Simvastatin Protects Against Long-Lasting Behavioral and Morphological Consequences of Neonatal Hypoxic/Ischemic Brain Injury

Walter Balduini, PhD; Valerio De Angelis, PhD; Erika Mazzoni, PhD; Mauro Cimino, PhD

Background and Purpose—Recent studies suggest that statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) not only reduce the incidence of stroke by lowering cholesterol levels but may also exert neuroprotective effects via a mechanism not related to their lipid-lowering effect. Despite the growing body of evidence, however, the neuroprotective effect of statins in stroke is still controversial. Herein, we studied whether a prophylactic administration of simvastatin (Sim) provides significant protection against brain damage, and we sought to determine its long-lasting behavioral consequences in a neonatal model of hypoxia/ischemia.

Methods—Newborn male rats were injected daily from postnatal days 1 to 7 with activated Sim (20 mg/kg) or an equivalent volume of vehicle. On postnatal day 7, the rats were subjected to ligation of the right common carotid artery, followed by 3 hours of hypoxia or by sham operation. The neuroprotective effect of Sim was evaluated after the rats had achieved adulthood by using a battery of behavioral tests and histological analysis.

Results—Sim-treated ischemic rats performed the circular water maze, the radial arm maze, and the multiple-choice water maze significantly better than did vehicle-treated ischemic rats. Furthermore, in contrast to the ischemic rats, hypoxia/ischemia-injured rats pretreated with Sim were not hyperactive at weaning and showed less behavioral asymmetry. Consistently, it was found that brain damage was significantly attenuated.

Conclusions—These findings indicate that prophylactic administration of statins may provide a potential neuroprotective strategy leading to an improvement in functional outcome in ischemic stroke. However, toxicity concern must be addressed before these agents can be directed to the asphyxiated fetus or newborn. (Stroke. 2001;32:2185-2191.)

Key Words: cerebral ischemia ■ HMG-CoA reductase inhibitors ■ neuroprotection ■ newborn ■ rats

Ischemic stroke is one of the leading causes of permanent disabilities and death in the elderly. Stroke also commonly occurs in the perinatal period and can have severe consequences on motor, cognitive, and behavioral functions that span the infant’s lifetime. The mechanisms of hypoxia/ischemia (HI)-induced brain damage, the behavioral outcome, and the effect of potential pharmacological treatments during development can be studied in 7-day-old rats subjected to unilateral carotid artery ligation and exposure to a hypoxic environment for 2 to 3 hours. In terms of brain development, this age of the rat is similar to that of near-term human babies. In a recent study, we characterized the long-lasting behavioral alterations occurring in this model of neonatal HI and found that these animals, when adults, show behavioral asymmetry and deficits in performing spatial learning tasks. Therefore, these behavioral abnormalities can be used as an end point to evaluate the efficacy of potential pharmacological treatments that may improve the consequences of a perinatal HI insult.

Recent clinical studies have reported that statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors), which are the most widely used cholesterol-lowering drugs, cause a significant reduction in the incidence of ischemic stroke in patients with and without high serum cholesterol levels. Thus, it has been suggested that statins may have neuroprotective properties that contribute to lessen the severity of the pathophysiological processes induced by the ischemic insult with a mechanism that is independent from their cholesterol-lowering effect. Animal studies have in fact shown that statins can be protective in ischemic stroke, probably because of their effects on different isoforms of NO synthase (NOS), which may lead to the restoration of cerebral blood flow. Furthermore, statins are able to reduce vascular inflammatory responses, modulate cytokine production, and promote angiogenesis. Nevertheless, the neuroprotective effect of statins in stroke is still controversial.

The behavioral and histological studies performed in the present study provide experimental evidence supporting the neuroprotective role of statins in ischemic stroke. We show that in a neonatal model of HI, a prophylactic treatment with simvastatin (Sim) not only reduces brain damage but also significantly improves its long-lasting consequences.
Materials and Methods

Animals, Drug Treatment, and Cerebral HI
All surgical and experimental procedures were carried out in accordance with the Italian regulations for the care and use of laboratory animals. Female Sprague-Dawley rats (Nossan, Italy) were housed with breeder males, and conception was determined by vaginal smear. Pregnant rats were then housed in individual cages, and the day of delivery was considered day 0 for the pups. On postnatal day (PN1), litters of pups born on the same day were randomized and reduced to 8 male rats. The experiment was performed with 2 different groups of animals (hereafter referred to as experiment 1 and experiment 2). Each experiment included the following groups (n = 8): (1) vehicle-treated control animals (control animals), (2) Sim-treated control animals, (3) vehicle-treated ischemic animals (ischemic animals), and (4) Sim-treated ischemic animals. Daily subcutaneous injections of activated Sim (20 mg/kg) were given to groups 2 and 4 from PN1 to PN7. Vehicle-treated control animals received a corresponding volume of PBS. Each litter contained both control (n = 4) and Sim-treated (n = 4) animals. The model of HI used was a slightly modified version of the model reported by Rice et al.1 On PN7, animals from groups 3 and 4 were anesthetized with ether and subjected to ligation of the right common carotid artery, followed by 3 hours of hypoxia (92% nitrogen and 8% oxygen).4 Animals in groups 1 and 2 were sham-operated. After surgery, each litter contained the 4 different groups: control rats (n = 2), ischemic rats (n = 2), Sim-treated control rats (n = 2), and Sim-treated ischemic rats (n = 2). Two ischemic animals from experiment 1 (1 from group 3 and 1 from group 4) died during the period of hypoxia. To avoid potential differences in the growth rate of the pups among litters within the experiment, the number of animals in the 2 control groups was also reduced to 7. All animals in experiment 2 survived the HI procedure.

Rectal temperatures were measured before surgery and at the end of the period of hypoxia. No differences were found among groups (data not shown).

**Experiment 1**

**Spontaneous Activity**
Spontaneous activity was measured in 23-day-old rats in a single session of 30 minutes by using an automated motor activity monitor (Automex).4

Left and Right Choices in a T-Maze
Each animal was given 3 trials in a T-maze, and the number of left or right choices was recorded.4 The test was repeated for 3 consecutive days (PN28 to PN30).

**Circular Water Maze**
In this task, animals must find a submerged 12-cm-diameter round pedestal located 2 cm below the surface of a circular water maze (1.5-m diameter). The maze was divided into 4 theoretical quadrants. The activity of the animal was recorded by using a digital camera connected to a VCR to allow a subsequent evaluation of behavioral patterns. The experiment was divided into 4 phases.

**Phase I: Acquisition**
Phase I consisted of 5 days of conditioning with 5 trials per day (PN42 to PN46). The animal was placed in the water within the quadrant opposite the one containing the pedestal (placed 30 cm from the edge of the tank equidistant from the edges of the quadrant) and facing the wall of the pool. The time taken to find the platform was recorded. If the animal located and climbed onto the pedestal, it was permitted 30 seconds on the pedestal before the next trial started. If the animal did not find the pedestal within 120 seconds, it was placed directly on the pedestal and allowed a 30-second rest period.

**Phase II: Probe Trials**
Phase II was aimed at determining the persistence of the conditioned response but also served as extinction trials in preparation for phase III. On PN47, the pedestal was removed, and the rat was placed in the maze at the same entry point as during the acquisition training. The time spent in an area of 30-cm diameter at the former location of the pedestal was measured for each of the 5 trials.

**Phase III: Reversal**
On PN49 to PN51, the pedestal was moved to the quadrant opposite the one used in phase I. Each rat was placed in the maze at the same location used in phase I. One session of 5 trials was conducted for each rat per day.

**Phase IV: Retention**
After 20 days (PN71), each rat was tested for 5 additional trials under the same conditions used in phase III to determine whether the groups differed with respect to recall of reversal training.

**Experiment 2**

**Radial Arm Maze**
This test was performed with 50-day-old animals. The 8-arm radial maze consisted of a central platform (30-cm diameter) from which 8 arms radiated symmetrically (50 cm long and 12 cm wide). A well was present at the outer end of each arm. Animals were deprived of water for 48 hours before testing. At the end of each daily session, the animals were individually placed in a cage, allowed to drink ad libitum for 30 minutes, and then put back into their home cages. Initially, animals were allowed free exploration sessions on 2 days in a row with all arms baited with water (50 μL per well). For spatial discrimination testing, only 3 arms were always baited, and the sequence of angles between them was 135°, 90°, and 135°. Rats were tested for acquisition over 3 daily sessions composed of 5 trials separated by 1-minute intervals. Each trial began with the placement of the animal on the central platform facing arm No. 3 and ended

Figure 2. Effect of treatment with Sim and neonatal HI on spontaneous activity. Animals were tested at 23 days of age (F3,27 = 3.7, \(P < 0.05\) by 1-way ANOVA), \(P < 0.05\) compared with control group (Newman-Keuls post hoc test). Error bars denote SE.
Figure 3. Effect of treatment with Sim and neonatal HI on left and right choices in a T-maze. Animals were tested at 28 to 30 days of age. NS indicates not significantly different (Mann-Whitney U test).

When the rat had visited the 3 baited arms. The following data were recorded: (1) time taken to visit the 3 baited arms; (2) number of working memory errors, ie, reentries into already visited baited arms; and (3) number of reference memory errors, ie, each entry into a nonbaited arm.

Multiple-Choice Water Maze
This test was performed with 80-day-old animals. The device and the procedure were reported in detail in our previous study. Animals were tested once a day for 7 days.

Histology
At the end of each experiment, animals were euthanized by decapitation, and the brain was immediately frozen in dry ice. To evaluate tissue injury, coronal sections (12 μm thick) of the brain of each animal were cut on a cryostat and thaw-mounted onto acid-washed subbed slides (gelatin and chrome alum). Sections were then postfixed with 4% paraformaldehyde in PBS and stained with toluidine blue. A computerized videocamera-based image analysis system (NIH Image software) was used to measure cross-sectional areas from the level of the anterior genu of the corpus callosum to the end of the gyrus dentatus. Measurements, based on the intensity and uniformity of the staining, were performed by an experimenter that was blinded to the conditions of the treatment; these measurements included only intact tissue. Regional volumes were estimated by summing areas and multiplying by the distance between sections (50 μm).

Data Analysis
Data are presented as mean±SE. Statistical analysis was performed by use of the Prism computer program (GraphPad Software, Inc).

Data on body weight collected during the pharmacological treatment were analyzed by a paired Student t test. Repeated-measures ANOVA was used for analyzing body weight data collected after HI and data on the multiple-choice water maze and on phases I and III in the circular water maze. Data from phases II and IV of the circular water maze, spontaneous activity, and radial arm maze were analyzed by 1-way ANOVA. The Mann-Whitney U test was used to analyze left and right choices in the T-maze and data from brain volume. Percent reduction in whole hemisphere or regional volumes was calculated by using the following formula: 100(left-sided volume−right-sided volume)/left-sided volume. Percent reduction was compared independently by the Mann-Whitney U test.15

The Newman-Keuls multiple comparison test was used to determine differences between single treatment groups.

Results
To determine whether Sim administration can protect against a neonatal ischemic stroke, the animals were treated with the drug from PN1 to PN7; the last injection was performed 30 minutes before surgery. We chose this protocol of drug administration on the basis of a previous report showing that the neuroprotective effect of Sim in adult mice was dependent on the dose and duration of treatment before the ischemic insult.

Experiment 1
Body weights, measured during treatment and at different times after HI, are shown in Figure 1. A significant difference was found in the growth rate between vehicle- and Sim-treated animals during treatment (Figure 1A, P<0.05). Significant differences among groups were also observed after HI throughout preweaning development (F3,4=19.9, P<0.001). On day 19, Sim-treated control, ischemic, and Sim-treated ischemic animals weighed 93%, 82%, and 78%, respectively, the control animal (Figure 1B). However, after weaning, a progressive recovery of body weight was observed, and in the adults, no differences were found among groups (Figure 1C, P=0.72).

On PN23, animals were tested in an automated motor activity apparatus (Figure 2). Spontaneous activity was significantly increased (F3,32=3.7, P<0.05), but only ischemic

Figure 4. Effect of treatment with Sim and neonatal HI on circular water maze performances. Animals were tested at 42 to 71 days of age. A, Acquisition (F3,4=29.2, P<0.0006 by repeated-measures ANOVA). B, Probe trials (F3,4=6.5, P<0.005 by 1-way ANOVA). *P<0.05 compared with both control groups and Sim-treated ischemic group; †P<0.05 compared with ischemic group. C, Reversal (F3,5=96.5, P<0.0001 by repeated-measures ANOVA). P<0.001 for comparison of ischemic group with both control groups. D, Probe trials (F3,5=9.7, P<0.0005 by 1-way ANOVA). ¶P<0.001 compared with both control groups and Sim-treated ischemic group; $P<0.01 for comparison of the Sim-treated ischemic group with both control groups and Sim-treated ischemic group; ¶P<0.01 for comparison of the Sim-treated ischemic group with both control groups and Sim-treated ischemic group; ¶P<0.01 for comparison of the Sim-treated ischemic group with both control groups. Symbols are defined in panel A. D, Retention (F3,27=9.7, P<0.0005 by 1-way ANOVA), ¶P<0.001 compared with both control groups; $P<0.05 compared with ischemic group. Symbols are defined in panel B. Error bars denote SE.
animals differed significantly from control animals \( (P<0.05) \).

In agreement with our previous data, we found that when tested in a T-maze, most ischemic rats preferentially chose the right arm of the maze \( (P<0.005) \), i.e., the arm ipsilateral to the damaged side (Figure 3). The number of right choices observed in Sim-treated ischemic animals, although higher, was not statistically different.

Figure 4 shows the results obtained in the circular water maze. Groups differed significantly in the mean time required to find the submerged pedestal over the training days \( (F_{3,6}=29.2, P<0.0005; \text{Figure 4A}) \). Both the control group and Sim-treated control group took the same time to find the pedestal, whereas the ischemic group needed a longer time \( (P<0.001 \text{ compared with both control groups}) \). The Sim-treated ischemic group, on the other hand, showed intermediate latencies. A statistically significant effect was observed between the 2 control groups and the Sim-treated ischemic group \( (P<0.05) \) but also between the ischemic and Sim-treated ischemic groups \( (P<0.05) \). Groups also differed significantly during phase II \( (F_{1,4}=6.5, P<0.005; \text{Figure 4B}) \). The ischemic group spent less time in the pedestal target area than did both control groups \( (P<0.05) \). The Sim-treated ischemic group, on the other hand, spent an intermediate time in the target area and did not differ significantly from either of the control groups. During phase III, very significant differences were found among groups \( (F_{1,3}=96.5, P<0.0001; \text{Figure 4C}) \). Significant effects were observed when the ischemic group was compared with both control groups or the Sim-treated ischemic group \( (P<0.001) \), as well as when the Sim-treated ischemic group was compared with both control groups \( (P<0.01) \). In the last learning session, the time taken to find the pedestal was as follows (mean±SE): control group, 9.0±2.4 seconds; Sim-treated control group, 4.3±0.6 seconds; ischemic group, 65.8±12.1 seconds; and Sim-treated ischemic group, 23.6±6.3 seconds. Twenty days after reversal training, the animals were tested for retention (Figure 4D). There were very significant group effects \( (F_{2,4}=10.8, P<0.0005) \), but only the ischemic animals differed significantly from both control groups \( (P<0.001) \) and the Sim-treated ischemic group \( (P<0.05) \). The Sim-treated ischemic group took only half the time needed by the ischemic group to find the pedestal \( (42.0±10.5 \text{ and } 21.1±5.1 \text{ seconds}, \text{respectively}) \).

**Experiment 2**

The growth rate of animals in experiment 2 was quite similar to that reported above for experiment 1 (data not shown).

Figure 5 shows the results obtained in the radial arm maze. The groups differed significantly in the mean time taken to perform the task \( (F_{3,56}=10.6, P<0.0001; \text{Figure 5A}) \). The ischemic group took more time than both control groups \( (P<0.001) \) and the Sim-treated ischemic group \( (P<0.001) \) in finding the 3 arms baited with water. Significant group effects were also found in both working memory \( (F_{3,56}=4.0, P<0.01) \) and reference memory \( (F_{3,56}=9.9, P<0.0001) \), but only the ischemic group differed significantly from both control groups \( (P<0.05 \text{ and } P<0.001 \text{ for working and reference memory, respectively}) \).

Very significant group effects \( (F_{3,6}=8.6, P<0.001) \) were also found in the multiple-choice water maze (Figure 6), but only the ischemic group differed significantly from both control groups.

**Histology**

At the end of the behavioral tests, the animals were euthanized and evaluated for histological damage. Figure 7 shows representative histological sections at the hippocampal level of the brain of the animals in experiment 1; a quantitative evaluation of brain damage in both experiments is reported in the Table. No difference in the volume of the whole brain was observed between control animals and Sim-treated control animals (data not shown). However, a remarkable difference in the extension of injury was found among ischemic animals. In experiment 1, 5 ischemic animals showed severely damaged brains with a marked degeneration of the hemisphere ipsilateral to the occluded carotid artery, whereas 2 animals showed only a reduction in the size of the ischemic region.
No injuries were observed in the left side of the brain, the cerebellum, or the brain stem (with the exception of dilatation of the lateral ventricle in some animals, Figure 7). The brain damage observed was less severe in Sim-treated ischemic animals. In these animals, the right hemisphere was mostly preserved, and injury was mainly limited to different sizes of cavitation in the cerebral cortex. Furthermore, a higher magnification of the 2 less damaged ischemic animals (Figure 8B and 8D) revealed a severe loss of pyramidal cells in the CA1 and in the CA2/CA3 fields (data not shown) of the hippocampus, which was markedly attenuated in Sim-treated ischemic animals (Figure 8H and 8F). The neuroprotective effect of Sim was confirmed by measuring the volume of the whole right and the left hemispheres of the brain as well as of the hippocampus and cerebral cortex (Table). Whole hemispheric and regional volumes were significantly reduced in both ischemic and Sim-treated ischemic animals. Compared with the left side, however, the infarct size was significantly smaller in the Sim-treated ischemic group than in the ischemic group.

Discussion
In the present study, we report experimental findings showing that prophylactic administration of Sim, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that crosses the blood-brain barrier, offers significant protection from brain damage and its behavioral consequences in a neonatal model of HI. The neuroprotective effect of Sim was long-lasting and occurred under conditions that resulted in severe tissue injury in most ischemic animals.

Animals that received HI insult on PN7 showed increased spontaneous activity at weaning, behavioral asymmetry, and a marked deficit in spatial learning in their adult life, all dysfunctions that were significantly attenuated by Sim treatment. These results are consistent with the finding that Sim-treated ischemic animals develop smaller infarctions. In agreement with data reported in our previous investigation and by other authors, we found that these ischemic animals, when adults, show different degrees of brain injury. Even animals without significant brain damage show a decreased...
number of cells in the hippocampus, particularly in the CA1 and CA2/CA3 fields, that was either not observed or markedly attenuated in Sim-treated ischemic animals. Furthermore, in most of these animals, the cerebral cortex was partially preserved. The reduction in neuronal damage in brain regions known to be essential for acquisition and retention of spatial memory tasks in rats, such as the hippocampus and the cerebral cortex,16,17 may account for the improvement in memory functions after Sim treatment.

The present study provides further experimental support to the hypothesis that statins exert a significant neuroprotective effect.8 Our results are consistent with those of Endres et al,9 showing that Sim was neuroprotective in adult mice after middle cerebral artery occlusion and reperfusion, and suggest that prophylactic treatment with this drug might also be useful in ischemic strokes occurring in infants. However, the use of pharmacological agents directed at preventing or reducing the effects of perinatal HI in humans needs to address specific toxicity concerns, and the balance between the beneficial effects and toxicity must be carefully evaluated. Several reports have shown that statins may induce apoptosis in cultures of cortical neurons of both humans and rat glioma cells18–20 and myopathy in young rats.21 However, a recent prospective study in humans found no relationship between exposure to Sim during pregnancy and adverse pregnancy outcome.22 In our experiments, the neuroprotective effect of Sim occurred without significant toxic effects, because the reduction of body weight observed during treatment and at weaning was completely recovered in adults, and both spontaneous activity and cognitive abilities in Sim-treated control animals did not differ from those of control animals.

In the present study, the mechanism(s) by which a prophylactic treatment with Sim is neuroprotective in neonatal HI was not investigated. In adult mice, statins have been found to improve brain blood flow, probably by endothelial NOS upregulation.9,10,23 Furthermore, statins can promote endothelial cell survival, NO production, and angiogenesis in vivo.13 In primary astrocytes, microglia, and macrophages,11 these drugs can reduce the induction of inflammatory mediators, such as tumor necrosis factor-α, interleukin-1β, and interleukin-6, and decrease the expression of the inducible form of NOS. Tumor necrosis factor-α and interleukin-1β expression, on the other hand, are increased after focal cerebral ischemia in the neonatal rat,24 and pharmacological strategies directed to reduce the activity of these proinflammatory cytokines attenuate tissue damage.25–27 Whether the neuroprotective effect of statins in neonatal rats is mediated by the same mechanisms found in adult animals remains to be determined.

In summary, the results of the present study indicate that prophylactic treatment with Sim is neuroprotective. Because statins are already used and known to be safe in humans, their administration may provide an important prophylactic therapeutic strategy for limiting the severity and the functional outcome of ischemic stroke. However, concerns with regard to toxicity must be addressed before these agents can be used in treating asphyxiated fetuses or newborns.

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References
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