Treatment With Statins After Induction of Focal Ischemia in Rats Reduces the Extent of Brain Damage

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Objective—In the present study, MRI has been used to investigate therapeutic intervention with statins in a model of permanent focal cerebral ischemia in rat.

Methods and Results—Brain ischemia was induced in rats by the permanent occlusion of middle cerebral artery (MCAO) and the brain infarct size followed up in alive animals 2, 24, and 48 hours after MCAO, using the trace of apparent diffusion coefficient [Tr(D)] maps and T2-weighted images. In vehicle-treated rats, the infarct volumes increased by 38.5% and 89% after 24 and 48 hours, respectively, compared with the damage detected at 2 hours after MCAO. Treatment with simvastatin (20 mg/kg) after MCAO prevented the increase in brain infarct volume occurring at 24 hours and induced a 46.6% reduction after 48 hours. This effect was similar to that observed when simvastatin was administered before the induction of focal ischemia. T2W-MRI images confirmed these findings. The neuroprotective effects of simvastatin were paralleled by an increase in endothelial NO synthase immunoreactivity, detectable in the brain of simvastatin-treated rats.

Conclusions—Statins, in addition to their preventive effect on cerebral ischemia, exert a neuroprotective role in the attenuation of brain damage after acute stroke. (Arterioscler Thromb Vasc Biol. 2003;23:322-327.)

Key Words: statins ■ cerebral ischemia ■ rat ■ MRI ■ endothelial nitric oxide synthase

Hypercholesterolemia is not a traditionally recognized risk factor in the pathogenesis of stroke.1,2 Recent studies, however, have shown that the HMG-CoA reductase inhibitors, or statins, which are the most widely used cholesterol-lowering drugs, significantly reduce the incidence of ischemic stroke in patients with and without high serum cholesterol levels.3,4

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It is likely that statins have pleiotropic effects that are beneficial in brain ischemia.5 Statin therapy may reduce stroke by ameliorating precerebral atherosclerosis in the carotid artery and the aorta.6–8 In addition to delaying atherosclerosis, there is emerging evidence indicating that statins have beneficial effects on cerebral vessels.6 These drugs possess antiinflammatory and antithrombotic activity in blood and plaques,9,10 and they reduce vascular inflammatory responses,10 modulate cytokine production,11 promote angiogenesis,12 and decrease oxidative stress.13

Previous studies, carried out in experimentally induced brain ischemia in normocholesterolemic mice, have shown that statins reduce cerebral infarct size, improve neurological function, and increase cerebral blood flow.14–17 These effects are dependent on the upregulation of endothelial nitric oxide synthase (eNOS), because they are not detectable in eNOS-deficient mice.14,15 The studies reported so far, however, refer to the prophylactic effects of statin treatment, but no information is available concerning its possible therapeutic efficacy in acute stroke.

Noninvasive MRI provides a new tool for monitoring the development of cerebral lesions as well as the effects of therapeutic strategies at different stages of developing brain damage.18–20 It also makes it possible to follow the onset (even before the occurrence of neurological symptoms), the development, and the outcome of brain damage in individual cases.18,21

Using a well-established rodent model of permanent middle cerebral artery occlusion (MCAO), we investigated with MRI the effects of simvastatin, administered to rats soon after the induction of focal cerebral ischemia, on brain infarct size and eNOS expression. For comparison, experiments were also carried out in rats treated with simvastatin 3 days before MCAO. The data show that the drug, when administered after the induction of ischemia, markedly reduces the extent of the brain damage and upregulates eNOS expression.

Methods

Drug Preparation

Simvastatin, kindly provided by Merck Sharp and Dohme (Rahway, NJ), was chemically activated by means of alkaline hydrolysis before

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subcutaneous injection. Atorvastatin, a gift of Parke-Davis, was dissolved in DMSO and diluted using sterile PBS.

Animals (Experimental Group) and Surgery
Male Sprague-Dawley rats (Charles River, Calco, Italy) weighing 200 to 250 g were allowed to have food and water ad libitum. The procedures involving the animals and their care at the Department of Pharmacological Sciences of the University of Milan respected the Institution’s guidelines, which comply with national and international rules and policies. The rats underwent permanent MCAO, as previously described. Briefly, they were intraperitoneally anesthetized with chloral hydrate (400 mg/kg) and the temporalsis muscle was dissected; after craniectomy, the right MCA was exposed and coagulated by means of microbipolar coagulation (SAMED MB122) to occlude it permanently from its stem to the lenticulostriate branch. It was then divided to ensure successful occlusion. The retracted temporalsis muscle was allowed to fall back into place and sutured. The animals were allowed to recover from anesthesia in warmed cages for 2 hours before the first MRI session, which was repeated 24 and 48 hours after MCAO. The rats showing in the first MRI evaluation a volume of the ischemic injury <20 mm³ were not included in the study. Their body temperature was continuously monitored during and after the occlusion and maintained at 37±0.5°C. The animals were randomly assigned to one of four groups. In groups A (n=12) and B (n=12), rats were treated with vehicle alone or simvastatin, respectively, for 3 days (48, 24, and 2 hours) before MCAO, and in groups C (n=12) and D (n=24), animals underwent MCAO and then were treated with vehicle (C) or simvastatin (D; 20 mg/kg, n=12; 2 mg/kg, n=6; and 0.2 mg/kg, n=6) at 3 and 25 hours after the induction of the injury; 1 hour later, each MRI session began. After the last MRI measurement, which was performed 48 hours post-MCAO, all the animals were anesthetized and their brains were removed and processed for histological and immunohistochemical evaluations. To assess the postischemia neuroprotective effect of atorvastatin in a separate set of experiments, Sprague-Dawley rats were subcutaneously injected with vehicle (n=6) or atorvastatin (10 mg/kg per day, n=6) at the times specified above for groups C and D.

Histology and Immunohistochemistry
For histological evaluations, the removed brains were fixed in Carnoy reagent (absolute ethanol:chloroform:glacial acetic acid, 6:3:1; Merck) embedded in paraplast (SIGMA), and 5-μm coronal sections were stained with H&E and examined by light microscopy. For the immunohistochemical studies of eNOS, paraffin-embedded slices were dewaxed in xylene and rehydrated using ethanol 100%, 96%, and 75% and tap water. Endogenous peroxidase was blocked by means of 1% H₂O₂ in H₂O for 20 minutes at room temperature and then washed in PBS. Nonspecific binding sites were blocked using 10% normal goat serum in 0.1% Triton/PBS for 45 minutes. Any excess serum was removed, and the sections were circled using a waterproof pen and then incubated overnight with primary Mab eNOS/NOS TypeIII (1:50, Transduction Laboratories) at 4°C. The control slides were incubated with diluting buffer alone. After washing in PBS, the slides were incubated with biotinylated secondary antibodies and streptavidine peroxidase (LSAB2 kit, peroxidase for use on RAT specimens; DAKO). After rinsing with PBS and 0.05 mol/L Tris-HCl pH 7.6 (5 minutes), HRP was detected by means of diaminobenzidine with H₂O₂ (SIGMA).

MRI
For MRI evaluations, rats were anesthetized with 2% isoflurane in 70% N₂/30% O₂, positioned on the animal holder by means of a rod held beneath the teeth, and placed into the 4.7T vertical 15-cm bore magnet of a Bruker spectrometer (AMX3 with microimaging accessory). A 6.4-cm-diameter birdcage coil was used for the imaging. A 3-orthogonal plane gradient echo scout acted as a geometric reference for locating the olfactory bulb, and then T2-weighted (T2W), reference, and diffusion-weighted (DW) images were acquired caudally. Turbo spin-echo T2W (Bruker RARE), with 16 echoes per excitation, 10 ms of interecho time, 85 ms of equivalent echo time, and 4 s of repetition time, allowed the acquisition of 16 contiguous 1-mm-thick slices. The spin-echo reference and DW images (TE=80 ms; TR=1 s) were acquired in 8 contiguous 2-mm-thick slices. The field of view of the DWI and T2W images was 4×4 cm² to ensure that the investigated volume was the same. The in-plane resolution of all of the images was 128×128 points. Diffusion weighting was obtained by adding 2 10-ms-long, 24.7-ms-spaced, and 8-gauss/cm rectangular gradients to a spin-echo multislice sequence, thus obtaining a b-value of ~1000 s/mm². Three different DW images were acquired using 3 orthogonal diffusion gradient directions; the reference images were identical but without diffusion gradients. Four 8° TEM² averages were acquired per gradient direction. ABC maps were computed for each gradient direction from the reference and DW images and then used to compute the map of the apparent diffusion tensor trace, Tr(D). Because it is rotationally invariant, the trace map has the advantage that it is free of anisotropy effects and thus offers a more precise definition of the lesions. The images were analyzed using homemade software (developed using IDL language by Research System) by measuring threshold image intensity values and interactively drawing outlines of the region of interest. The areas of ischemic damage were used to determine the total volume of ischemic tissue in each brain. We did not analyze the T2WI images taken 2 hours after ischemia, because no vasogenic edema was detectable. Together with a decrease in the diffusion coefficient indicating cytotoxic edema, in ~80% of the animals, the Tr(D) maps of the 48-hour images also showed regions in which the diffusion coefficient was higher than in healthy tissue; these regions were interpreted as cellular lysis areas. In such cases, the reported damaged area is the sum of the areas of cytotoxic edema and cell lysis.

Image Analysis and Correlation Studies
The extent of cytotoxic brain damage was obtained by delineating the cortical hypointensity region from Tr(D) maps obtained at 2, 24, and 48 hours after the MCAO by using homemade software. The areas of ischemic damage were used to determine the total volume of ischemic tissue in each brain by integration of areas with the distance between each level. To estimate the damage on histological preparations, after last MRI interrogation the brain were removed, 5-μm coronal sections were stained with H&E and scanned with a video camera, and the size of damage was measured using a computer analysis package (NIH Image 1.52 analysis software). A group of animals (n=10) was subjected to MCAO and killed after MRI interrogation at 24 (n=5) and 48 (n=5) hours to estimate the correlation between damage measured with MRI and histology. The size of ischemic brain damages (3 slices at different levels for each individual animal) from MR images and the corresponding histology sections were plotted onto scale diagrams.

Cholesterol Assay
Serum total cholesterol was assayed using a standard enzymatic method (Diagnostic Cholesterol kit; Sigma).

Data Analysis
The progression of the ischemic damage over time was studied using ANOVA for repeated measure. Comparison between the variation of the ischemic volume expressed as percentage to the 2-hour group was performed considering the mean value of the 2-hour group as 100%. Each single value of all groups was proportionally recalculated. The data are expressed as mean values±SEM. P<0.05 was considered statistically significant.

Results
Plasma total cholesterol levels in the vehicle-treated rats were 75±7 mg/dL. Simvastatin pretreatment (20 mg/kg for 3 days before MCAO) or posttreatment (20 mg/kg for 2 days after MCAO) did not affect the plasma cholesterol levels measured
at the time of death, which were 76±3 and 73±8 mg/dL, respectively.

**MRI Studies of the Rats Treated With Simvastatin After MCAO**

To evaluate whether treatment with simvastatin after the induction of focal brain ischemia confers neuroprotection, male Sprague-Dawley rats (n=48) were subjected to permanent MCAO, and the brain damage was followed up in each animal by MRI techniques. At 2 hours after MCAO, the Tr(D)-derived average volume of the ischemic lesions (detectable as hypointense areas in the cerebral cortex) was 32.2±4.4 mm³ (n=48). After this first determination of the brain damage, 2 groups of animals received the first administration of simvastatin (20 mg/kg; n=12) or vehicle alone (n=12), respectively. The size of the Tr(D)-derived volumes in the vehicle-treated rats increased by 38.5% and 89% after 24 and 48 hours, respectively (P<0.01). In rats treated with simvastatin (20 mg/kg) after MCAO, however, the infarcted area did not increase, but rather it decreased by 7.6% and 46.6% (P<0.01) at 24 and 48 hours, respectively (Figures 1A and 1B). Correlation studies showed a good agreement between the infarct size measured at 24 hours (r=0.87; slope 0.85, intercept 2.4) and 48 hours (r=0.76; slope 0.75, intercept 2.5) by Tr(D) maps and that measured by histology (Figure 1C).

Similarly, T2W-derived ischemic lesion volumes, assessed 24 and 48 hours after MCAO, increased over time in the vehicle-treated animals (+21±3% at 48 hours versus 24 hours, P<0.05), but they significantly decreased in the animals posttreated with simvastatin (−21.7±2.7% after 48 hours; P<0.05) (Figure 2). Two other groups of animals were treated after MCAO (at 2, 24, and 48 hours), with lower doses of simvastatin (0.2 mg/kg, n=6 or 2 mg/kg, n=6). Even a dose of 0.2 mg/kg completely prevented the progression of brain injury for up to 48 hours. Furthermore, the 2-mg/kg

**Figure 1.** Evolution of focal cerebral ischemia in rats treated with vehicle or simvastatin (20 mg/kg) after MCA occlusion. A, Tr(D) images of a coronal brain section taken 2, 24, and 48 hours after the induction of ischemia in rats treated with the vehicle alone (top) or simvastatin (bottom). B, Bar graph showing the changes in infarct volume measured at different times in the Tr(D) images of vehicle (n=12) or simvastatin-treated rats (n=12). **P<0.01 vs volume at 2 hours.** C, Correlation curves for the size of damage measured using histology and Tr(D) maps at 24 and 48 hours after MCAO.

**Figure 2.** Coronal brain T2-weighted MR images taken 24 and 48 hours after MCA occlusion in rats treated with vehicle (upper rows) or simvastatin 20 mg/kg (lower rows) after (A) or before (B) the induction of ischemia. The T2W signal was negligible 2 hours after the induction of ischemia, but the region of hyperintensity indicating the area of ischemic tissue can be clearly seen after 24 hours.
dose induced a significant reduction of the damaged area at 48 hours (Figure 3).

To assess whether the neuroprotection offered by simvastatin could be attributed to a class effect, studies were carried out in 2 additional groups of rats treated with atorvastatin 10 mg/kg (n = 6) or vehicle (n = 6), starting 2 hours after MCAO. Atorvastatin almost completely prevented the increase in Tr(D)-derived ischemic volume at 24 hours (7 ± 7%). At 48 hours, a statistically significant 37 ± 6% (P < 0.05) reduction compared with ischemic volume measured at 2 hours was observed in rats treated with atorvastatin after MCAO.

MRI Studies of the Rats Treated With Simvastatin Before MCAO
Two groups of rats underwent MCAO after treatment for 3 days with simvastatin (20 mg/kg, n = 12) or the corresponding vehicle (n = 12). Tr(D)s were acquired 2, 24, and 48 hours after induction of brain infarct. Brain damage was detected in the vehicle-treated rats as early as 2 hours after MCAO [Tr(D)-derived infarct volume: 37 ± 5 mm3; n = 12; mean ± SE], and the size of the damaged volume increased by 47% after 24 hours and 83% after 48 hours (P < 0.05 and P < 0.01; n = 12). The 2-hour Tr(D)-derived infarct volume in the simvastatin-pretreated rats was similar (40.9 ± 9 mm3, n = 12) to that of vehicle-treated rats but became significantly less after 24 hours (−33.3%; P < 0.05) and 48 hours (−47.2%; P < 0.01) (see online Figure I, available at http://atvb.ahajournals.org).

The T2W-derived volume of the lesions at 48 hours, with respect to damaged volume recorded at 24 hours with T2W-MRI contrast, was 21 ± 3% larger in the vehicle-treated rats (P < 0.05) but considerably smaller (−30 ± 3%; P < 0.05) in the simvastatin-pretreated rats (Figure 2B).

Immunohistochemical Localization of eNOS
Because previous studies have suggested that the neuroprotective effects of statins may be attributable to a putative increase in eNOS expression, we compared the localization of eNOS immunoreactivity in the brain of rats treated with vehicle or simvastatin before or after the induction of focal ischemic insult. To this end, the rats after last MRI section were killed and brain slices subjected to immunohistochemical analysis (see Methods section). In vehicle-treated rats, either before or after MCAO, eNOS immunostaining was negligible, suggesting that ischemia did not influence the expression of eNOS. Immunostaining in rats treated with simvastatin, however, either before or after MCAO, showed an increased eNOS reactivity, which localized with blood vessels in both the ipsilateral and contralateral sides of the occluded artery (Figure 4).

Discussion
This study shows that the administration of statins after an ischemic insult reduces the extent of the brain damaged areas and this effect is accompanied by eNOS upregulation in cerebral blood vessels. In addition to these findings, which suggest that statins may have therapeutic potential in acute stroke, we confirm the preventive effects of these drugs on experimental brain ischemia by using MRI techniques.

MRI is a noninvasive technique that allows an accurate assessment of the state of the brain tissue in living animals. In addition to T2W-MRI measurements, diffusion-weighted images (DWI), from which trace map values [Tr(D)] can be computed, provide useful information concerning postischemic brain homeostasis. Many studies have compared the relationship between brain lesions determined after MCAO using DWI with histology. A good agreement exists on the good spatial correspondence between the 2 measurements when they are performed in the early (2- to 4-hour) and late (24- and 48-hour) phase after vascular occlusion. Our data are in line with these findings.

During the early stages of ischemic injury, MRI discriminates ischemia-induced brain damage from normal tissue on the basis of the changes in spatial Tr(D) maps, in particular, energy impairment and membrane pump failure allow the osmotic drainage of water from extracellular to intracellular spaces that leads to cytotoxic edema.

By unmasking very early metabolic changes in brain tissue, Tr(D) allowed us to detect the appearance of edematous tissue at the beginning of its formation after vessel occlusion. Therefore, we started the treatment with simvastatin immediately after the earliest determination of the brain damage. Instead, T2W images are sensitive to the changes in water motility that reveal vasogenic edema, which starts only 12 to 24 hours after the induction of brain ischemia. Simvastatin administered after permanent MCA occlusion dose-
dependently protected brain tissue against the early ischemic damage, and a second dose administered after 24 hours almost completely prevented the naturally occurring growth of the injury. The fact that similar data were obtained with atorvastatin suggests that the protection against brain ischemia is probably a class effect. Thus, statins not only are effective in preventing acute stroke, as previously described,14–16 but they are also of potential benefit to treat it. The administration of statins after ischemic stroke in the rat, therefore, protects some of the tissue otherwise destined to infarction, limiting or even reducing brain tissue damage. If confirmed by clinical studies, this finding may open up new avenues for the therapeutic treatment of brain ischemia. A protective effect of simvastatin in mice treated with the drug before the induction of MCAO has been previously reported using conventional histological approaches.14 Under these experimental conditions, however, information on the onset of the injury, as well as on the time course of brain damage in the same animal, cannot be obtained. For this reason, we have investigated, with MRI, the effects of simvastatin administered before the induction of MCAO. Simvastatin pretreatment did not affect the infarct volume determined 2 hours after MCAO as judged by Tr(D) values, but it prevented its worsening up to 48 hours. At this time, Tr(D) measurements may no longer be reliable, and the extent of the damage is better detected by means of T2W analysis.18

Clinical trials suggest that HMG-CoA reductase inhibitors at doses that reduce plasma cholesterol levels also reduce the risk of stroke, despite the lack of any clear association between stroke and serum cholesterol levels.2,7,31,32 These findings in clinical studies raise the question of how statins, a class of cholesterol-lowering drugs, can reduce ischemic stroke when this disease is not related to cholesterol levels. Several data indicate that statins have cholesterol-independent or pleiotropic effects, many of which are mediated by the ability of statins to block the isoprenoid synthesis. These have been shown to play an important role in modifying several of the proteins involved in a variety of cell functions.8 In particular, experimental data indicate that the beneficial effects of statins on ischemic damage may be consequent to the upregulation of eNOS expression.14 Indeed, the drug has no neuroprotective effects in eNOS-deficient mice undergoing MCAO.14 The involvement of NO in the neuroprotection is corroborated by evidence showing that treatment of animals with permanent occlusion of MCA with NO donors induced a reduction of infarct volume by ameliorating cerebral blood flow in ischemic tissue.33

In this study, we show that treatment with simvastatin as early as 2 hours after MCAO increases eNOS immunoreactivity in the ischemic and contralateral brain hemispheres of rats. Thus, we hypothesize that a common mechanism, possibly the NO-mediated increase in blood flow, may likely result in the brain protection, which occurs in rats either posttreated or pretreated with statins.16 Studies aimed to dissect these complex mechanisms are presently ongoing. In conclusion, our study strongly supports the hypothesis that statins not only have a marked prophylactic effect against brain ischemia but may also control, at least in part, acute stroke. Appropriate studies are now needed to extend this observation to clinical settings.

References


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Figure I. Evolution of focal cerebral ischemia in rats treated with vehicle or simvastatin 20 mg/kg before MCA occlusion. Panel A shows the Tr(D) images of a coronal section taken 2, 24 and 48 hours after the induction of ischemia in rats treated with the vehicle alone (upper row) or simvastatin (lower row). Panel B: Bar graph showing the changes in infarct volume measured at different times in the Tr(D) images of vehicle (n=12) or simvastatin-treated rats (n=12). (*p<0.05; **p<0.01 vs volume at 2 hr).
Figure A shows images of brain sections labeled 'vehicle' and 'simvastatin' at 2, 24, and 48 hours. Figure B illustrates the percentage variation in infarct volume, comparing 'vehicle' and 'simvastatin' at 2, 24, and 48 hours.