HMG-CoA Reductase Inhibitor Has Protective Effects Against Stroke Events in Stroke-Prone Spontaneously Hypertensive Rats

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Background and Purpose—Recent clinical studies suggest that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exert protective effects against nonhemorrhagic stroke. In a murine cerebral ischemia model produced by occlusion of the middle cerebral artery, statins were shown to reduce infarct size. However, the effect of statins on hypertension-based stroke is unknown. The purpose of this study is to clarify the effect of a statin on stroke in stroke-prone spontaneously hypertensive rats (SHR-SP), in which both cerebral hemorrhage and infarction occur.

Methods—We treated SHR-SP chronically from 4 weeks of age with cerivastatin (2 mg/kg per day by gavage) or vehicle. The physiological parameters, the incidence of stroke-associated symptoms, and mortality were assessed.

Results—At 14 weeks of age, the incidence (13 ± 3% versus 37 ± 8%; P < 0.01) and the size of stroke (1.6 ± 0.2 versus 2.2 ± 0.1 arbitrary units; P < 0.01) were significantly decreased by cerivastatin, although blood pressure and plasma cholesterol levels were not different. Moreover, stroke-associated symptoms and early mortality of SHR-SP were markedly reduced in the statin-treated group (mortality at the age of 15 weeks: 15% versus 50%; P < 0.05). Statin treatment significantly reduced superoxide production from nonstroke parenchyma of brain and infiltration of inflammatory cells to the stroke lesions.

Conclusions—Our data show that a high dose of statin exerts protection against hypertension-based stroke and ameliorates the disease severity via inhibition of superoxide production and modulation of inflammation in brain. (Stroke. 2003;34:157-163.)

Key Words: mortality ■ rats, inbred SHR ■ statins ■ stroke ■ superoxides

Current epidemiological evidence failed to demonstrate a clear relationship between the risk of stroke and serum cholesterol levels.1 However, recent clinical trials and meta-analyses of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have shown a significant reduction in the incidence of ischemic stroke in patients with a history of coronary artery disease.2-3 As in the prevention of coronary artery disease, statins are reported to reduce the incidence of stroke by approximately 30%. Statins block the conversion of HMG-CoA to mevalonate, which is not only a precursor of cholesterol but also a source of molecules associated with intracellular signal transduction. Therefore, statins exert beneficial effects on vascular disorders including atherosclerosis, which are independent of lipid-lowering effects.4,5 In a murine model of acute cerebral ischemia produced by ligation of the cerebral artery, statins are reported to reduce the size of infarction and exert neuroprotective actions.6,7 These beneficial effects are mediated mainly by upregulation of endothelial nitric oxide synthase (eNOS) by statins, which results in augmentation of cerebral blood flow. These effects are shown to occur in the absence of changes in serum cholesterol levels.

Hypertension is one of major risk factors for stroke. Long-lasting hypertension results in dysfunction and injury of cerebral arteries, and the features of stroke based on risk factors such as hypertension are not identical to those of ischemic stroke produced by acute occlusion of the cerebral artery. In hypertension-associated cerebral events, not only cerebral infarction but also cerebral hemorrhage occurs, and multiple lacunar infarctions are commonly seen. It is important to investigate whether statins exert beneficial effects on incidental stroke that develops as a result of risk factors such as hypertension. Stroke-prone spontaneously hypertensive rats (SHR-SP) develop severe hypertension from an early
age, which causes stroke consisting of hemorrhage and infarction. SHR-SP have been used extensively in assessing pharmacological agents that are putatively capable of protection against stroke. In the present study we chronically treated SHR-SP with cerivastatin and evaluated its effects on the incidence of stroke, the size of stroke lesions, and early mortality. We found the protective effects of cerivastatin against stroke and examined the mechanisms of its beneficial actions.

Materials and Methods

Materials
Cerivastatin sodium was obtained from Bayer Yakuhin, Ltd. Other drugs were purchased from Sigma Chemical Co.

Animal Preparation and Experimental Design
Male SHR-SP, 4 weeks of age, were obtained from SLC (Shizuoka, Japan) and used in this study. They were randomly divided into 2 groups (statin-treated group and vehicle-treated group). Animals were provided standard chow and water ad libitum and maintained on a 12-hour light/dark cycle. Preliminary experiments indicated that 0.5 mg/kg cerivastatin does not affect the mortality and the occurrence of stroke signs in SHR-SP compared with untreated rats. Therefore, we chose the comparatively high dose of 2.0 mg/kg by referring to previous reports. The statin-treated group was treated with cerivastatin (2 mg/kg by gavage once per day) and the vehicle-treated group with vehicle (0.3% sodium carboxymethylcellulose) throughout the experiments. Each group was further divided into 2 subgroups. One subgroup (n=20 for each vehicle-treated group and statin-treated group) of rats was monitored on a daily basis for behavioral signs of stroke. Major neurological findings and symptoms of stroke were the occurrence of seizures, paralysis, and low-spirited conditions. If 1 of these symptoms occurred in SHR-SP, they were regarded as stroke sign positive. The follow-up was terminated when mortality of the vehicle-treated group reached 50%. Another subgroup (15 rats for each vehicle-treated group and statin-treated group) was killed at 10 weeks after treatment (14 weeks of age) and used for morphological analysis of brain and other assays. All animals were euthanized by overdose of pentobarbital sodium (Abbott Laboratories) given intraperitoneally. After anesthesia, blood was obtained by cardiac puncture and then perfused for 5 minutes with physiological salt saline via cardiac puncture. The excised brains were used for histological analysis, protein extraction, and an assay for superoxide. Aortas and carotid arteries were excised and used for eNOS analysis. Brain samples from Wistar-Kyoto rats (WKY) at the age of 14 weeks (SLC, Japan) were used as normal controls for several assays. The breeding of animals was conducted at Shin Nippon Biomedical Laboratories Ltd (Japan), and analysis of samples was performed at Kobe University. This study conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996).

Blood Pressure Measurement and Plasma Chemical Analysis
Blood pressure was measured by the tail-cuff method (model MK-1100; Muromachi Kikai). Blood was collected from tail veins, and plasma creatinine, blood urea nitrogen, total cholesterol, and triglyceride levels were determined with the use of an automated clinical chemistry analyzer. HDL cholesterol levels were quantified by enzymatic reaction with a commercially available kit (Wako).

Detection of Cerebral Damage and Determination of Incidence of Stroke
The brains removed from 14-week-old SHR-SP were each divided into 10 coronal 2-mm-thick sections with a rat brain matrix (Bio Research Center). Those were stained in 2% 2,3,5-triphenyltetrazo-

lium chloride (TTC) and fixed in a 10% neutralized formalin solution. Photographs of gross view of the brain were taken with a stereoscope (Olympus SZX12). The presence of TTC-negative stained area and the existence of extravascular erythrocytes or hemosiderin indicated stroke-positive areas. The incidence of stroke was analyzed with the use of 10 coronal sections in each brain. In each brain, the number of sections in which stroke lesions were detected was divided by 10, and the incidence was expressed as a percentage. Moreover, we performed computer-assisted volumetry to qualify the stroke volume. The perimeter of each stroke lesion was traced, and the lesion area was measured with the use of National Institutes of Health imaging software. The stroke volume for each lesion was calculated by multiplying the lesion area and the thickness (2 mm). However, because the interobserver differences were comparatively large, we applied another method to quantify the size of stroke lesions. The size of the largest lesion in each stroke-positive section was assessed semiquantiitatively by the following scoring: grade 1, small stroke (<2×2 mm); grade 2, medium-sized stroke (2- to 3-mm diameter); and grade 3, large stroke (>3×3 mm).

Histological and Immunohistological Examinations in Brain
The stroke-positive coronal sections, described above, were equilibrated in 20% sucrose and embedded in OCT compounds (Tissue-Tek). Cryosections (8 μm in thickness) of the brain were cut and collected. Those were stained with an anti-rat CD45 monoclonal mouse antibody (OX-1; Pharmingen; 1:100 dilution) followed by detection with biotinylated secondary antibodies and streptavidin–horseradish peroxidase. Quantitative analysis of the positively stained cell number in ×200 magnification was performed. In each brain, stroke lesions excluding pure bleeding lesions were used for the analysis because such lesions usually contained no leukocyte infiltration. At least 5 fields per lesion were used to count the number of leukocytes. A total of 41 lesions in the vehicle-treated group and 14 lesions in the statin-treated group were analyzed.

eNOS Expression in Vessels
The thoracic aorta was used for protein extraction for Western blotting and an assay for eNOS activity. Immunoblotting of eNOS was performed with a rabbit polyclonal eNOS antibody (Transduction Laboratories; dilution 1:500) as previously described. Using the same membrane, we also performed immunoblotting of β-actin with a mouse anti-β-actin monoclonal antibody (AC-15, Sigma Chemical; 1:200 dilution) to standardize the expression of eNOS. Enzymatic activity of eNOS was determined by the conversion of L-arginine to L-citrulline as described previously. Carotid arteries were fixed in 10% formaldehyde solution and embedded in OCT compounds. To determine the eNOS expression in the endothelium, cryosections of carotid arteries were stained with the rabbit anti-eNOS antibody (1:100 dilution) as described previously.

Detection of Superoxide Anion Production in Brain
Dihydroethidium was used to evaluate levels of superoxide in situ as described previously. Unfixed frozen brain parenchymas from SHR-SP in both the vehicle- and statin-treated groups were cut into 20-μm-thick sections and placed on same glass slides. Dihydroethidium (2 μmol/L) was applied to each tissue, and slides were incubated in a dark humidified chamber at 37°C for 20 minutes. Images were obtained with a confocal microscope equipped with an argon laser. Fluorescence was detected with a 585-nm long-pass filter.

A piece of brain sample taken from the stroke-negative area was incubated with a Cu-Zn superoxide dismutase inhibitor (diethyldithiocarbamate) for 30 minutes at 37°C. Brain superoxide levels were measured with the use of lucigenin chemiluminescence according to the method modified from that of Münzel et al. We used 5 μmol/L lucigenin; this concentration of lucigenin accurately reflects levels of ambient superoxide and is not subject to the redox cycling and artifactual production of superoxide observed with higher concentrations of the agent.
with the dose used in this experiment did not alter the elevation of blood pressure. This drug did not modify total and HDL cholesterol levels, but it significantly reduced triglyceride levels. However, triglyceride levels in both vehicle- and statin-treated groups remained within the normal range for SHR (60 to 100 mg/dL).

**Statin Reduced Cerebral Damage**

At 14 weeks of age, 10 weeks after the drug or vehicle treatment, 11 of 15 vehicle-treated rats and 12 of 15 statin-treated rats survived and were used for histological analysis. Figure 1A and 1B show representative photographs of the stroke-positive coronal brain sections of SHR-SP in vehicle-treated and statin-treated groups, respectively. The mean incidence of stroke and stroke size was estimated as described in Materials and Methods. The incidence was significantly decreased in statin-treated group compared with vehicle-treated group (13 vs 37%; \( P < 0.01 \); Figure 1C). Moreover, the stroke lesion size detected by both semiquantitative method and quantitative volumetry was markedly smaller in the statin-treated group than in the vehicle-treated group (Figure 1D and 1E).

**Statin Delayed the Appearance of Stroke-Associated Symptoms and Early Death**

Previous reports demonstrated that SHR-SP developed stroke within 24 weeks of age, and the majority of them died within 15 months.\(^8,17\) In contrast to those reports, SHR-SP of the colony used in our study exhibited a severe phenotype similar to that of high-salt–loaded SHR-SP.\(^9,18\) Indeed, 50% of the SHR-SP in the vehicle-treated group died by 16 weeks of age (Figure 2A). High-dose (2.0 mg/kg) statin treatment delayed early death and reduced the occurrence of stroke-associated symptoms (Figure 2B), although low-dose (0.5 mg/kg) cerivastatin did not affect these parameters (data not shown).
Statin Increased eNOS Expression in Vessels
Statin treatment increased eNOS protein levels and eNOS activity in aortas of SHR-SP (Figure 3A and 3B). Consistent with the results, eNOS expression in carotid arteries was increased in statin-treated SHR-SP compared with vehicle-treated SHR-SP (Figure 3C and 3D).

Statin Reduced Superoxide Production
Superoxide production from the SHR-SP brain parenchyma detected by lucigenin chemiluminescence was increased compared with that from the brain parenchyma of age-matched WKY (977±279 versus 205±108 arbitrary units per minute per milligram protein; P<0.01; Figure 4C). Superoxide production detected by both dihydroethidium (Figure 4A and 4B) and lucigenin chemiluminescence (Figure 4C) was significantly reduced in statin-treated SHR-SP compared with vehicle-treated SHR-SP. Although cellular types responsible for superoxide production could not be identified, leukocyte infiltration was not seen in the area we examined (data not shown). In addition, from the distribution of the dihydroethidium-induced signals, both vascular and nonvascular cells seemed to be responsible for superoxide production.

Statin Decreased Stroke-Associated Infiltration of Inflammatory Cells
As shown in Figure 5, CD45-positive cells were observed in the stroke and peristroke lesions. Those cells were located mainly in the brain parenchyma. In the nonstroke area, inflammatory cells were not detected (data not shown). Quantitative analysis revealed that statin treatment markedly reduced the infiltration of inflammatory cells to the stroke lesions (Figure 5C).

Discussion
The major finding of the present study is that a statin reduced cerebral damage in SHR-SP apart from its effects on cholesterol levels and blood pressure. The reduced cerebral damage was associated with delay of both the appearance of neurologically abnormal signs and early death. Consistent with previous reports, chronic statin treatment increased eNOS levels in vessels. We also showed that statin treatment reduced superoxide production from the brain parenchyma and modulated inflammatory responses at the stroke lesions.
Recently, statins were shown to have a protective effect toward focal cerebral transient ischemia in mice produced by occlusion of the cerebral artery followed by reperfusion.\textsuperscript{6,7} However, these reports were limited to the acute onset ischemia model, and there are no experimental reports of the effects of statins on spontaneously developed stroke. This is the first report that demonstrates the protective effects of a statin against hypertension-based incidental stroke and associated symptoms in an animal model.

In SHR-SP, stroke begins as very minor cerebral lesions typically consisting of small pinpoint-sized hemorrhage.\textsuperscript{18} Then, although diversity exists among colonies, those rats exhibit multiple cerebral hemorrhage/infarct several weeks after the first signs of stroke and have a fatal outcome. We assessed the severity of cerebral damage of SHR-SP at 14 weeks of age when most vehicle-treated SHR-SP exhibited neurological signs of stroke. Since lesions of stroke in SHR-SP are nonhomogeneous and include both infarct and hemorrhage of variable sizes, we assessed the extent of cerebral damage by 2 methods. We first evaluated the incidence of cerebral lesions in each animal and then performed semiquantitative assessment of the TTC-negative area of all coronal sections of the brain examined. Both methods clearly demonstrated the protective effects of cerivastatin against cerebral damage. These protective effects were associated with a marked delay of the appearance of neurological abnormality in the statin-treated SHR-SP. Chronic treatment with statin also delayed early death in SHR-SP.

The beneficial effects of statins on prevention of coronary artery disease have been established. In addition to their lipid-lowering effects, it has been suggested that the so-called pleiotropic effects of statins play important roles in prevention.\textsuperscript{4} Those beneficial effects include modification of endothelial function, reduction of inflammatory responses, increase in plaque stability, and inhibition of thrombus formation.\textsuperscript{19,20} However, the protective mechanisms of statins against stroke are not well understood. In a murine transient ischemia stroke model, chronic administration of statins augmented cerebral blood flow and reduced infarct size. The protective effects of statins are mainly attributed to their increasing effects on NO production\textsuperscript{5} because eNOS deficiency canceled the protective effects of statins against stroke.\textsuperscript{6,7} The mechanisms of spontaneously developed stroke in SHR-SP are not identical to those of the transient cerebral ischemia model; more complex mechanisms are involved. Therefore, statins may exert beneficial effects on stroke in SHR-SP by different mechanisms. In the transient ischemia model, blood flow to the lesion is critical in determination of infarct size, and increased cerebral blood flow by statins plays a major role in protection. On the other hand, dysfunction/injury of cerebral vessels precedes the occurrence of incidental stroke in SHR-SP, and prevention of vessel dysfunction/injury is important for stroke protection.

Although elevated blood pressure is primarily responsible for dysfunction/injury of cerebral vessels and the occurrence of stroke in SHR-SP, the renin-angiotensin system also plays an important role. This model is associated with high levels of plasma renin activities and angiotensin II,\textsuperscript{21} and it has been
reported that angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists reduce cerebral damage and prolong life span.17,22 The beneficial effects of these drugs are independent of a reduction in blood pressure. Although details are unknown, several mechanisms are involved in the deleterious effects of angiotensin II in SHR-SP. Those include arteriolar hypertrophy, infiltration of macrophages, and increases in vascular permeability.22 In addition to the direct effects of angiotensin II, the deleterious effects are likely mediated by oxidative stress. Angiotensin II has been shown to produce superoxide, which plays an important role in tissue damage, via activation of NADH/NADPH oxidase.23 Indeed, as we demonstrated in the present study, oxidative stress has been shown to be increased in organs, including brains, in SHR-SP.24

To elucidate the mechanisms of the protective effects of cerivastatin against spontaneously developed stroke, we first measured eNOS levels in vessels. We found increases in eNOS protein levels and activity. We also confirmed by isometric tension measurement on the isolated carotid arteries that vasorelaxation to acetylcholine was increased in the statin-treated group compared with the vehicle-treated group (data not shown). Since blood pressure was not changed by statin treatment, counterregulatory factors against augmented NO production might be operating to maintain blood pressure. Furthermore, we revealed that superoxide production in brains was significantly reduced in the statin-treated rats. In addition to the interaction with NO, the reduced superoxide production is likely caused by the direct effects of statins. Statins have been demonstrated to scavenge superoxide25 and may directly inhibit superoxide production by acting on NADH/NADPH oxidase.26 In a recent report of Wassmann et al, atorvastatin was shown to improve endothelial dysfunction in SHR via reduction of reactive oxygen species. Therefore, chronic treatment with statin increased eNOS-derived NO production and decreased superoxide production in SHR-SP. These effects would result in the protection of endothelial function and serve to preserve vascular integrity in the face of elevated blood pressure and stimulation by angiotensin II. We also examined inflammatory cell infiltration and found that the number of leukocytes in the stroke lesion was significantly reduced in the statin-treated brain of SHR-SP. Although the mechanisms of the reduced inflammatory cell infiltration were not clarified in the present study, cerivastatin might inhibit leukocyte-endothelial adhesion, as reported previously.28 Thus, statin treatment decreased inflammatory responses associated with stroke.29,30

In the murine transient cerebral ischemia model, it was suggested that the protective effects of statins are partly mediated by their inhibitory effects on platelet activation and thrombus formation.31 Multicenter clinical studies showed that statins reduced the incidence of nonhemorrhagic stroke, but their effects on hemorrhagic stroke remain unclear.3 In hemorrhagic stroke, statins may have adverse effects by inhibiting thrombus formation. However, we found that statin treatment reduced the incidence of stroke, which consists of both hemorrhage and infarction, in SHR-SP. Therefore, the protection against vascular dysfunction/injury by statins may lead to protection from the occurrence of cerebral hemorrhage; further studies are needed.

In conclusion, we showed the protective effects of a statin against hypertension-based stroke in SHR-SP. The increase in eNOS levels and the reduction of superoxide production caused by statins are at least partly involved in the protective mechanisms. Although clinical studies have reported the protective effects of statins against stroke, those studies were conducted in patients with hyperlipidemia and coronary artery disease.2,3 Hyperlipidemia is associated with atherosclerotic lesions in the carotid artery or the aorta, which can be an origin of thrombotic emboli to the brain. Coronary artery disease causes ventricular dysfunction, which results in thrombus formation. Therefore, treatment by statins of hyperlipidemia and coronary artery disease itself would exert protection against stroke, particularly ischemic stroke. In the present study we clearly demonstrated that a statin exerts protective effects against stroke in SHR-SP, which occurred independently of the presence of atherosclerotic vessels or coronary artery disease. Although these beneficial effects were obtained by a high dose of cerivastatin (2.0 mg/kg) and the present data may not be directly applicable to human cases, our findings may help to clarify the role of statins in protection against hypertension-based stroke.

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