a norepinephrine precursor in the brain and may increase the cerebral blood flow and locomotor activity via the activation of central noradrenergic neurons. Benserazide, an extracerebral decarboxylase inhibitor, at a high dose (1 mg/kg or more) may penetrate the blood-brain barrier and inhibit the decarboxylation of DOPS to norepinephrine. An addition of DOPS increases nerve growth factor (NGF) synthesis in cultured mouse L-M fibroblast and astroglial cells, and this effect is not blocked by treatment with decarboxylase inhibitor. This suggests that the increased NGF level is due to the direct effect of DOPS but not the secondary effect by conversion into norepinephrine.

Selective vulnerability of the brain neurons after ischemia is found especially in the hippocampal CA1, the dorsolateral striatal, certain layers of the cerebral cortical, and the cerebellar cortical cells. Among these neurons, hippocampal CA1 pyramidal cells are the most vulnerable. In rodents, a transient global cerebral ischemia causes delayed neuronal death of the CA1 cells found more than 2 days after recirculation. However, the exact mechanism has not been fully understood.

NGF is known to be required for the development, survival, and maintenance of special neuronal cell populations and is present at a high level in the normal hippocampus and cerebral cortex. On the other hand, NGF receptor, both the low (p75) and the high (trkA) affinity, is not normally present in the hippocampal and cerebral cortical neurons. Among these neurons, hippocampal CA1 pyramidal cells are the most vulnerable. In rodents, a transient global cerebral ischemia causes delayed neuronal death of the CA1 cells found more than 2 days after recirculation. However, the exact mechanism has not been fully understood.

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bound low-affinity form is required for the receptor to convert into the high-affinity form.10,17 These NGF-receptor complexes are then retrogradely transported to the cholinergic cell body where NGF exerts its biological actions.10 The monoclonal antibody (192-IgG) we used for immunohistochemical study has been reported to stain the low-affinity NGF receptor.18 However, other data17,19 (including our Western blot study) suggest that this 192-IgG can recognize both the low- and the high-affinity NGF receptors. For this reason, the reader should understand that the term “NGF-receptor immunoreactivity” implies “NGF-receptor-like immunoreactivity.”

NGF is effective in protecting rat hippocampal neurons against hypoglycemia.20 In gerbils, the delayed hippocampal CA1 neuronal death after ischemia can be ameliorated by an intraventricular injection of NGF.21 Studies using 192-IgG antibody found that the immunoreactive NGF receptor can be induced in hippocampal neurons after exposure to β-amyloid in vitro or after treatment with colchicine in vivo.15 Also, redistribution of NGF-receptor-positive immunostained fibers can be found in the dentate gyrus after ablation of the entorhinal cortex, which follows the reorganization pattern of septal fibers.22 Therefore, activation of NGF and the receptor system should be involved in the survival of neurons after brain injury such as ischemia. The 70-kD heat-shock protein (HSP70) is an inducible species of the “HSP70 family” under stressful conditions and has been recognized as a marker of cell injury.24,25 Using HSP70 gene expression as a marker of neuronal cell injury, the protective effect of DOPS itself on the hippocampal CA1 cells after transient forebrain ischemia was examined in relation to the change of immunoreactive NGF and its receptor.

**Materials and Methods**

Male Mongolian gerbils (*Meriones unguiculatus*, Seiwa Experimental Animals), aged 9 to 11 weeks and weighing 60 to 70 g, were given an intraperitoneal injection of DOPS (suspended in 0.5% methylcellulose, *n*=28) or vehicle (0.5% methylcellulose, *n*=30) 30 minutes before the ischemia. The DOPS was injected at a dose of 30, 100, or 300 mg/kg (*n*=9 to 10 for each dose) in each group. Benserazide, at a dose of 1 mg/kg, is known to penetrate the blood-brain barrier and significantly inhibit the rise of brain norepinephrine concentration induced by 400 mg/kg DOPS in norepinephrine-depleted mice.5 We used 10 mg/kg benserazide injected 30 minutes before the administration of DOPS to inhibit the conversion of DOPS into norepinephrine. Also, we used 10 mg/kg benserazide injected 30 minutes before the administration of vehicle in another group of gerbils (*n*=6) for comparison. Then the animals were lightly anesthetized by inhalation of a nitrous oxide/oxygen/halothane (69%:30%:1%) mixture. Both common carotid arteries were exposed, and anesthesia was discontinued. When the animals began to regain consciousness, the arteries were occluded for 3.5 minutes with surgical clips.26 Body and subtemporal temperatures were monitored in all animals and were maintained at around 37°C using a heating pad and overhead lamp during surgical preparation. After the restoration of blood flow, no attempt was made to maintain constant body and subtemporal temperature. Animals exhibiting convulsion or with loss of righting reflex after ischemia were excluded from the study. Sham-operated control animals (*n*=5) were killed just after exposing the carotid arteries without clamping the vessels. The animal study was approved by the animal committee of the Tohoku University School of Medicine.

For histopathologic study, the gerbils were anesthetized with sodium pentobarbital (50 mg/kg IP) at 7 days after recirculation, and their brains were perfused transcardially with 200 to 300 mL of 4% phosphate-buffered paraformaldehyde after a flush of 100 mL heparinized saline. The brains were removed, placed in 4% phosphate-buffered paraformaldehyde solution for 2 days, and then embedded in paraffin. Coronal sections of 5-μm thickness were cut using a microscope, stained with cresyl violet, and examined under a light microscope. The average value of neuronal density from both sides of hippocampal CA1 areas was counted for each animal by a person who was blind to the study.

For immunohistochemical study, another group of gerbils (*n*=34) treated with 300 mg/kg DOPS plus benserazide or vehicle was recovered after ischemia for 1, 2, and 7 hours and 1, 2, and 7 days (*n*=2 to 5 for each time point) at ambient temperature (21°C to 23°C) and then decapitated. The dissected brains were frozen in powdered dry ice and stored at −80°C. Sections (10-μm) at the dorsal hippocampal level were cut on a cryostat at −18°C and collected on glass slides coated with Histostick (Accurate Chemical and Scientific Corp.). Immunostaining against NGF, NGF receptor, and HSP70 in gerbil-brain sections was performed by avidin-biotin-peroxidase method (ABC) using a kit (PK-6101 for NGF, PK-6102 for NGF receptor and HSP70, Vector Laboratories). For NGF, the fresh-frozen sections were fixed for 30 minutes in 4% phosphate-buffered paraformaldehyde. For NGF receptor and HSP70, the fresh-frozen sections were fixed for 30 minutes in ice-cold acetone and air-dried. Then the sections were rinsed in 0.02 mol/L phosphate buffer containing 0.15 mol/L...
TABLE 1. Neuronal Density in the Hippocampal CA1 Area After Treatment With Vehicle or L-Threo-3,4-dihydroxyphenylserine Plus Benserazide (10 mg/kg) in 3.5-Minute Forebrain Ischemia in Gerbils

<table>
<thead>
<tr>
<th>Neuronal density, cells/mm</th>
<th>Control</th>
<th>Vehicle</th>
<th>Benserazide Plus Vehicle</th>
<th>30 mg/kg</th>
<th>100 mg/kg</th>
<th>300 mg/kg</th>
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<tbody>
<tr>
<td>(n=5)</td>
<td>220±27</td>
<td>49±11</td>
<td>45±15</td>
<td>40±7</td>
<td>66±24</td>
<td>125±24*</td>
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<td>(n=7)</td>
<td></td>
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<td>(n=6)</td>
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<td>(n=9)</td>
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<td>(n=9)</td>
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<td>(n=10)</td>
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DOPS indicates L-threo-3,4-dihydroxyphenylserine. Values expressed as mean±SEM.

*D<.01, compared with the vehicle group.

NaCl (pH 7.4). After blocking with 10% normal serum (normal goat serum for NGF and normal horse serum for NGF receptor and HSP70) for 2 hours, the slides were washed and incubated for 12 to 15 hours at 4°C with the first antibody: a rabbit polyclonal antibody against NGF (AB927, Chemicon), a mouse monoclonal antibody against NGF receptor (192-IgG, Oncogene Science), and a mouse monoclonal antibody against the stress-inducible species of the HSP70 family (RPNI197, which was originally designated as clone C92, Amersham), diluted in the phosphate buffer (1/200) containing 10% normal serum and 0.3% Triton X-100. Some sections were treated simultaneously without the first antibody. Specificity of the HSP70 antibody has been described elsewhere.25-27 Endogenous peroxidase was blocked for 30 minutes with 0.1% H2O2, and 100% methanol. The sections were then washed and incubated for 2 hours with the second antibody (1/200), biotinylated anti-rabbit IgG made in goat for NGF and anti-mouse IgG made in horse for NGF receptor and HSP70, in the buffer, followed by incubation for 30 minutes with avidin-biotin-horseradish peroxidase complex. Staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mmol/L Tris-HCl buffer, pH 7.4) in the presence of 0.02% H2O2.

Western blot analysis was performed to ensure the specificity of the first antibodies for NGF and NGF receptor according to a previous method.29-30 For NGF, normal gerbil brains (n=2), age 11 weeks, were homogenized in autoclaved double-distilled water and centrifuged at 12,000 g for 1 hour. For NGF receptor, normal gerbil brains (n=2), age 11 weeks, were homogenized in 10.5% sucrose and centrifuged at 3000 g for 5 minutes. The pellet was dissolved in 0.1 mol/L phosphate-buffered saline with 0.5% Nonidet P-40 (Sigma), incubated on ice for 10 minutes, and microcentrifuged for 10 minutes at 4°C. The absorbance at 560 nm was read to estimate the protein content of the supernatant (S1) for NGF and the Nonidet P-40 soluble fraction (S2) for NGF receptor using a BCA protein assay reagent kit (Pierce). About 12 µg for NGF or 7 µg for NGF receptor was applied per lane on a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis using Phastsystem (Pharmacia). A premixed size marker (Rainbow marker, Amersham) was also applied to indicate the molecular weight. After transfer to a nitrocellulose membrane with semidy transfer kit (Pharmacia), the membrane was stained with or without the first antibody by avidin-biotin-peroxidase method using the ABC kit. The submaxillary glands of normal mice (n=2, age 10 weeks) were analyzed with the same method for NGF receptor. Normal rat brain (n=1), age 11 weeks, was analyzed with the same method for NGF receptor for comparison.

Optical density of NGF, NGF receptor, and HSP70 immunoreactivities at 1 day after recirculation (n=5) was measured using an Image Analyzer (Kontron, Karl Zeiss) by a person who was blind to the study. After subtraction of the background density that was measured on the sections without the first antibody, the optical density was measured and expressed as a ratio to sham-operated control. The data were statistically compared between the vehicle- and 300-mg/kg-DOPS-plus-benserazide–treated groups.

Results

The histological examination with cresyl violet staining revealed that most of the hippocampal CA1 cells in the group treated with 300 mg/kg DOPS plus benserazide were preserved with a clear and round appearance (Fig ID) as compared with the almost destroyed or disappeared CA1 cells in the vehicle-treated group (Fig IB). Neuronal density of the CA1 area in the sham-operated control gerbils was 220±27 cells per millimeter (mean±SEM). The 300-mg/kg-DOPS-plus-benserazide group had a significantly higher neuronal density in the CA1 area (125±24 cells per millimeter) than the vehicle group (49±11 cells per millimeter) (P<.01). However, no significant difference was found in the comparison of the vehicle- and 300-mg/kg-DOPS-plus-benserazide–treated groups.
Fig 3. Representative photomicrographs of immunostaining against nerve growth factor (NGF) receptor in the hippocampal CA1 areas. Left and right sides are the vehicle-treated and 300-mg/kg L-threo-3,4-dihydroxyphenylserine (DOPS)-plus-benserazide-treated brains, illustrating the sham-operated control (A) and reperfused brains at 8 hours (B and E), 1 day (C and F), and 2 days (D and G), respectively. The NGF receptor immunoreactivity is induced in the vehicle-treated brains with a peak at 1 day (C) and is only slightly induced at 8 hours (E) in the DOPS-plus-benserazide-treated ones. Original magnification, ×50.

groups treated with DOPS 30 mg/kg (40±7 cells per millimeter) or 100 mg/kg (66±24 cells per millimeter) plus benserazide, or benserazide plus vehicle (45±15 cells per millimeter) as compared with the vehicle group (P>.05) (Table 1). None of the areas in the hippocampal formation except the CA1 subfield was found to have neuronal change after treatment with vehicle or DOPS plus benserazide.

Northern blot analysis and enzyme-linked immunosassay for rat NGF showed that NGF and its messenger RNA (mRNA) were present in a relatively high level in normal hippocampal formation and cerebral cortex. However, the fixation-dependent lability of NGF antigen hampered attempts to localize NGF by immunohistochemical methods. Previous studies showed that prolonged fixation might result in a marked reduction of the NGF immunoreactivity. We attempted to fix our fresh-frozen sections briefly for 30 minutes in 4% phosphate-buffered paraformaldehyde, which to our experience gave the best preservation of the NGF antigen. We found that in the sham-operated control brain sections, the NGF immunoreactivity was present mostly in the dentate gyrus and pyramidal cell layer of hippocampal formation (Fig 2A) and in the neurons of cerebral cortex. Also, the dentate gyrus had a denser NGF immunoreactivity than the CA1-CA3 pyramidal cells. Our finding was compatible with the in situ hybridization studies in rats that demonstrated that the NGF mRNA hybridizing neurons were densely present in the dentate gyrus and were also present in the pyramidal cell layer of hippocampal formation and in the cerebral cortex. However, the immunohistochemical studies in rats using the same rabbit polyclonal antibody against mouse submaxillary gland NGF showed that the NGF immunoreactivity was primarily associated with fibers located in the hilus of dentate gyrus and in CA2-CA3 areas. In these studies, they used perfusion/immersion fixation with formaldehyde for longer than 2 hours and then cryoprotected the brain specimens in the sucrose solution overnight. Also, in these studies, Conner et al found that the NGF immunoreactivity was not seen in the cerebral cortex and that the localization of NGF immunoreactivity within the fiber pathway could be obtained when using the preimmune IgG fraction instead of the first antibody or using a higher concentration of the second antibody with the first antibody omitted. Nevertheless, our study showed that after transient forebrain ischemia, the NGF immunoreactivity in the CA1 area decreased significantly from 3 hours after recirculation in the vehicle group (Fig 2, B through D). However, in the 300-mg/kg-DOPS-plus-benserazide group, there was only slight reduction of NGF immunoreactivity as compared with the vehicle group (Fig 2, E through G).

The NGF receptor mRNA was not detectable or present in a very small level in the normal hippocampal and cortical neurons. Immunohistochemical studies using the same 192-IgG antibody also showed that immunoreactive NGF receptor was not present in the hippocampal cell bodies in normal rats. Conner et al, on the other hand, found that the immunostaining for NGF receptor was intense within the supragranular
zone and molecular layer of the dentate gyrus and within the pyramidal cell layer of CA1-CA3 in normal rats. However, our study showed that the immunoreactivity for NGF receptor was present in very small amounts in control hippocampal CA1-CA3 pyramidal cells (Fig 3A) and dentate gyrus. After transient forebrain ischemia, the immunoreactive NGF receptor in the CA1 area was gradually induced from 1 hour after recirculation with the peak at 1 day in the vehicle group (Fig 3, B through D). However, it was only slightly induced at 8 hours in the group treated with 300 mg/kg DOPS plus benserazide (Fig 3E) and was not induced at 1 to 2 days (Fig 3, F and G).

HSP70 immunoreactivity was rarely present in the normal hippocampal CA1 cells (Fig 4A). It was greatly induced from 3 hours after recirculation and reached the peak at 1 day (Fig 4C) in the vehicle group. However, in the 300-mg/kg-DOPS-plus-benserazide group, the induction of HSP70 was found from 8 hours and was much less intensive (Fig 4, E through G) as compared with the vehicle group. The CA3 and dentate granule cells of hippocampus showed a similar pattern of the HSP70 induction to those in the CA1 area, but there was no immunoreactivity visible in the CA3 subfield in the group treated with 300 mg/kg DOPS plus benserazide (data not shown). The sections stained without the first antibody against NGF, NGF receptor, and HSP70 showed no immunoreactivity (data not shown).

Statistical analysis of the optical densities for NGF, NGF receptor, and HSP70 immunoreactivities showed a significant difference between the vehicle-treated and 300-mg/kg-DOPS-plus-benserazide–treated groups (P<.01) (Table 2).

The rabbit anti-NGF polyclonal antibody (Chemicon) was raised against purified NGF obtained from mouse submaxillary gland and was affinity purified. This antibody is known to react with the β-subunit of 7S NGF and the 7S NGF itself in the solid phase of two-site enzyme-linked immunosorbant assay. We used the mouse submaxillary gland for comparison with gerbil brain. The mouse submaxillary gland is known to contain abundant NGF, and its homogenate extract consists exclusively of the 7S form. Our Western blot analysis for NGF showed that there was a band for gerbil (Fig 5, NGF, G) that had a similar size as the mouse submaxillary gland (molecular weight, 140 kD; Fig 5, NGF, M). The monoclonal antibody (192-IgG) was generated by immunization of mice with solubilized PC12 cell membranes and was suggested to identify the low-affinity receptor only. However, other studies reported that this 192-IgG antibody could recognize both the low and high affinity of NGF receptor, which might possess a common epitope on their receptor site. Nevertheless, our Western blot analysis for NGF receptor showed that there were two bands visible for gerbil (Fig 5, NGF-R, G) that were similar to the low and high affinity of NGF receptor for rat, 80 and 133 kDa, respectively (Fig 5, NGF-R, R). The control membranes stained without the polyclonal antibody against NGF or the monoclonal antibody against NGF receptor showed no such bands (Fig 5, NGF, C, and NGF-R, C).
DOPS is reported to increase NGF synthesis in cultured mouse L-M fibroblast and astroglial cells primarily by increasing the cellular content of NGF mRNA, and this is a dose-dependent effect. Because this effect was not blocked by a decarboxylase inhibitor, DOPS may have direct effect to increase the NGF content.

NGF is present at a high level in normal hippocampal formation. Shozuhara and Hashimoto reported that in the CA1 area, the level of NGF protein decreased initially but became significantly increased 2 weeks after ischemia or later. This increase of NGF content after ischemia is suggested to be due to the enhanced NGF synthesis by reactive astrogliosis. Northern blot analysis using the same rat model as Shozuhara's on the other hand, showed that in the CA1 area, NGF mRNA increased markedly initially but decreased to below the sham-operated control level by 2 days after ischemia. These data may suggest that in the CA1 area, there is a translation defect for NGF synthesis in the early stage after ischemia. The low- and high-affinity NGF receptors are absent or present in very small amounts in normal hippocampal formation. However, recent immunocytochemical and Western blot studies showed that both the low- and the high-affinity NGF receptor could be expressed in cultured hippocampal neurons, and the immunoreactive NGF receptor is also inducible in the hippocampal neurons after exposure to β-amyloid in vitro or after treatment with colchicine in vivo. Expression of low-affinity NGF receptor may induce neural cell death constitutively when low-affinity receptor is unbound, and binding by NGF or the monoclonal antibody against NGF receptor can inhibit the cell death. These data explain the dependence of some neural cells on NGF for survival and suggest that under stressful conditions, the cells that can express NGF receptor may die eventually if the NGF protein decreases markedly after the injury. In fact, our present study demonstrates that in the vehicle group, which has significant CA1 neuronal death, NGF immunoreactivity decreases markedly (Fig 2, B through D), and NGF-receptor immunoreactivity increases greatly in the CA1 area after ischemia (Fig 3, B through D). However, treatment with DOPS may activate the NGF synthesis to support the CA1 cells for survival (Fig 2, E through G) and decrease the demand to induce the NGF receptor (Fig 3, E through G).

HSP70 is a protein that is induced under various stressful conditions including ischemia. Once HSP70 is induced, it may prevent certain proteins from being denatured and protect cells from subsequent severe injury. HSP70 is also suggested as a marker for neuronal circuitry involved in the excitotoxic mechanisms after ischemia and other stresses. In the neurons most susceptible to ischemic injury (eg, the hippocampal CA1 neurons), the expression of HSP70 protein occurred after a brief period of ischemia. In contrast, in more resistant neurons (eg, dentate granular neurons) the expression of HSP70 was less inducible and occurred only after longer duration of ischemia. In our study, the expression of HSP70 was substantially reduced after treatment with DOPS. This suggests that treatment with DOPS was protective to the hippocampal CA1 cells, which reduced the subsequent induction of a putative cell-protective protein such as HSP70 in the CA1 cells.

Our study shows that there is a protective effect of DOPS itself on the ischemic hippocampal CA1 cells. This protective effect of DOPS on the hippocampal CA1 cells may be related to the role of the NGF-receptor system as well as HSP70.

Acknowledgments

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References


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