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Pitavastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, reduces hippocampal damage after transient cerebral ischemia in gerbils

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Summary. Pitavastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, is a potent cholesterol-lowering drug that reduces the risk of myocardial infarction and stoke. In this study, we examined its neuroprotective effects against hippocampal CA1 neuronal damage following transient cerebral ischemia in gerbils. Forebrain ischemia was induced by occlusion of bilateral common carotid arteries for 5 min. Pitavastatin, at a dose of 3, 10 or 30 mg/kg, was administered orally twice a day for 5 consecutive days and transient cerebral ischemia was induced in mice 1 h after the last treatment with pitavastatin. Histopathological observations showed that neuronal damage to the hippocampal CA1 neurons, which was observed 5 days after ischemia in animals, was prevented by pitavastatin treatment. Immunohistochemical staining for copper/zinc superoxide dismutase (SOD) and manganese SOD decreased in the hippocampal CA1 sector of gerbils 2 days after ischemia when histological neuronal destruction was not yet found, but was clearly observed in pitavastatin-treated animals. These results indicate that pitavastatin can protect dose-dependently against ischemia-induced neuronal damage and that the mechanism of the neuroprotection may be related to the preservation of SODs, especially copper/zinc-SOD. This in part explains how pitavastatin therapy, which targets free radicals, has beneficial effects against disorders including ischemic stroke.

Keywords: Cerebral ischemia, superoxide dismutase, neuronal damage, immunohistochemistry, free radicals, gerbils.

Introduction

A brief period of global cerebral ischemia in the gerbil causes delayed neuronal death of selectively vulnerable CA1 neurons of the hippocampus (Kirino, 1982; Araki et al., 1990), but the detailed mechanisms of such neuronal death in the hippocampal CA1 sector remain elusive. Reactive oxygen species (ROS) are well known to be excessively induced during reperfusion after global cerebral ischemia. These radicals initiate a radical chain reaction and/or signaling pathways that involve mitochondria and lead to neuronal death. Some endogenous antioxidant enzymes like superoxide dismutases (SODs), glutathione peroxidases and catalases can eliminate ROS, but excessive ROS produced following reperfusion and cerebral ischemia will exceed the capacity of these enzymes and lead to neuronal cell loss. Several studies have suggested that transgenic animals that overexpress copper/zinc-SOD (Cu/Zn-SOD), mitochondrial manganese SOD (Mn-SOD) or extracellular SOD show reduced damage after focal cerebral ischemia (Yang et al., 1994; Keller et al., 1998; Sheng et al., 1999). In contrast, the infarct in Cu/Zn-SOD, mitochondrial Mn-SOD or extracellular SOD knockout mice is increased after transient focal cerebral ischemia (Kondo et al., 1997; Sheng et al., 1999; Kim et al., 2002). Thus, much of the evidence about the roles of ROS and SOD against brain damage after cerebral ischemia is contradictory. However, some natural antioxidants such as vitamin C, vitamin E and uric acid have been demonstrated to be protective in animal models of stroke or in clinical experiments (Gilgun-Sherki et al., 2002). From these observations, it is conceivable that reducing excessive ROS may ameliorate oxidative damage after cerebral ischemia.

Recent studies have demonstrated that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors can reduce focal cerebral ischemia and infarct size in mice (Endres et al., 1998; Laufs et al., 2002). The HMG-CoA reductase inhibitors are known to upregulate endothelial nitric oxide synthase (eNOS) expression and activity and protect against focal cerebral ischemia (Laufs et al., 2002). In contrast, Kawashima et al. (2003) suggested that an HMG-CoA reductase inhibitor, cerivastatin, can exert protection against hypertension-based stroke and ameliorate the disease severity via inhibition of superoxide production and modulation of inflammation in the brain. Therefore, ROS may play a key role in the pathogenesis of ischemic brain damage.

Pitavastatin is a new synthetic potent and selective inhibitor of HMG-CoA reductase (Aoki et al., 1997). This drug is known to lower plasma total cholesterol levels and decrease triglyceride levels (Kajinami et al., 2000). Furthermore, this drug is known to cross the blood-brain barrier and distribute in the brain (Fujino et al., 1998). However, little is known about the neuroprotective effect of pitavastatin against transient global ischemia. The aim of the present study, therefore, was to determine utilizing immunohistochemistry whether treatment with pitavastatin can decrease the neuronal damage of the hippocampal CA1 sector after transient cerebral ischemia in gerbils via inhibition of superoxide production.

Materials and methods

Animals

Male Mongolian gerbils weighing 50–70 g (Nihon SLC Co., Shizuoka, Japan) were used in this study. The animals were kept in a controlled environment ($23 \pm 1°C$, $50 \pm 5\%$ humidity) and were allowed food and tap water *ad libitum*. The room lights were on between 8:00 and 20:00. The animals were anesthetized with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. The bilateral carotid arteries were exposed and anesthesia was discontinued to minimize its effect. The carotid arteries were clamped with aneurysm clips for 5 min, and then the gerbils were allowed to survive for 1 or 5 h, or 1, 2, 5 or 14 days after the cerebral ischemia. Sham-operated animals were treated in the same manner, except for clipping of the bilateral carotid arteries. Body temperature was maintained at 37–38°C with a heating pad equipped with a thermostat until the animals started moving after the operation.

Drugs

To evaluate the prophylatic effect of drug treatment, pitavastatin at a dose of 3, 10 or 30 mg/kg was administered orally twice a day for 5 consecutive days. Transient cerebral ischemia was induced in mice 1 h after the last treatment with pitavastatin. Vehicle (0.5% carboxy-methylcellulose, CMC) was also administered to animals under the same conditions. To examine the mechanisms of neuroprotection with pitavastatin, we also investigated the effect of this compound at a dose of 10 mg/kg 5 h, 1 and 5 days after transient cerebral ischemia. Pitavastatin was generously provided by Kowa Company, Ltd. Tokyo, Japan.

Immunohistochemistry

The animals were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) 1 or 5 h, or 1, 2, 5 or 14 days after ischemia, and the brains were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) following a heparinized saline flush. The brains were removed 1 h after perfusion fixation at 4°C and were immersed in the same fixative overnight at 4°C. Then the brain sections were embedded in paraffin using standard procedures. Paraffin sections of the hippocampus, 5 μm in thickness, were stained with Cresyl violet and used for immunohistochemistry. Each group consisted of 5–8 gerbils. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Tohoku University School of Medicine.

For immunohistochemical studies, a Vectastain elite ABC kit (Vector Labs., Burlingame, CA, USA), a Vectastain M.O.M. Kit (Vector Labs., Burlingame, CA, USA), mouse anti-microtubule-associated protein 2a,b (MAP2) monoclonal antibody (Chemicon International Inc., Temecula, CA, USA), rabbit anti-Cu/Zn superoxide dismutase (Cu/Zn-SOD) polyclonal antibody (Stressgen, Victoria, BC, Canada), rabbit anti-manganese superoxide dismutase (Mn-SOD) polyclonal antibody (Stressgen, Victoria, BC, Canada) and mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) were used. For histochemical staining, alpha-D-galactosyl-specific isolectin B_4 conjugated with horseradish peroxidase derived from *Griffonia simplicifolia seeds* (Isolectin B_4) (Sigma, St Louis, MO, USA) was used. The staining with anti-MAP2 antibody (1:500), anti-Cu/Zn-SOD antibody (1:100), anti-Mn-SOD antibody (1:250), anti-GFAP antibody (1:200) and isolectin B_4 (20 μl/ml) was performed as described previously (Kato et al., 1995; Muramatsu et al., 2003). Negative control sections were treated in the same way except that each antibody or isolectin B_4 was omitted. Immunoreactions for MAP2, Cu/Zn-SOD and Mn-SOD were visualized using a Vector DAB (3′,3′-diaminobenzidine) substrate kit (Vector Labs, Burlingame, CA, USA). The immunostaining for MAP2, Cu/Zn-SOD and Mn-SOD was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0) as described previously (Kato et al., 1995; Muramatsu et al., 2003). Values were expressed as means ± S.D. Statistical significance was evaluated using the non-parametric Dunnett's multiple comparison test and Mann-Whitney U-test.

Double-labeled immunostaining with anti-Cu/Zn-SOD and anti-GFAP antibodies or anti-Cu/Zn-SOD antibody and isolectin B_4 was performed for some sections of gerbil hippocampus

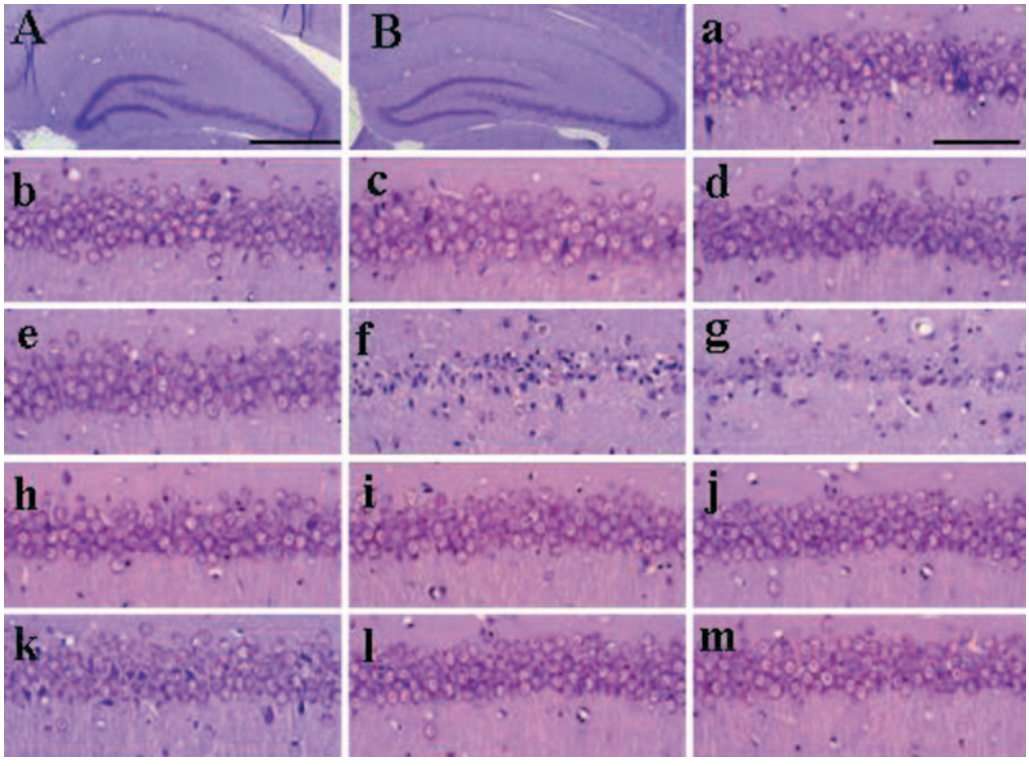

Fig. 1

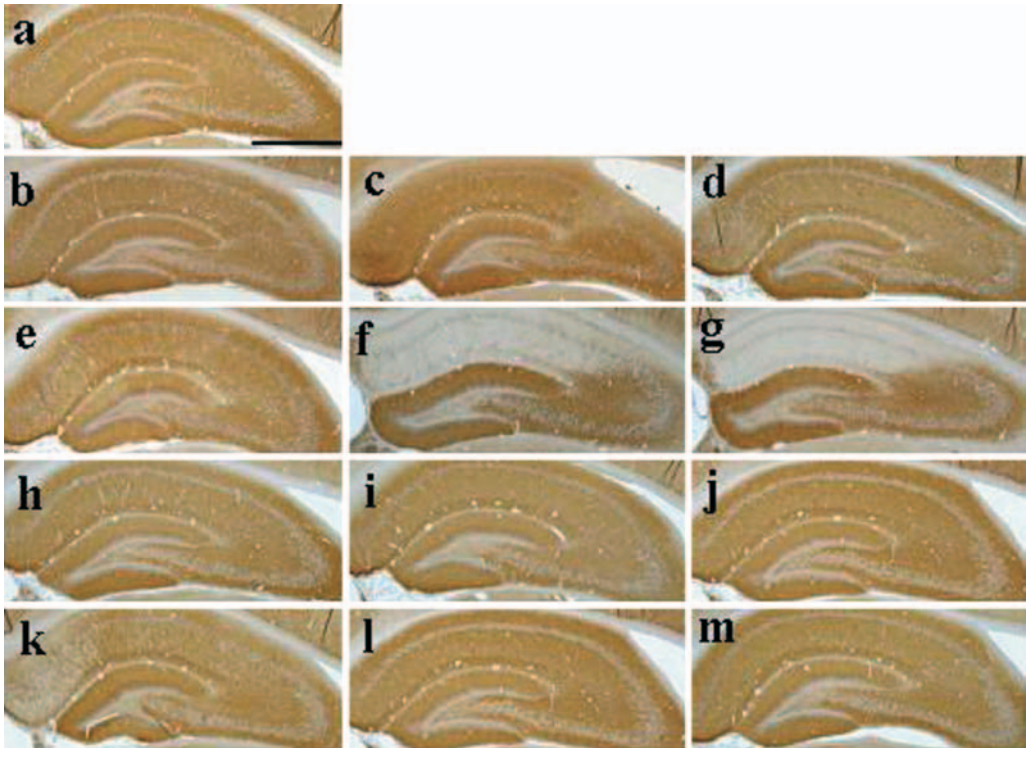

Fig. 2

after transient cerebral ischemia. Furthermore, double-labeled immunostaining with anti-Mn-SOD and anti-GFAP antibodies or anti-Mn-SOD antibody and isolectin B_4 was performed for some sections of gerbil hippocampus after transient cerebral ischemia, as described previously (Muramatsu et al., 2003). Immunoreactions for double-labeled staining were visualized using a Vector DAB substrate kit (brown) and a Vector SG substrate kit (blue).

Results

Neuronal cell death in the hippocampus after transient cerebral ischemia

Representative photographs of cresyl violet staining in the hippocampus and hippocampal CA1 sector after transient cerebral ischemia are shown in Fig. 1. Sham-operated gerbils showed no neuronal damage throughout the experiments in the hippocampus. Gerbils subjected to ischemia showed no neuronal damage in the hippocampus up to 2 days after ischemia. Five and fourteen days after ischemia, severe neuronal damage was observed in the hippocampal CA1 pyramidal neurons. However, neuronal damage was not found in the hippocampal CA3 sector or dentate gyrus. Prophylactic treatment with pitavastatin dose-dependently prevented the neuronal cell loss in the hippocampal CA1 sector 5 days after ischemia (Table 1).

Representative photographs of MAP2 immunostaining in the hippocampus after transient cerebral ischemia are shown in Fig. 2. MAP2-positive neurons were easily detectable in the hippocampus of sham-operated gerbils. The bodies and fibers of hippocampal neurons were intensely stained with evident immunopositive processes. Gerbils subjected to ischemia showed no damage in MAP2-positive neurons in the hippocampus 5 h after ischemia. One and two

Fig. 1. Representative light micrographs of cresyl violet staining in the gerbil hippocampus (**A** and **B**) and hippocampal CA1 sector (**a**–**m**) after transient cerebral ischemia. **A** Sham-operated gerbil hippocampus; **B** vehicle-treated gerbil hippocampus 5 days after ischemia. **a** Sham-operated gerbil brain; **b** vehicle-treated gerbil brain 1 h after ischemia; **c** vehicle-treated gerbil brain 5 h after ischemia; **d** vehicle-treated gerbil brain 1 day after ischemia; **e** vehicle-treated gerbil brain 2 days after ischemia; **f** vehicle-treated gerbil brain 5 days after ischemia; **g** vehicle-treated gerbil brain 14 days after ischemia; **h** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 h after ischemia; **i** pitavastatin (10 mg/kg, po)-treated gerbil brain 1 day after ischemia; **j** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **k** pitavastatin (3 mg/kg, po)-treated gerbil brain 5 days after ischemia; **l** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **m** pitavastatin (30 mg/kg, po)-treated gerbil brain 5 days after ischemia. Bar = 1 mm (**A** and **B**). Bar = 100 μm (**a**–**m**). n = 7–8 animals

Fig. 2. Representative photographs of MAP2 immunostaining in the gerbil hippocampus after transient cerebral ischemia. **a** Sham-operated gerbil brain; **b** vehicle-treated gerbil brain 1 h after ischemia; **c** vehicle-treated gerbil brain 5 h after ischemia; **d** vehicle-treated gerbil brain 1 day after ischemia; **e** vehicle-treated gerbil brain 2 days after ischemia; **f** vehicle-treated gerbil brain 5 days after ischemia; **g** vehicle-treated gerbil brain 14 days after ischemia; **h** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 h after ischemia; **i** pitavastatin (10 mg/kg, po)-treated gerbil brain 1 day after ischemia; **j** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **k** pitavastatin (3 mg/kg, po)-treated gerbil brain 5 days after ischemia; **l** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **m** pitavastatin (30 mg/kg, po)-treated gerbil brain 5 days after ischemia. Bar = 1 mm (**a**–**m**). n = 5–8 animals

Table 1. Effect of pitavastatin on neuronal densities in the CA1 sector of the hippocampus of the gerbil brain 5 days after transient forebrain ischemia

	Number of CA1 neurons/mm
Sham	$262 \pm 52^*$
Ischemia + 0.5% CMC	56 ± 53
Ischemia + pitavastatin	
Pitavastatin (3 mg/kg)	$153 \pm 75^*$
Pitavastatin (10 mg/kg)	$232 \pm 33^*$
Pitavastatin (30 mg/kg)	$243 \pm 15^*$

Values are expressed as means ± S.D. Statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (* $p < 0.01$ compared with ischemia +0.5% CMC group). Each group contained 7–8 gerbils

days after ischemia, mild neuronal damage in MAP2-positive neurons was observed in the hippocampal CA1 sector. Thereafter, severe damage in MAP2-positive neurons was found in the hippocampal CA1 sector, but not in the hippocampal CA3 sector or dentate gyrus 5 and 14 days after ischemia (Table 2). Prophylactic treatment with pitavastatin significantly attenuated the decreases in the number of MAP2-positive neurons in the hippocampal CA1 sector 1 and 5 days after ischemia (Tables 2 and 3).

Cu/Zn-SOD immunostaining

Representative photographs of Cu/Zn-SOD immunostaining in the hippocampus and hippocampal CA1 sector after transient cerebral ischemia are shown in Fig. 3. In sham-operated animals, intense Cu/Zn-SOD immunoreactivity was found in the cytoplasm of pyramidal neurons and interneurons in the hippocampal CA1 and CA3 sectors, but the staining was moderate in dentate granule cells. Scattered glial cells were also stained. The gerbils subjected to ischemia showed reduced Cu/Zn-SOD immunoreactivity by 1 day after ischemia and the reduction of the immunoreactivity in the hippocampal CA1 neurons never recovered up to the 14th day when the CA1 neurons had completely disappeared. In contrast, the hippocampal CA3 neurons and dentate granule cells showed no significant changes in Cu/Zn-SOD immunoreactivity up to 2 days after ischemia. Thereafter, intense Cu/Zn-SOD immunoreactivity was observed in the dentate granule cells 5 and 14 days after ischemia. The hippocampal CA3 neurons also showed a tendency of increased Cu/Zn-SOD immunoreactivity 5 and 14 days after ischemia. On the other hand, a large number of Cu/Zn-SOD-immunopositive glial cells were observed in the hippocampal CA1 sector after 5 and 14 days (Tables 4 and 5). Prophylactic treatment with pitavastatin significantly attenuated the decreases in the number of Cu/Zn-SOD-immunopositive neurons in the hippocampal CA1 sector 5 hr, 1 day and 5 days after ischemia (Table 4). Furthermore, this drug reduced the significant increase in the number of Cu/Zn-SOD-immunoreactive glial cells in the hippocampal CA1 sector 5 days after ischemia (Table 5). In addition, pitavastatin also prevented the increases in the Cu/Zn-SOD immunoreactivity of the dentate granule cells and hippocampal CA3 cells 5 days after ischemia (Table 5).

Table 2. Effect of pitavastatin on MAP2 immunoreactivity in the gerbil hippocampus after transient cerebral ischemia

	After ischemia						
	Sham	1 h	5 h	1 day	2 days	5 days	14 days
Ischemia CA1 pyramidal cells	2.8 ± 0.2	2.7 ± 0.3	2.6 ± 0.3	2.1 ± 0.4	2.1 ± 0.2	$0.7 \pm 0.7^{*}$	$0.7 \pm 0.6^{*}$
CA3 pyramidal cells	2.6 ± 0.1	2.5 ± 0.2	2.5 ± 0.3	2.7 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.2
Dentate granule cells	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.2	2.2 ± 0.1
Ischemia + Pitavastatin (10 mg/kg)							
CA1 pyramidal cells	–	–	2.9 ± 0.1	$2.7 \pm 0.2^{\dagger}$	–	$2.6 \pm 0.3^{\dagger}$	–
CA3 pyramidal cells	–	–	2.6 ± 0.1	2.6 ± 0.1	–	2.6 ± 0.2	–
Dentate granule cells	–	–	2.1 ± 0.1	2.1 ± 0.1	–	2.1 ± 0.2	–

The immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0). Values are expressed as means ± S.D. For the time course study, statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (* $p < 0.01$ compared with sham-operated control). For the evaluation of drugs, statistical significance was performed with Mann-Whitney U-test for non-parametric analysis († $p < 0.05$ compared with each day after cerebral ischemia). Each group contained 5–8 gerbils. –: not test

Table 3. Effect of pitavastatin on MAP2 immunoreactivity in the gerbil hippocampus 5 days after transient cerebral ischemia

	Sham	Ischemia	Ischemia + pitavastatin		
			Pitavastatin (3 mg/kg)	Pitavastatin (10 mg/kg)	Pitavastatin (30 mg/kg)
CA1 pyramidal cells	$2.8 \pm 0.2^*$	0.7 ± 0.7	1.8 ± 0.7	$2.6 \pm 0.3^*$	$2.4 \pm 0.3^*$
CA3 pyramidal cells	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.2	2.6 ± 0.2	2.5 ± 0.2
Dentate granule cells	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	2.1 ± 0.2

The immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0). Values are expressed as means ± S.D. For the time course study, statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (* $p < 0.01$ compared with ischemia group). Each group contained 7–8 gerbils

Mn-SOD immunostaining

Representative light micrographs of Mn-SOD immunostaining in the hippocampus and hippocampal CA1 sector after transient cerebral ischemia are shown in Fig. 4. In sham-operated gerbils, the hippocampal CA1 neurons showed more intense Mn-SOD immunoreactivity than the hippocampal CA3 neurons. Furthermore, a small number of non-pyramidal neurons, which appeared morphologically to be interneurons, in or near the CA1 and CA3

Fig. 3. Representative photographs of Cu/Zn-SOD immunostaining in the gerbil hippocampus (**A** and **B**) and hippocampal CA1 sector (**a**–**m**) after transient cerebral ischemia. **A** Sham-operated gerbil hippocampus; **B** vehicle-treated gerbil hippocampus 5 days after ischemia. **a** Sham-operated gerbil brain; **b** vehicle-treated gerbil brain 1 h after ischemia; **c** vehicle-treated gerbil brain 5 h after ischemia; **d** vehicle-treated gerbil brain 1 day after ischemia; **e** vehicle-treated gerbil brain 2 days after ischemia; **f** vehicle-treated gerbil brain 5 days after ischemia; **g** vehicle-treated gerbil brain 14 days after ischemia; **h** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 h after ischemia; **i** pitavastatin (10 mg/kg, po)-treated gerbil brain 1 day after ischemia; **j** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **k** pitavastatin (3 mg/kg, po)-treated gerbil brain 5 days after ischemia; **l** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **m** pitavastatin (30 mg/kg, po)-treated gerbil brain 5 days after ischemia. Bar = 1 mm (**A** and **B**). Bar = 100 μm. n = 5–8 animals

Fig. 4. Representative light micrographs of Mn-SOD immunostaining in the gerbil hippocampus and hippocampal CA1 sector after transient cerebral ischemia. **a** Sham-operated gerbil brain; **b** vehicle-treated gerbil brain 1 h after ischemia; **c** vehicle-treated gerbil brain 5 h after ischemia; **d** vehicle-treated gerbil brain 1 day after ischemia; **e** vehicle-treated gerbil brain 2 days after ischemia; **f** vehicle-treated gerbil brain 5 days after ischemia; **g** vehicle-treated gerbil brain 14 days after ischemia; **h** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 h after ischemia; **i** pitavastatin (10 mg/kg, po)-treated gerbil brain 1 day after ischemia; **j** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **k** pitavastatin (3 mg/kg, po)-treated gerbil brain 5 days after ischemia; **l** pitavastatin (10 mg/kg, po)-treated gerbil brain 5 days after ischemia; **m** pitavastatin (30 mg/kg, po)-treated gerbil brain 5 days after ischemia. Bar = 1 mm (**A** and **B**). Bar = 100 μm (**a**–**m**). n = 5–8 animals

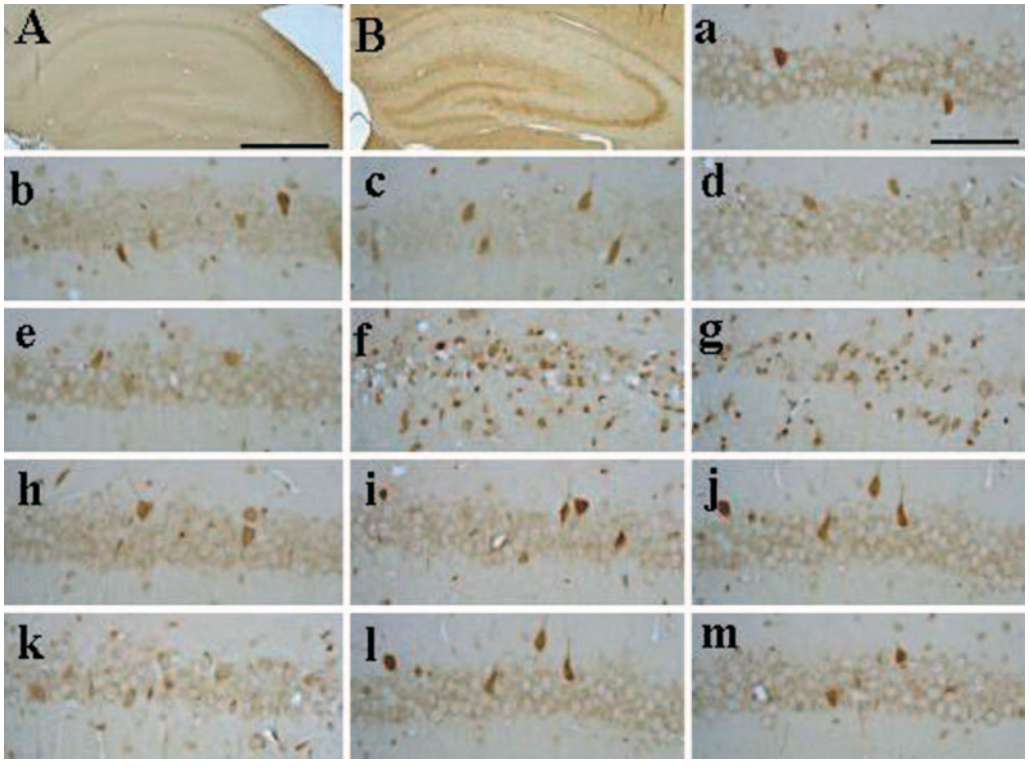

Fig. 3

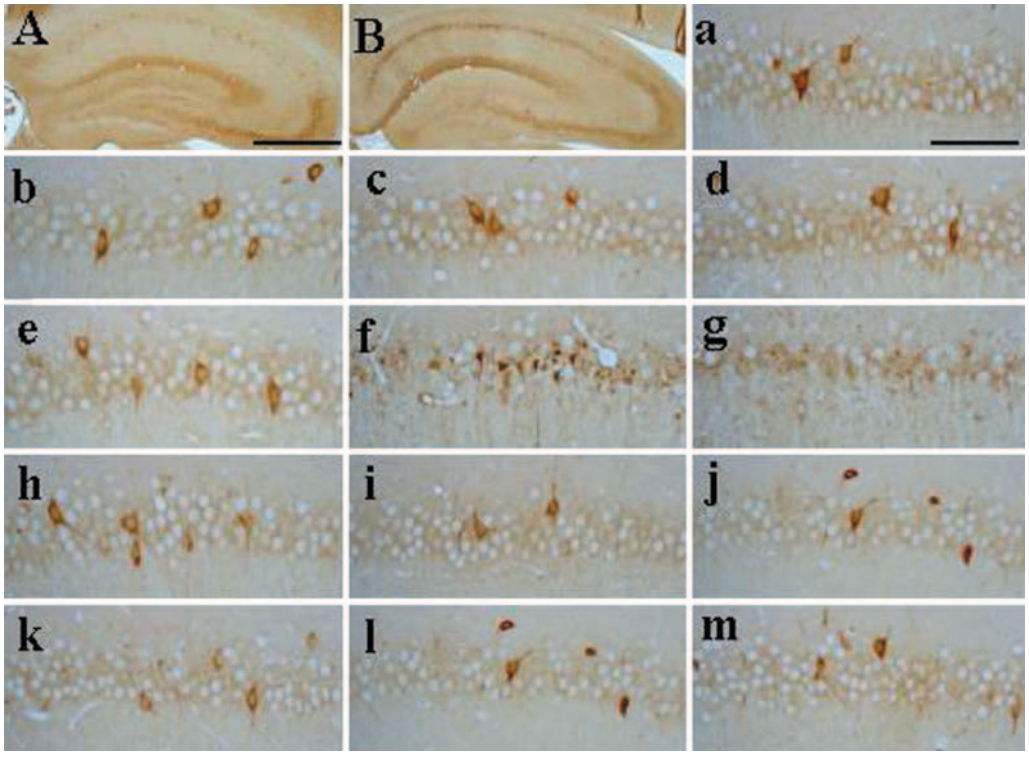

Fig. 4

Table 4. Effect of pitavastatin on Cu/Zn-SOD and Mn-SOD immunoreactivity in the gerbil hippocampus after transient cerebral ischemia

	After ischemia						
	Sham	1 h	5 h	1 day	2 days	5 days	14 days
Cu/Zn-SOD							
Ischemia CA1 pyramidal cells	2.4 ± 0.3	1.8 ± 0.3	1.3 ± 0.3	$1.2 \pm 0.4^{*}$	$0.8 \pm 0.3^{**}$	$0.3 \pm 0.4^{**}$	$0.4 \pm 0.4^{**}$
CA3 pyramidal cells	1.7 ± 0.3	1.5 ± 0.3	1.6 ± 0.2	1.4 ± 0.2	1.4 ± 0.4	2.2 ± 0.5	2.1 ± 0.4
Dentate granule cells	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	$2.1 \pm 0.4^{**}$	$2.2 \pm 0.3^{**}$
CA1 glial cells	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.2	$2.5 \pm 0.5^{**}$	$2.7 \pm 0.3^{**}$
Ischemia + Pitavastatin (10 mg/kg)							
CA1 pyramidal cells	–	–	$2.3 \pm 0.5^{\dagger}$	$2.5 \pm 0.4^{\dagger}$	–	$2.4 \pm 0.6^{\dagger}$	–
CA3 pyramidal cells	–	–	1.9 ± 0.4	1.7 ± 0.3	–	1.9 ± 0.4	–
Dentate granule cells	–	–	1.5 ± 0.4	1.2 ± 0.3	–	$1.2 \pm 0.3^{\dagger}$	–
CA1 glial cells	–	–	1.0 ± 0.0	1.0 ± 0.0	–	$1.0 \pm 0.1^{\dagger}$	–
Mn-SOD							
Ischemia CA1 pyramidal cells	2.5 ± 0.4	2.2 ± 0.5	1.6 ± 0.4	1.7 ± 0.3	1.4 ± 0.4	$0.2 \pm 0.3^{**}$	$0.2 \pm 0.3^{**}$
CA3 pyramidal cells	1.5 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.3	1.6 ± 0.2
Dentate granule cells	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	0.9 ± 0.2	1.0 ± 0.1
CA1 glial cells	0.1 ± 0.2	0.3 ± 0.3	0.4 ± 0.2	0.3 ± 0.3	0.3 ± 0.3	$2.3 \pm 0.3^{**}$	$1.2 \pm 0.3^{**}$
Ischemia + Pitavastatin (10 mg/kg)							
CA1 pyramidal cells	–	–	2.0 ± 0.4	2.2 ± 0.4	–	$2.2 \pm 0.3^{\dagger}$	–
CA3 pyramidal cells	–	–	1.4 ± 0.1	1.4 ± 0.1	–	1.6 ± 0.1	–
Dentate granule cells	–	–	1.0 ± 0.1	0.9 ± 0.1	–	1.0 ± 0.1	–
CA1 glial cells	–	–	0.1 ± 0.2	0.2 ± 0.3	–	$0.1 \pm 0.2^{\dagger}$	–

The immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0). Values are expressed as means ± S.D. For the time course study, statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$ compared with sham-operated control). For the evaluation of drugs, statistical significance was performed with Mann-Whitney U-test for non-parametric analysis († $p < 0.01$ compared with each day after cerebral ischemia). Each group contained 5–8 gerbils. –: not test

Table 5. Effect of pitavastatin on Cu/Zn-SOD and Mn-SOD immunoreactivity in the gerbil hippocampus 5 days after transient cerebral ischemia

	Sham	Ischemia	Ischemia + pitavastatin		
			Pitavastatin (3 mg/kg)	Pitavastatin (10 mg/kg)	Pitavastatin (30 mg/kg)
Cu/Zn-SOD					
CA1 pyramidal cells	$2.4 \pm 0.3^{**}$	0.3 ± 0.4	$1.7 \pm 0.8^{*}$	$2.4 \pm 0.6^{**}$	$2.4 \pm 0.6^{**}$
CA3 pyramidal cells	1.7 ± 0.3	2.2 ± 0.5	1.8 ± 0.3	1.9 ± 0.4	$1.6 \pm 0.3^{**}$
Dentate granule cells	$1.2 \pm 0.3^{**}$	2.1 ± 0.4	$1.2 \pm 0.3^{**}$	$1.2 \pm 0.3^{**}$	$1.0 \pm 0.3^{**}$
CA1 glial cells	$1.0 \pm 0.0^{**}$	2.5 ± 0.5	1.7 ± 0.7	$1.0 \pm 0.1^{**}$	$1.0 \pm 0.1^{**}$
Mn-SOD					
CA1 pyramidal cells	$2.5 \pm 0.4^{**}$	0.2 ± 0.3	1.2 ± 1.0	$2.2 \pm 0.4^{**}$	$2.1 \pm 0.4^{**}$
CA3 pyramidal cells	1.5 ± 0.1	1.5 ± 0.3	1.5 ± 0.2	1.6 ± 0.1	1.5 ± 0.2
Dentate granule cells	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
CA1 glial cells	$0.1 \pm 0.2^{**}$	2.3 ± 0.3	1.3 ± 1.2	$0.1 \pm 0.2^{**}$	$0.0 \pm 0.0^{**}$

The immunoreactivity was semiquantitatively graded as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0). Values are expressed as means ± S.D. For the time course study, statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$ compared with ischemia group). Each group contained 7–8 gerbils

pyramidal layers and dentate hilus showed the strongest Mn-SOD immunostaining. Scattered glial cells were also stained. The gerbils subjected to ischemia showed reduced Mn-SOD immunoreactivity by 5 days after ischemia and the reduction of the immunoreactivity in the hippocampal CA1 neurons never recovered up to 14 days after ischemia. In contrast, the hippocampal CA3 neurons and dentate granule cells showed no significant changes in Mn-SOD immunoreactivity throughout the experiments. On the other hand, Mn-SOD immunopositivity was observed in the hippocampal CA1 sector 5 and 14 days after ischemia (Tables 4 and 5). Prophylactic treatment with pitavastatin significantly attenuated the decreases in the number of Mn-SOD-immunopositive neurons in the hippocampal CA1 sector 5 days after ischemia (Table 5).

Double-labeled immunostaining with anti-Cu/Zn-SOD and anti-GFAP antibodies or anti-Cu/Zn-SOD antibody and isolectin B_4

Representative light micrographs of double-labeled immunostaining with anti-Cu/Zn-SOD and anti-GFAP antibodies or anti-Cu/Zn-SOD antibody and isolectin B_4 in the hippocampal CA1 sector 5 days after transient cerebral ischemia are shown in Fig. 5a, b. Cu/Zn-SOD immunoreactivity was found mainly both in the cytoplasm of GFAP-positive astrocytes and isolectin B_4-positive microglia. The damaged neurons in the hippocampal CA1 sector were also stained.

Representative light micrographs of double-labeled immunostaining with anti-Mn-SOD and anti-GFAP antibodies or anti-Mn-SOD antibody and isolectin B_4 in the hippocampal CA1 sector 5 days after transient cerebral ischemia are

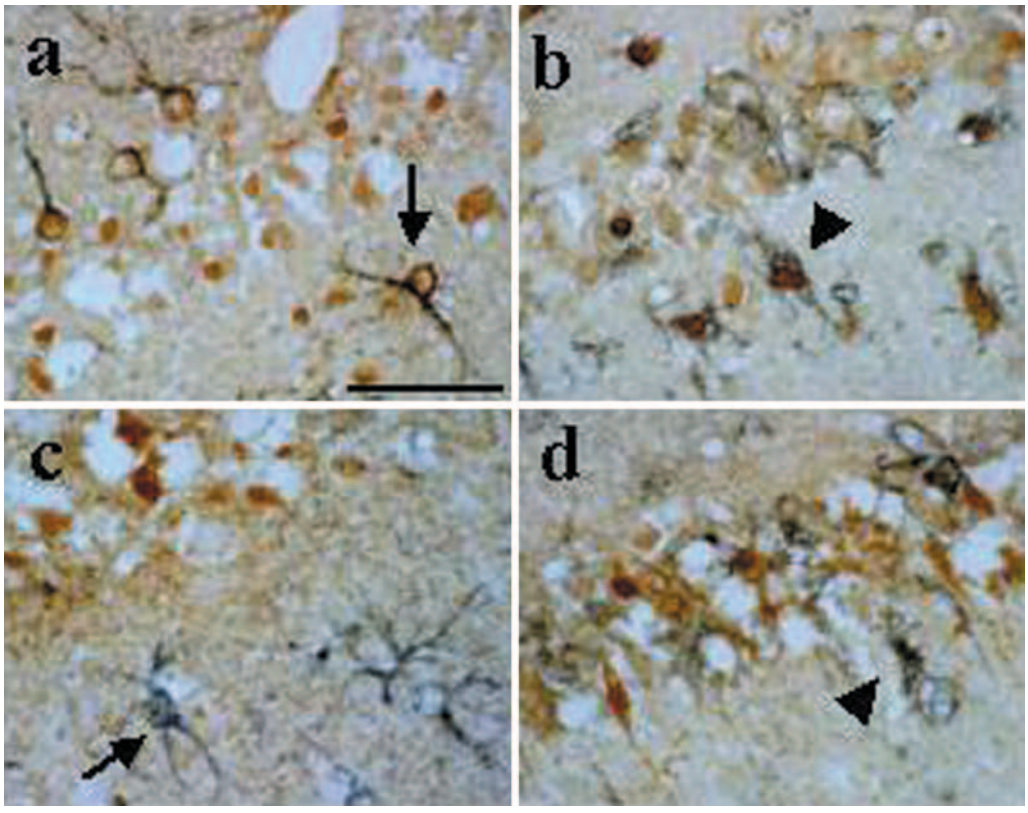

Fig. 5. Representative light micrographs of double-labeled immunostaining with anti-Cu/Zn-SOD and anti-GFAP antibodies (**a**), anti-Cu/Zn-SOD antibody and isolectin B_4 (**b**), anti-Mn-SOD and anti-GFAP antibodies (**c**) or anti-Mn-SOD antibody and isolectin B_4 (**d**) in the hippocampal CA1 sector 5 days after transient cerebral ischemia. Arrow: GFAP-positive astrocytes; arrowhead: isolectin B_4-positive microglia. Bar = 100 μm. n = 5 animals

shown in Fig. 5c, d. Mn-SOD immunoreactivity was not observed in the GFAP-positive astrocytes or isolectin B_4-positive microglia. In contrast, Mn-SOD immunoreactivity was found mainly in the damaged neurons of the hippocampal CA1 sector.

Discussion

Cerebral ischemia results in a selective pattern of neuronal degeneration with the central nervous system in both humans and animals (Pulsinelli et al., 1982; Zola-Morgan et al., 1986). This phenomenon has been termed selective neuronal vulnerability. In particular, the hippocampus exhibits the highest sensitivity to ischemia throughout the brain. Many studies of the mechanism and possible therapeutic amelioration of ischemic hippocampal damage have been carried out over a period of many years.

Oxidative damage to neurons in cerebral ischemia has received much attention since the hypothesis of free radical toxicity was proposed (Demopoulos, 1973; Fridovich, 1978). Free radicals have been demonstrated to be massively

produced during ischemia and reperfusion, and to cause a detrimental chain reaction in the brain. Oxidative damage not only causes changes in many biological molecules, such as lipids, proteins and nucleic acids (Oliver et al., 1990; Robert and John, 1992), but also causes the release of excitatory amino acids and overload of intracellular Ca^{2+}, which are other pivotal factors contributing to the development of delayed neuronal damage (Fridovich, 1978; Oliver et al., 1990; Robert and John, 1992). Therefore, free radical scavenging has been considered to be one of the important principle for neuroprotection against cerebral ischemia (Siesjö, 1992).

Recent large clinical trials have been demonstrated that a class of cholesterol-lowering agents called statins decrease the incidence of myocardial infarctions and ischemic stroke in hypercholesterolemic and atherosclerotic individuals. Pitavastatin is a new potent HMG-CoA reductase inhibitor with prolonged action and has been approved for treatment of hyperlipoproteinemia (Aoki et al., 1997; Fujino et al., 1999). This compound is known to lower plasma total cholesterol levels and reduce triglyceride levels (Kajinami et al., 2000). Furthermore, pitavastatin possesses a 10-fold higher cholesterol-lowering activity than other HMG-CoA reductase inhibitors such as pravastatin and simvastatin (Aoki et al., 1997; Suzuki et al., 1999). A recent study demonstrated that long-term treatment with pitavastatin is effective and safe for patients with heterozygous familial hypercholesterolemia (Noji et al., 2002). From these observations, it is conceivable that pitavastatin is a potentially superior therapeutic compound in comparison with currently available statins. It is, however, well known that HMG-CoA reductase inhibitors affect the expression and activity of eNOS, increasing bioavailability of NO (Laufs et al., 1997, 1998; John et al., 1998; Kaesemeyer et al., 1999). Furthermore, a previous study demonstrated that prophylactic treatment with HMG-CoA reductase inhibitors decreased the severity of cerebral focal ischemic damage irrespective of serum cholesterol levels in mice (Endres et al., 1998). In contrast, a recent interesting study suggested that an HGM-CoA reductase inhibitor, cerivastatin, can exert protection against hypertension-based stroke and ameliorate the disease severity via inhibition of superoxide production and modulation of inflammation in the brain (Kawashima et al., 2003). Furthermore, most studies have explored the antioxidant properties of HMG-CoA reductase inhibitors in relation to LDL (Chen et al., 1997; Vaugham and Delantry, 1999). Based on these observations, it has been suggested that pitavastatin may also prevent the neuronal damage after cerebral ischemia. However, little is known about the neuroprotective effects and mechanisms of pitavastatin against transient forebrain ischemia.

In the present study, we showed that pitavastatin can protect hippocampal CA1 neurons against ischemia-induced neuronal damage. As the body temperature of all animals was strictly maintained during the surgery and ischemia, the neuroprotective effects observed in this study can not be attributed to drug-induced hyperthermia, which is powerful way of protecting against ischemic neuronal damage (Busto et al., 1987; Buchan and Pulsinelli, 1990).

In this study, Cu/Zn-SOD immunoreactivity in the hippocampal CA1 sector was significantly decreased 1 day after ischemia in gerbils when neuronal

destruction was not completed, whereas the neurons of pitavastatin-treated animals retained the immunoreactivity. Thereafter, the reduction of Cu/Zn-SOD immunoreactivity in the hippocampal CA1 neurons never recovered up to the 14th day, when the CA1 neurons had completely disappeared. In contrast, a large number of Cu/Zn-SOD-immunopositive glial cells were found in the hippocampal CA1 sector after 5 and 14 days. Pitavastatin dose-dependently prevented the decrease in the number of Cu/Zn-SOD-immunopositive neurons and the increase in the number of Cu/Zn-SOD-immunoreactive glial cells in the hippocampal CA1 sector 5 days after ischemia.

Mn-SOD immunoreactivity in the hippocampal CA1 sector was significantly decreased 5 and 14 days after ischemia, when the neurons were severely destroyed. In addition, the hippocampal CA1 neurons also showed a tendency to decrease Mn-SOD immunoreactivity 2 days after ischemia ($p = 0.0576$). Pitavastatin dose-dependently prevented the decreases in the number of Mn-SOD-immunopositive cells of the hippocampal CA1 sector 5 days after ischemia. It is known that SOD is a potent free radical scavenger of superoxide radicals. This enzyme may play a key role in protection of the CNS when the CNS is exposed to oxidative stresses, such as ischemia. There are many reports about the extent of infarction in focal cerebral ischemia models and the number of damaged CA1 pyramidal neurons in transient global ischemia models (Imaizumi et al., 1990; Uyama et al., 1992). Endogenously induced Cu/Zn-SOD in transgenic mice also caused resistance against ischemic injury (Kinouchi et al., 1991). Furthermore, a previous study indicated that loss of Cu/Zn-SOD and Mn-SOD immunoreactivities in the hippocampal CA1 neurons occurred prior to morphological changes after cerebral ischemia in rats (Liu et al., 1993). Therefore, impairment of the free radical scavenging system may be one of the causal factors leading to the hippocampal CA1 damage. Thus, preservation of SOD in pitavastatin-treated gerbils may be one of the mechanisms of the neuroprotection.

It is well known that cerebral vascular tone and blood flow are regulated by endothelium-derived NO (Dalkara et al., 1994). Mutant mice lacking eNOS are relatively hypertensive and develop greater profilerative and inflammatory responses to vascular injury (Huang et al., 1995). Mice lacking eNOS develop larger cerebral infarcts after cerebrovascular occlusion (Huang et al., 1996). Therefore, the beneficial effects of HMG-CoA reductase inhibitors in ischemic stroke may be due, at least in part, to their ability to upregulate eNOS expression and activity.

A recent interesting study demonstrated that HMG-CoA reductase inhibitors can also activate eNOS via the phosphatidylinositol 3-kinase/protein kinase Akt pathway (Kureishi et al., 2000). However, direct inhibition of geranylgeranylated protein Rho or the actin cytoskeleton mimics the effect of HMG-CoA reductase inhibitors on eNOS upregulation and stroke protection (Laufs et al., 2000), indicating that inhibition of isoprenoid synthesis and Rho is the key mechanism by which HMG-CoA reductase inhibitors exert their neuroprotective effects (Liao et al., 2002). Interestingly, several studies have demonstrated that therapy with HMG-CoA reductase inhibitors may decrease lipoprotein oxidation and ameliorate free radical injury. In addition to having

favorable antioxidant effects (as measured using several ex vivo system) such as increased lag time of Cu-induced LDL oxidation (Hussein et al., 1997) and reduced leukocyte-induced LDL oxidation (Chen et al., 1997), HMG-CoA reductase inhibitors may have broader antioxidant effects. Hydroxy metabolites of atorvastatin have also been shown to inhibit oxidation in an in vitro model (Aviram et al., 1998). Together with these observations, therefore, our findings suggest that pitavastatin may have exerted broader antioxidant effects through preservation of SOD activity in the present study. However, it has been suggested that therapies which target the endothelial actin cytoskeleton may have beneficial effects in ischemic stroke (Laufs et al., 2000). Furthermore, it is well known that the different isoforms of NOS play important but opposing roles in cerebral ischemia. Therefore, further studies are required to clarify our findings.

The present results with double-labeled immunostaining showed that a large number of glial cells proliferated and displayed intense Cu/Zn-SOD immunoreactivity when the hippocampal CA1 neurons had completely disappeared. In our double-labeled immunostaining with anti-Cu/Zn-SOD and anti-GFAP antibodies or anti-Cu/Zn-SOD antibody and isolectin B_4 in the hippocampal CA1 sector 5 days after transient cerebral ischemia, Cu/Zn-SOD immunoreactivity was observed mainly in GFAP-positive astrocytes and isolectin B_4-positive microglia. In contrast, for double-labeled immunostaining with anti-Mn-SOD and anti-GFAP antibodies or anti-Mn-SOD antibody and isolectin B_4 in the hippocampal CA1 sector 5 days after transient cerebral ischemia, Mn-SOD immunoreactivity was not observed mainly in GFAP-positive astrocytes and isolectin B_4-positive microglia. Furthermore, Cu/Zn-SOD and Mn-SOD immunoreactivity was also stained in the damaged hippocampal CA1 neurons. In our findings, the appearance of the activated glial cells following ischemia suggests a connection between ultrastructural injury in the neurons and the proliferation of glial cells. Based on the previous studies of reactive glial cells after ischemia showing that some signals may be transmitted from indisposed neurons to glial cells for initiating their function (Tanaka et al., 1992), we suggest that some signals following ischemia that are transmitted to the glial cells cause them to be transformed from resting forms to activated ones and produce the synthesis of Cu/Zn-SOD lately. Therefore, it is conceivable that the glial Cu/Zn-SOD may help the surviving neurons to resist attack from superoxide radicals. In the present study, however, we can not rule out that the neuroprotective effect of pitavastatin is mediated through the expression of eNOS in the hippocampal CA1 neurons after transient cerebral ischemia. Therefore, further studies are needed to investigate the precise biochemical mechanisms for our finding.

In conclusion, we showed that HMG-CoA reductase inhibitor pitavastatin can protect the hippocampal CA1 neurons after ischemia-induced cell damage. The many pleiotropic actions of HMG-CoA reductase inhibitors demonstrated that the neuroprotective effects of pitavastatin may, at least in part, be due to its free radical scavenging activity. These findings support the idea that pitavastatin therapy, which targets free radicals, will have beneficial effects against disorders including ischemic stroke.

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