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RESEARCH PAPER

Immunohistochemical Study on Distribution of NF-κB and p53 in Gerbil Hippocampus after Transient Cerebral Ischemia: Effect of Pitavastatin

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Abstructs We investigated the immunohistochemical alterations of the transcription nuclear factor kappa-B (NF-κB) and transcription factor p53 in the hippocampus after transient cerebral ischemia in gerbils. We also examined the effect of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor pitavastatin against the alterations of NF-κB, p53 and neuronal nuclei in the hippocampus after ischemia. Severe neuronal damage was observed in the hippocampal CA1 neurons 5 and 14 days after ischemia. In the present study, the increase of NF-κB immunoreactivity in glial cells and p53 immunoreactivity in neurons preceded neuronal damage in the hippocampal CA1 sector after ischemia. Thereafter, NF-κB immunoreactivity was induced highly in reactive astrocytes and microglia of the hippocampal CA1 sector where severe neuronal damage was observed. Our immunohistochemical study showed that pitavastatin prevented the alterations of NF-κB and p53 in the hippocampal CA1 sector 5 days after transient ischemia. Furthermore, our results with neuronal nuclei immunostaining indicate that pitavastatin dose-dependently prevented the neuronal cell death in the hippocampal CA1 sector 5 days after transient cerebral ischemia. These results suggest that the up-regulations of NF-κB in glia and p53 in neurons can cause neuronal cell death after ischemia. Our findings also support the hypothesis that NF-κB- and/or p53-mediated neuronal cell death is prevented through decreasing oxidative stress by pitavastatin. Thus, NF-κB and p53 may provide an attractive target for the development of novel therapeutic approaches for brain stroke.

Keywords Immunohistochemistry · NF-κB · p53 · Neuronal nuclei · Pitavastatin · Gerbil

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Introduction

It is well known that apoptotic cell death plays a key role in brain development, neuronal damage and disease. In the developing nervous system, apoptosis is required for the establishment of appropriate cell numbers and for the elimination of improperly connected neurons (Pettmann and Henderson, 1998). In the mature nervous system, the inappropriate induction of apoptotic cell death contributes to the neuropathology associated with neurodegenerative diseases (Portera-Gailliau et al., 1995; Smale et al., 1995) and acute neurological insults (Nitatori et al., 1995; Yakovlev et al., 1997). From these findings, the molecular mechanisms that regulate apoptosis are essential for the development of therapeutic strategies for the treatment of neurodegenerative diseases.

NF-κB, which is crucial for inflammation reactions in the periphery (Makarov, 2000) and regulates expression of both apoptotic and anti-apoptotic genes (Mattson and Camandola, 2001), is thought to be sensor of oxidative stress and is activated in the brain after focal cerebral ischemia (Schneider et al., 1999; Stephenson et al., 2000). The role of NF-κB in the brain is presently unclear, as the lack of the p50 subunit of NF-κB lessens ischemia-induced neuronal damage (Schneider et al., 1999; Nurmi et al., 2004). However, NF-κB increases the excitotoxic damage of the hippocampus (Yu et al., 1999). Furthermore, the activation of NF-κB has been reported to promote neuronal survival in various models (Guo et al., 1998; Bhakar et al., 2002) and complete blockade of neuronal NF-κB activity leads to a loss of neuroprotection in kainite-treated hippocampal slices (Fridmacher et al., 2003). Thus whether the inhibition of NF-κB reduces inflammation after stroke and whether NF-κB is a relevant target for stroke therapy is presently unclear.

Several lines of evidence demonstrate that p53 is a key upstream initiator of the cell death process after neuronal damage. P53 expression has been reported to be up-regulated in response to excitotoxins, hypoxia and ischemia (Xiang et al., 1996; Banasiak and Haddad, 1998; McGahan et al., 1998). Furthermore, it has been demonstrated that brain damage induced by ischemia and kainic acid excitotoxicity is significantly reduced in mice carrying a null mutation for the p53 gene (Crumrine et al., 1994; Morrison et al., 1996). Furthermore, cultured neurons derived from p53-deficient mice have been shown to be resistant to excitotoxins (Xiang et al., 1998), DNA damaging agents (Morris et al., 2001) and hypoxia (Halterman et al., 1999). Therefore, these findings demonstrate that p53 plays a pivotal role in the regulation of neuronal cell death and apoptosis.

Pitavastatin is a new synthetic potent and selective inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and the rate-limiting enzyme in cholesterol biosynthesis (Aoki et al., 1997). This agent has been reported to lower total cholesterol and total triglyceride levels in patients with hypercholesterolemia in Japan (Kojima et al., 1999). A previous interesting study has demonstrated that HMG-CoA inhibitors can decrease cerebral ischemia and infarct size by up-regulating eNOS expression in normocholesterolemic mice (Endres et al., 1998). We recently reported that pitavastatin can reduce the neuronal damage of the hippocampal CA1 sector in gerbils after transient cerebral ischemia (Himeda et al., 2005). However, little is known about the effect of pitavastatin against the expression of NF-κB and p53 in the hippocampus after transient cerebral ischemia.

In this study, therefore, we investigated the changes of NF-κB and p53 proteins in gerbil hippocampus after transient cerebral ischemia using an immunohistochemical study. We also examined the effect of pitavastatin against the changes of NF-κB, p53 and neuronal damage in gerbil hippocampus after transient cerebral ischemia.

Materials and methods

Experimental animals and treatments

Male adult Mongolian gerbils, weighing 60–90 g, were used. The animals were housed in an air-conditioned environment with constant temperature and a standardized light/dark schedule, food and water *ad libitum*. They were anesthetized with 2% halothane in a mixture of 70% N_2O and 30% O_2. The bilateral common carotid arteries were exposed, anesthesia was discontinued, and the arteries were clamped with aneurysm clips for 5 min. After occlusion, the aneurysm clips were removed and ischemic animals were allowed to survive for 1 and 5 h and 1, 2, 5 and 14 days after transient forebrain ischemia. Sham-operated animals were treated in the same manner, except for the clipping of the bilateral carotid arteries. Body temperature was maintained at 37–38°C, using a heating pad with a thermostat until the animals started moving. Pitavastatin at a dose of 3, 10 and 30 mg/kg was administered orally for 5 days before ischemic insult. Transient forebrain ischemia was induced in gerbils 1 h after the last treatment with pitavastatin. Vehicle (0.5% carboxymethylcellulose) was also administered in sham-operated gerbils under the same conditions. Pitavastatin was generously provided by Kowa Company, Ltd., Tokyo, Japan. All experiments were performed in accordance with the Guidelines for Animal Experiments for the Tokushima University School of Medicine.

Immunohistochemistry

The gerbils were anesthetized with pentobarbital (50 mg/kg, i.p.) at 1 and 5 h and 1, 2, 5 and 14 days after transient forebrain ischemia. For pitavastatin treatment, the animals were anesthetized with pentobarbital (50 mg/kg, i.p.) at 5 days after transient forebrain ischemia. 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. The brain sections were then dehydrated with graded ethanol, passed through chloroform, and embedded in parafiin. Paraffin sections, 5 μm in thickness, of the hippocampus were used for immunohistochemistry.

For immunohistochemical studies, a Vectastain *elite* ABC Kit (Vector Labs., Burlingame, CA, USA), mouse anti-NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology Inc., CA, USA), mouse anti-p53 monoclonal antibody (NeoMarkers, Fremont, CA, USA) and mouse anti-NeuN (neuronal nuclei) monoclonal antibody (Chemicon International, Temecula, CA, USA) were used.

Briefly, the paraffin sections were washed twice for 5 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 10% methanol/0.3% H_2O_2 in 0.01 M PBS for 20 min to quench endogenous peroxidase activity. The paraffin sections were then washed for 2 min in 0.01 M PBS, followed by 60 min of pre-incubation with 10% normal horse or goat serum. The brain sections were then incubated with anti-NF-κB p65 monoclonal antibody (1:100), anti-p53 monoclonal antibody (1:100) and anti-NeuN antibody (1:200) overnight at 4°C. After a 5 min rinse in 0.01M PBS, the sections were incubated with secondary antibody for 60 min at room temperature. The sections were washed twice for 5 min in 0.01 M PBS and then with avidin-biotin peroxidase (ABC) complex for 30 min at room temperature. Immunohistochemical staining with anti-NF-κB monoclonal antibody and anti-p53 monoclonal antibody was performed using an enzyme substrate kit (Vector Labs., Burlingame, CA, USA), (Araki et al., 2001; Kurosaki et al., 2002). A negative control study was performed using non-specific IgG or by omission of the primary antibody, which showed no notable staining.

The immunostaining for NF-κB or p53 antibody was graded semi-quantitatively as intense (grade 3), moderate (grade 2), weak (grade 1) and not detectable (grade 0), without the examiner knowing the experimental protocols, as described previously (Kato et al., 1995; Muramatsu et al., 2003; Himeda et al., 2005). For NeuN immunostaining, changes in number of immunopositive cells in stained sections were examined with a light microscope at magnification of X 400 without the examiner knowing the experimental protocols, using a computer associated image analyzer software (WinRoof Version 5, Mitani Corporation, Fukui, Japan). Values were expressed as means $\pm$ S.D., and statistical significance was performed with one-way ANOVA followed by Fisher's PLSD multiple comparison test. For the evaluation of drug, statistical significance was performed with F-test followed by Dunnett's multiple comparison test for non-parametric analysis. Each groups consisted of 5–8 gerbils. In addition, double-labeled immunostainings with anti-NF-κB and anti-glial fibrillary acidic protein (GFAP, Chemicon International, Temecula, CA, USA, 1:200) antibodies and anti-NF-κB antibody and isolectin *B4* (Sigma, St Louis, MO, USA, 20 μg/ml) were performed in some brain sections of gerbils after transient forebrain ischemia as described previously (Muramatsu et al., 2003; Kumagai et al., 2004). Immunoreactions were visualized using an enzyme substrate kit (brown and blue).

Result

NF-κB p65 immunostaining

Representative photomicrographs are presented in Figs. 1 and 2. The changes of NF-κB p65 immunoreactivity after transient forebrain ischemia are summarized in Tables 1 and 2. NF-κB p65 immunoreactivity was mainly observed in glial cells of the hippocampus in sham-operated animals. No change of NF-κB p65 immunoreactivity was detected in glial cells 5 h after transient cerebral ischemia. A significant increase of NF-κB p65 immunoreactivity was evident in glial cells of the hippocampal CA1 sector 1 and 2 days after cerebral ischemia. Five and fourteen days after ischemia, a marked increase of NF-κB p65-immunopositive glial cells was found in the hippocampal CA1 sector where severe neuronal damage was observed. On the other hand, no change of NF-κB p65 immunoreactivity was found in the hippocampal CA3 sector and dentate gyrus throughout the experiments except for a transient increase in dentate gyrus. In contrast, the administration of pitavastatin at two doses (10 and 30 mg/kg) prevented a significant increase of NF-κB p65-immunoreactive glial cells in the hippocampal CA1 sector 5 days after ischemia.

p53 immunostaining

Representative photomicrographs are presented in Figs. 3 and 4. The changes of p53 immunoreactivity after transient cerebral ischemia are summarized in Tables 1 and 2. p53 immunoreactivity was observed mainly in hippocampal neurons of sham-operated animals. A significant increase of p53 immunoreactivity was detected in the hippocampal CA 1 sector 1 h after transient ischemia. Furthermore, a significant increase of p53 immunoreactivity was evident in the hippocampal CA1 sector 1 and 2 days after cerebral ischemia. Five and fourteen days after ischemia, thereafter, a significant decrease of p53 immunoreactivity was observed in the hippocampal CA1 neurons where severe neuronal damage was observed. In contrast, no change of p53 immunoreactivity was observed in the hippocampal CA3 sector and dentate gyrus throughout the experiments except for a transient increase in dentate

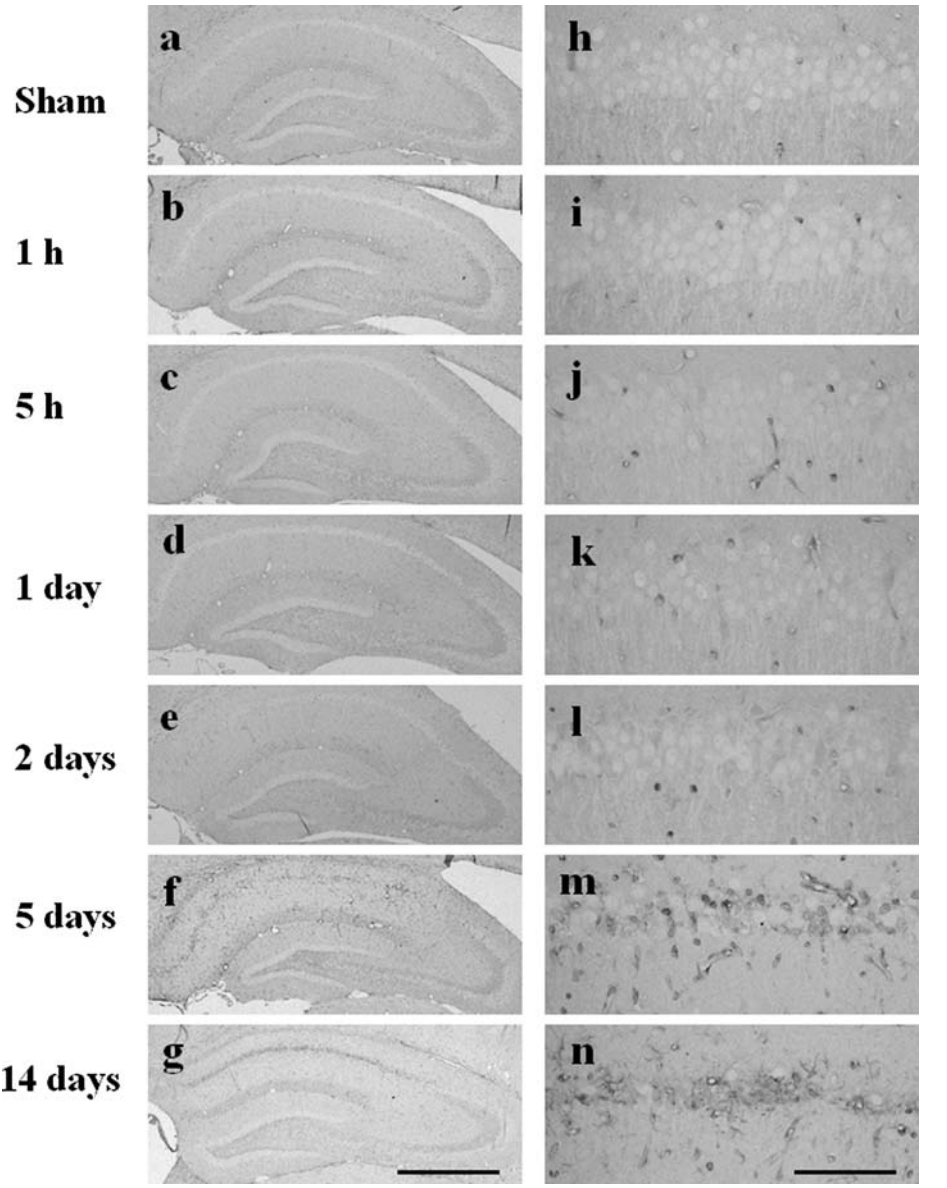

Fig. 1 Representative photomicrographs with NF-κB immunostaining of the gerbil hippocampus (**a–g**) and CA1 sector (**h–n**) after transient cerebral ischemia. (**a, h**): sham-operated gerbil brain, (**b, i**): vehicle-treated gerbil brain 1 h after ischemia, (**c, j**): vehicle-treated gerbil brain 5 h after ischemia, (**d, k**): vehicle-treated gerbil brain 1 day after ischemia, (**e, l**): vehicle-treated gerbil brain 2 days after ischemia, (**f, m**): vehicle-treated gerbil brain 5 days after ischemia, (**g, n**): vehicle-treated gerbil brain 14 days after ischemia. Each group contained 5–8 animals. Bar (**a–g**) = 1 mm, Bar (**h–n**) = 100 μm

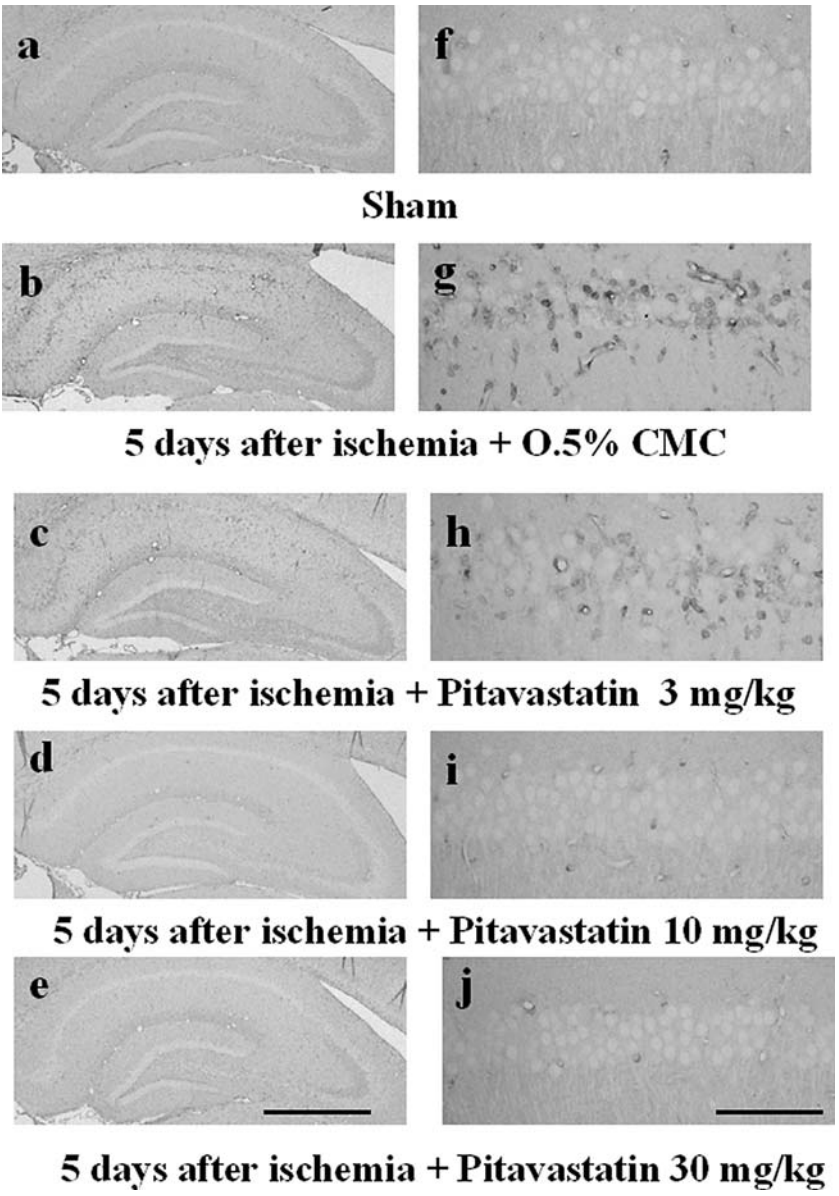

Fig. 2 Effect of pitavastatin on NF-κB immunoreactivity of the gerbil hippocampus (**a–e**) and CA1 sector (**f–j**) 5 days after transient cerebral ischemia. (**a, f**): sham-operated gerbil brain, (**b, g**): vehicle-treated gerbil brain 5 days after ischemia, (**c, h**): pitavastatin (3 mg/kg)-treated gerbil brain 5 days after ischemia, (**d, i**): pitavastatin (10 mg/kg)-treated gerbil brain 5 days after ischemia, (**e, j**): pitavastatin (30 mg/kg)-treated gerbil brain 5 days after ischemia. Each group contained 7–8 animals. Bar (**a–e**) = 1 mm, Bar (**h–j**) = 100 μm

Table 1 NF-κB and p53 immunoreactivity in the gerbil hippocampus after transient cerebral ischemia

	CA1 sector	CA3 sector	Dentate gyrus
NF-κB (glia)			
Sham	0.54 ± 0.09	1.68 ± 0.37	0.79 ± 0.17
1 h	0.75 ± 0.22	1.79 ± 0.19	0.92 ± 0.26
5 h	0.80 ± 0.21	1.85 ± 0.38	0.90 ± 0.14
1 day	0.85 ± 0.29*	1.75 ± 0.35	0.95 ± 0.21
2 days	1.20 ± 0.33**	1.65 ± 0.14	0.95 ± 0.27
5 days	2.19 ± 0.37**	1.50 ± 0.30	1.16 ± 0.30**
14 days	1.50 ± 0.18**	1.35 ± 0.42	0.65 ± 0.22
p53 (neuron)			
Sham	0.61 ± 0.13	0.71 ± 0.27	0.61 ± 0.13
1 h	1.00 ± 0.32**	0.63 ± 0.14	0.54 ± 0.25
5 h	0.75 ± 0.31	0.55 ± 0.11	0.55 ± 0.11
1 day	1.05 ± 0.33**	0.60 ± 0.14	0.55 ± 0.11
2 days	1.35 ± 0.29**	0.65 ± 0.22	0.95 ± 0.27**
5 days	0.16 ± 0.19**	0.59 ± 0.19	0.47 ± 0.09
14days	0.15 ± 0.22**	0.60 ± 0.14	0.50 ± 0.18

Note. 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.. Each group contained 5–8 animals. Statistical significance was evaluated by Fisher's PLSD multiple comparison test (*$P < 0.05$,**$P < 0.01$ compared with sham operated control group).

gyrus. In contrast, the administration of pitavastatin prevented a significant decrease of p53 immunoreactivity in the hippocampal CA1 neurons 5 days after ischemia.

NeuN immunostaining

Representative photomicrographs are presented in Figs. 5 and 6. The changes in number of NeuN immunopositive cells after transient cerebral ischemia are summarized in Table 3. NeuN immunoreactivity was observed in the hippocampal neurons of sham-operated animals. No change of NeuN immunoreactivity was detected in the hippocampal CA1 neurons up to 2 days after transient forebrain ischemia. Thereafter, a marked decrease of NeuN immunoreactivity was evident in the hippocampal CA1 neurons 5 and 14 days after ischemia. On the other hand, no change of NeuN immunoreactivity was observed in neurons of the hippocampal CA3 sector and dentate gyrus, throughout the experiments. In contrast, the administration of pitavastatin dose-dependently prevented a significant decrease of NeuN immunoreactivity in the hippocampal CA1 neurons 5 days after ischemia.

Table 2 Effect of pitavastatin on NF-κB and p53 immunoreactivity in the gerbil hippocampal CA1 sector 5 days after transient cerebral ischemia

	NF-κB (glia)	p53 (neuron)
Sham	0.54 ± 0.09*	0.61 ± 0.13*
Ischemia + 0.5% CMC	2.19 ± 0.37	0.16 ± 0.19
Ischemia + Pitavastatin		
3 mg/kg	1.71 ± 1.10	0.86 ± 0.52*
10 mg/kg	0.68 ± 0.19*	0.64 ± 0.32*
30 mg/kg	0.61 ± 0.13*	0.68 ± 0.35*

Note. 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.. Each group contained 7–8 animals. Statistical significance was evaluated by non-parametric Dunnett's multiple comparison test (*$P < 0.01$ compared with ischemia + 0.5% CMC group).

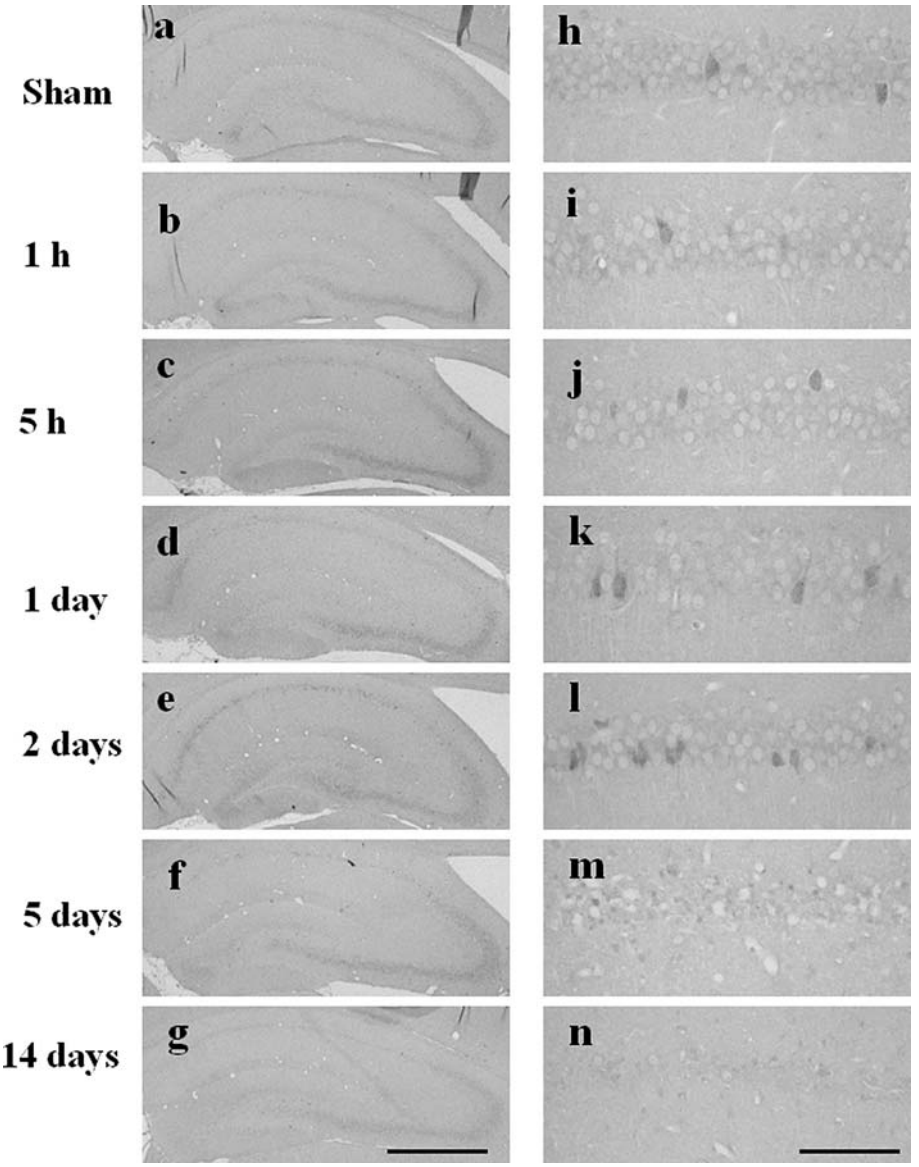

Fig. 3 Representative photomicrographs with p53 immunostaining of the gerbil hippocampus (**a–g**) and CA1 sector (**h–n**) after transient cerebral ischemia. (**a, h**): sham-operated gerbil brain, (**b, i**): vehicle-treated gerbil brain 1 h after ischemia, (**c, j**): vehicle-treated gerbil brain 5 h after ischemia, (**d, k**): vehicle-treated gerbil brain 1 day after ischemia, (**e, l**): vehicle-treated gerbil brain 2 days after ischemia, (**f, m**): vehicle-treated gerbil brain 5 days after ischemia, (**g, n**): vehicle-treated gerbil brain 14 days after ischemia. Each group contained 5–8 animals. Bar (**a–g**) = 1 mm, Bar (**h–n**) = 100 μm

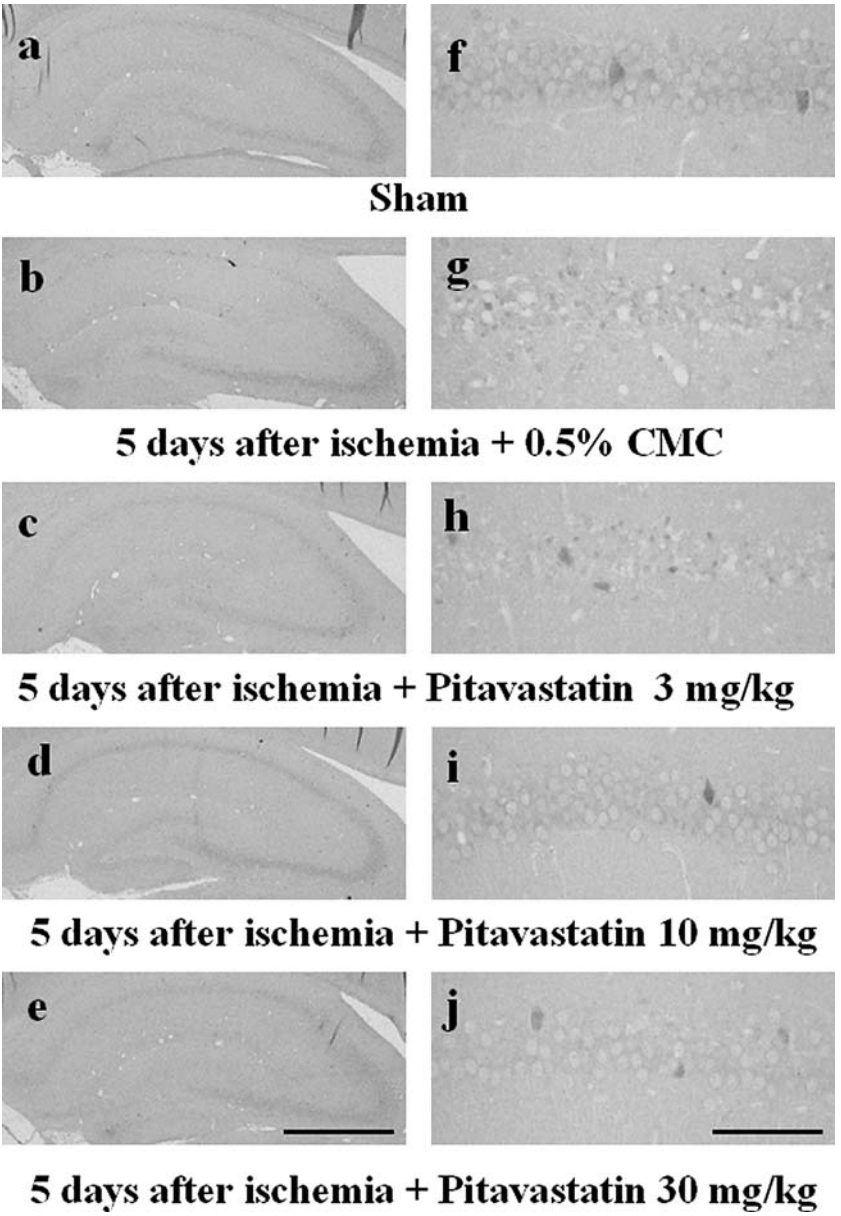

Fig. 4 Effect of pitavastatin on p53 immunoreactivity of the gerbil hippocampus (**a–e**) and CA1 sector (**f–j**) 5 days after transient cerebral ischemia. (**a, f**): sham-operated gerbil brain, (**b, g**): vehicle-treated gerbil brain 5 days after ischemia, (**c, h**): pitavastatin (3 mg/kg)-treated gerbil brain 5 days after ischemia, (**d, i**): pitavastatin (10 mg/kg)-treated gerbil brain 5 days after ischemia, (**e, j**): pitavastatin (30 mg/kg)-treated gerbil brain 5 days after ischemia. Each group contained 7–8 animals. Bar (**a–e**) = 1 mm, Bar (**f–j**) = 100 μm

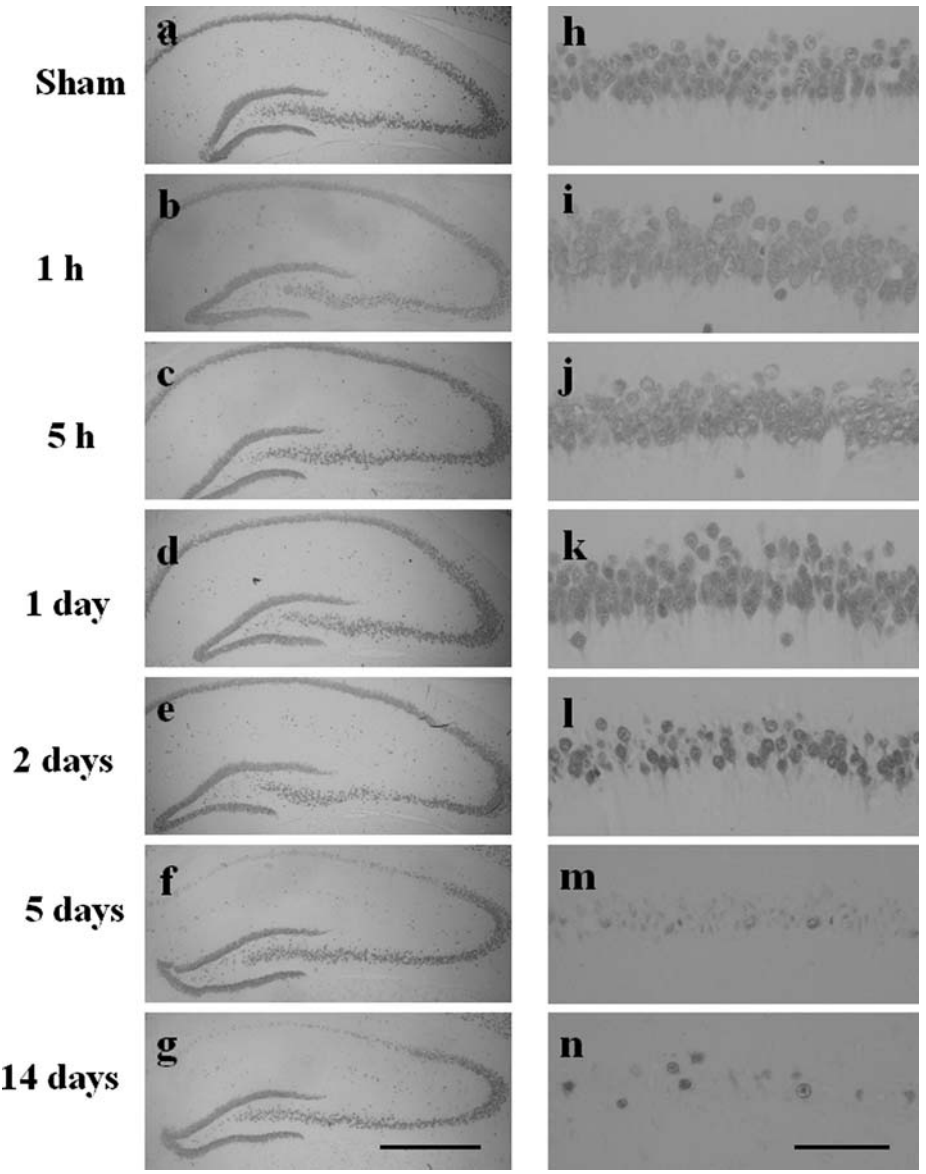

Fig. 5 Representative photomicrographs with NeuN immunostaining of the gerbil hippocampus (**a–g**) and CA1 sector (**h–n**) after transient cerebral ischemia. (**a, h**): sham-operated gerbil brain, (**b, i**): vehicle-treated gerbil brain 1 h after ischemia, (**c, j**): vehicle-treated gerbil brain 5 h after ischemia, (**d, k**): vehicle-treated gerbil brain 1 day after ischemia, (**e, l**): vehicle-treated gerbil brain 2 days after ischemia, (**f, m**): vehicle-treated gerbil brain 5 days after ischemia, (**g, n**): vehicle-treated gerbil brain 14 days after ischemia. Each group contained 5–8 animals. Bar (**a–g**) = 1 mm, Bar (**h–n**) = 100 μm

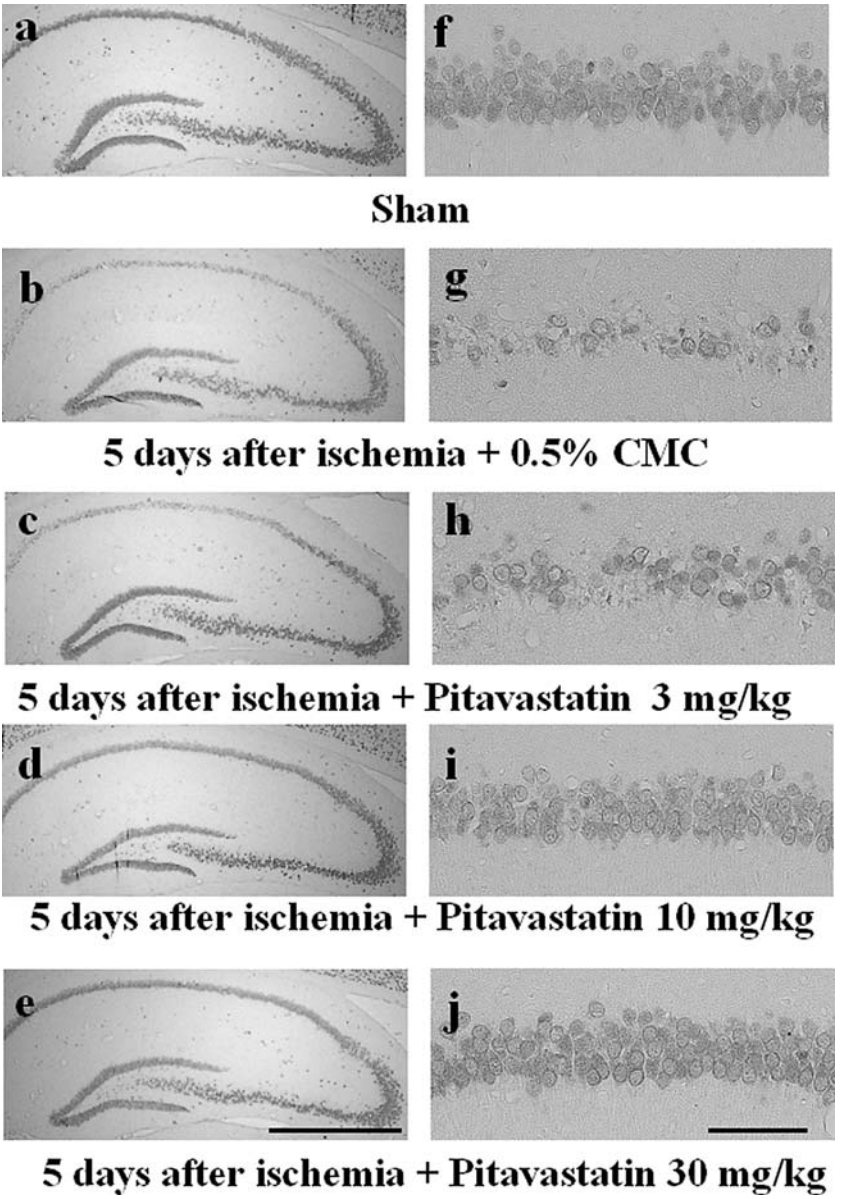

Fig. 6 Effect of pitavastatin on NeuN immunoreactivity of the gerbil hippocampus (**a–e**) and CA1 sector (**f–j**) 5 days after transient cerebral ischemia. (**a, f**): sham-operated gerbil brain, (**b, g**): vehicle-treated gerbil brain 5 days after ischemia, (**c, h**): pitavastatin (3 mg/kg)-treated gerbil brain 5 days after ischemia, (**d, i**): pitavastatin (10 mg/kg)-treated gerbil brain 5 days after ischemia, (**e, j**): pitavastatin (30 mg/kg)-treated gerbil brain 5 days after ischemia. Each group contained 7–8 animals. Bar (**a–e**) = 1 mm, Bar (**f–j**) = 100 μm

Table 3 Effect of pitavastatin on neuronal nuclei immunoreactive cells in the gerbil hippocampal CA1 sector 5 days after transient cerebral ischemia

	Neuronal nuclei positive cells/mm
Sham	$183 \pm 12.4^{**}$
Ischemia + 0.5% CMC	50.7 ± 21.4
Ischemia + Pitavastatin	
3 mg/kg	102 ± 57.0
10 mg/kg	$169 \pm 12.0^{*}$
30 mg/kg	$180 \pm 7.93^{**}$

Note. Values are expressed as means ± SD of the number of cells per 1mm. Each group contained 5–6 animals. Statistical significance was evaluated by non-parametric Dunnett's multiple comparison test ($^{*}P < 0.05$,$^{**}P < 0.01$ compared with ischemia + 0.5% CMC group).

Double-labeled immunostaining

Representative photomicrographs of double-labeled immunostainings are presented in Fig. 7. In double-labeled immunostaining with anti-NF-κB and anti-GFAP antibody, NF-κB immunostaining was observed both in GFAP-positive astrocytes and isolectin *B4*-positive microglia in the hippocampal CA1 sector after transient cerebral ischemia.

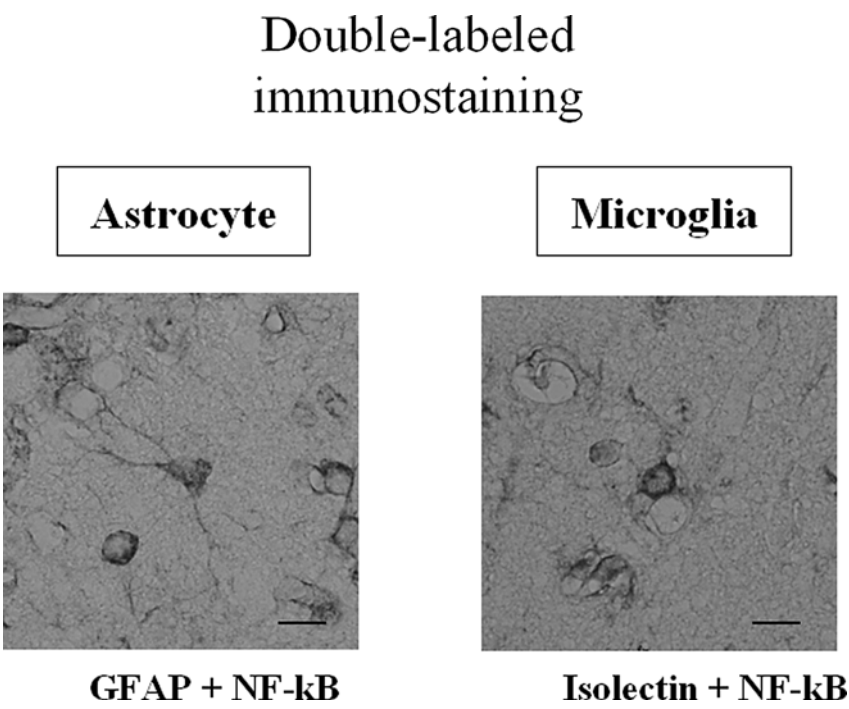

Fig. 7 Representative photomicrographs of double-labeled immunostainings with anti-NF-κB and anti-GFAP antibody (left panel) and anti-NF-κB antibody and Isolectin *B4* (right panel) in the CA1 sector after transient cerebral ischemia. Bar = 20 μm

Discussion

Neuronal cell loss following ischemic brain injury is a delayed process in which the primary injury is followed by a prolonged period of secondary neurodegeneration resulting in neurological dysfunction. It has been shown that pyramidal neurons in the hippocampal CA1 sector in gerbils are particularly vulnerable to injury due to transient cerebral ischemia, and neuronal cell death occurs within days after cerebral ischemia/reperfusion (Kirino, 1982; Araki et al., 1989; Schmidt-Kastner and Freund, 1991). This phenomenon is referred to as delayed neuronal death. Although not fully understood, ischemia-induced delayed neuronal death is probably associated with a myriad of biochemical events initially triggered by the extracellular accumulation of glutamate. This leads to membrane depolarization, increased concentrations of intracellular calcium, overproduction of reactive oxygen species and oxidative damage (Urabe et al., 2000; Chan, 2001). The effects of oxidative stress are mediated through numerous intracellular signaling molecules. Amongst them is NF-κB (Schreck et al., 1991). Furthermore, stimuli such as those elicited by tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) can cause activation of NF-κB in cells of glial origin (Moynagh et al., 1993; Carter et al., 1996). Some of those factors are released by activated glial cells after injury to the central nervous system (Buttini et al., 1994; Feuerstein et al., 1994). A previous study also suggests that NF-κB is involved in the inflammatory responses that take place in the brain after transient cerebral ischemia (Gabriel et al., 1999). Therefore, it is suggested that NF-κB becomes activated under inflammatory conditions and the activation of NF-κB is putatively involved in the cerebral post-ischemic reaction.

In the present study, no neuronal damage in the hippocampus was observed up to 2 days after transient cerebral ischemia. Five and fourteen days after ischemia, severe neuronal damage was found only in the hippocampal CA1 sector, as illustrated in Figs. 5 and 6. These findings were consistent with our previous results of Cresyl violet staining (Kumagai et al., 2004).

NF-κB p65 immunoreactivity was found weakly in the gerbil brain under normal conditions and the NF-κB positive cells were mainly glial cells. A significant increase of NF-κB p65 immunoreactivity was evident in glial cells of the hippocampal CA1 sector 1 and 2 days after cerebral ischemia. Five and fourteen days after ischemia, thereafter, a marked increase of NF-κB p65-immunopositive glial cells was found in the hippocampal CA1 sector where severe neuronal damage was observed. Therefore, the present study demonstrates that the increase of NF-κB p65 immunoreactivity in glial cells precedes neuronal damage in the hippocampal CA1 sector after cerebral ischemia. Furthermore, our findings suggest that NF-κB p65 immunoreactivity was induced highly in reactive astrocytes and microglia/macrophages of the hippocampal CA1 region where severe neuronal damage was observed, as illustrated in Fig. 7. To our knowledge, however, the exact role of NF-κB in relation to cell death or survival is presently unclear. Interestingly, a recent study demonstrates that activation of NF-κB in neurons can promote their survival by inducing the expression of genes encoding anti-apoptotic proteins such as Bcl-2 and the antioxidant enzyme Mn-superoxide dismutase. On the other hand, by inducing the production and release of inflammatory cytokines, reactive oxygen molecules and excitotoxins, activation of NF-κB in glial cells such as microglia and astrocytes may be contribute to neuronal degeneration (Mattson, 2005). In the present study, therefore, we speculate that the activation of NF-κB p65 in glial cells may contribute to neuronal cell death of the hippocampal CA1 region after transient cerebral ischemia. However, the precise mechanisms responsible for such findings should be investigated in further studies.

p53 has been recognized a key regulator of cell death after neuronal injury (Morrison and Kinoshita, 2000). In the present study, the increase of p53 immunoreactivity was observed in

hippocampal CA 1 neurons at an early stage after transient ischemia. Furthermore, a significant increase of p53 immunoreactivity was evident in the hippocampal CA1 sector 1 and 2 days after cerebral ischemia. Five and fourteen days after ischemia, thereafter, a significant decrease of p53 immunoreactivity was observed in the hippocampal CA1 neurons where severe neuronal damage was observed. Based on these findings, the present study shows that the increase of p53 immunoreactivity precedes neuronal damage in the hippocampal CA1 sector after cerebral ischemia. Furthermore, our findings suggest that p53 immunoreactivity was decreased significantly in scars of damaged neurons in the hippocampal CA1 region after cerebral ischemia. These results indicate that p53 plays a key role in development of neuronal death of the hippocampal CA1 neurons after cerebral ischemia.

Pitavastatin is a new potent HMG-CoA reductase inhibitor with prolonged action and has been approved for treatment of hyperlipoproteinaemia (Fujimoto et al., 1999). This drug is known to lower plasma total cholesterol levels and reduce triglyceride levels (Kajinami et al., 2000). A previous study demonstrates that prophylactic treatment with HMG-CoA reductase inhibitors can decrease the severity of cerebral focal ischemic damage irrespective of serum cholesterol levels (Endres et al., 1998). Several studies also suggest that HMG-CoA reductase inhibitors can reduce ischemic brain injury of gerbils and rats through decreasing oxidative stress on neurons (Kumagai et al., 2004; Hayashi et al., 2005). Furthermore, clinical studies demonstrate that statins can reduce the incidence of stroke (Rosenson, 2000). Therefore, it is conceivable that HMG-CoA reductase inhibitor pitavastatin is effective against neuronal damage after transient cerebral ischemia. Our immunohistochemical study shows that pitavastatin prevented the alterations of NF-κB and p53 in the hippocampal CA1 sector 5 days after transient cerebral ischemia. Furthermore, our results indicate that pitavastatin dose-dependently prevented the neuronal cell death in the hippocampal CA1 sector 5 days after transient cerebral ischemia. These results suggest that the up-regulations of NF-κB in glia and p53 in neuron can cause neuronal cell death after cerebral ischemia. Furthermore, our findings support the hypothesis that NF-κB- and/or p53-mediated neuronal cell death is prevented through decreasing oxidative stress by HMG-CoA reductase inhibitors. Therefore, NF-κB and p53 may provide an attractive target for the development of novel therapeutic approaches for brain stroke, although further studies are needed to investigate the exact mechanisms for our findings.

A recent interesting study demonstrates a reciprocal regulation of the transcription factors NF-κB and p53 in neurons undergoing cell death by DNA damage and ischemia (Culmsee et al., 2003). Both p53-mediated block of NF-κB activity and neuronal cell death are attenuated by the p53 inhibitor pifithrin-κ in vitro and in vivo (Culmsee et al., 2003). However, the precise relationship between NF-κB and p53 is not fully understood. Therefore, further studies are needed to investigate the exact mechanisms for the relationship between NF-κB and p53.

In conclusion, the present study demonstrated that the increase of NF-κB p65 in glial cells and the expression of p53 immunoreactivity in neurons precede neuronal damage in the hippocampal CA1 sector after cerebral ischemia. Our findings also suggest that NF-κB p65 immunoreactivity is induced highly in reactive astrocytes and microglia/macrophages of the hippocampal CA1 region where severe neuronal damage was observed. Furthermore, the present study demonstrated that pitavastatin can prevent the alterations of NF-κB and p53 immunoreactivity in the hippocampal CA1 sector after transient cerebral ischemia. These results suggest that the up-regulations of NF-κB in glia and p53 in neuron can cause neuronal cell death after cerebral ischemia. Thus, NF-κB and p53 may provide an attractive target for the development of novel therapeutic approaches for brain stroke.

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