Prophylactic but Not Delayed Administration of Simvastatin Protects Against Long-Lasting Cognitive and Morphological Consequences of Neonatal Hypoxic-Ischemic Brain Injury, Reduces Interleukin-1β and Tumor Necrosis Factor-α mRNA Induction, and Does Not Affect Endothelial Nitric Oxide Synthase Expression

Walter Balduini, PhD; Erika Mazzoni, PhD; Silvia Carloni, PhD; Maria Grazia De Simoni, PhD; Carlo Perego; Luigi Sironi, PhD; Mauro Cimino, PhD

Background and Purpose—Prophylactic administration of simvastatin has been shown to protect against brain damage and its long-lasting behavioral consequences in neonatal rats. To establish the drug treatment window, we evaluated the effectiveness of simvastatin administered at different intervals before and after stroke. Furthermore, we determined whether simvastatin affected endothelial nitric oxide synthase (eNOS) or inflammatory cytokines in brain tissue or cholesterol levels in serum.

Methods—On postnatal day 7, male rats were subjected to hypoxia-ischemia (HI). The experiment included sham-operated controls and HI animals receiving daily saline or activated simvastatin (20 mg/kg) injections from postnatal day 1 to day 7 (HI-simvastatin 1–7 group), from postnatal day 4 to day 11 (HI-simvastatin 4–11 group), or from postnatal day 7 to day 14 (HI-simvastatin 7–14 group). The neuroprotective effect of simvastatin was evaluated at adulthood by means of behavioral and histological analyses. Cytokines and eNOS expression were assessed by reverse transcriptase–polymerase chain reaction and Western blotting.

Results—Animals in both the HI-simvastatin 1–7 and HI-simvastatin 4–11 groups performed better than HI rats in either the T-maze or the circular water maze and showed significantly attenuated brain damage. Expression of interleukin-1β and tumor necrosis factor-α mRNA in cortex was significantly increased in HI but not in HI-simvastatin 1–7 animals. In the same brain area, simvastatin treatment did not affect the increase of eNOS expression observed after HI**.**

Conclusions—These findings indicate that prophylactic but not delayed administration of simvastatin improves functional outcome in neonatal rat stroke. The reduced induction of cytokines suggests that the neuroprotective effect of simvastatin may be related to a dampening of the inflammatory response. **(*Stroke*. 2003;34:2007-2012.)**

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

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are widely prescribed to lower cholesterol in hyperlipidemic patients at risk of cardiovascular diseases. A growing body of experimental and clinical evidence, however, suggests that statins possess additional properties that may confer to these drugs a direct neuroprotective effect in stroke.[1–4]

In adult animal models of experimental stroke, statins were found to be neuroprotective, and this effect has been ascribed to their ability to modulate endothelial nitric oxide synthase (eNOS).[5,6] Indeed, chronic treatment with simvastatin or mevastatin upregulates eNOS activity in cells and improves cerebral blood flow.[5,6] Further important evidence of the role of eNOS in the effect of statins is the finding that simvastatin is not neuroprotective in eNOS-deficient mice, indicating that increased eNOS activity may represent a mechanism against cerebral injury.[5] However, statins also possess additional properties that could be related to their neuroprotective effect in stroke. They were found to reduce vascular inflammatory responses[2,7]; to ameliorate endothelial function[4]; to modulate cytokine responses that occur during ischemia and reperfusion by reducing the induction of inducible nitric oxide

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From the Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino, Urbino (W.B., E.M., S.C., M.C.); Istituto di Ricerche Farmacologiche "Mario Negri," Milano (M.G. De S., C.P.); and Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, Milano (L.S.), Italy.

Correspondence to Dr Walter Balduini, Istituto di Farmacologia e Farmacognosia, Università di Urbino, via S. Chiara 27, 61029 Urbino (PS), Italy. E-mail balduini@uniurb.it

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synthase (iNOS), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) in astrocytes and macrophages[8]; and to promote angiogenesis.[9]

Stroke can also occur in the perinatal period and generally results in severe neurological deficits that span the individual's lifetime, including delayed mental and motor development, epilepsy, and major cognitive deficits. Because of the variations in the clinical presentation and diagnosis, the exact incidence of perinatal stroke remains to be determined, although estimates indicate that it can occur in approximately 1 in 4000 term births.[10] Using a well-established animal model for studying perinatal stroke, originally developed by Rice et al,[11] we recently reported that a prophylactic administration of simvastatin, a statin that crosses the blood-brain barrier, significantly protected against brain damage and its long-lasting behavioral consequences, indicating that the drug is also effective in perinatal stroke.[12] In the present report we extend evidence of the neuroprotective effect of simvastatin in this model of stroke. The aim of the study was 2-fold: (1) to determine the effectiveness of simvastatin administered at different times before and after the ischemic insult to establish a drug treatment window and (2) to evaluate whether a prophylactic administration of simvastatin affected brain eNOS, inflammatory cytokines, and serum cholesterol levels.

Materials and Methods

Animals, Drug Treatment and Cerebral Hypoxia-Ischemia

All surgical and experimental procedures were performed in accordance with the Italian regulations for the care and use of laboratory animals. On postnatal day 7, Sprague-Dawley (Charles River, Italy) pup rats 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), as previously described.[12,13] Activated simvastatin (20 mg/kg) was injected daily (subcutaneously) from postnatal day 1 to day 7 (hypoxia-ischemia [HI]–simvastatin 1–7 group), from postnatal day 4 to day 11 (HI-simvastatin 4–11 group), or from postnatal day 7 to day 14 (HI-simvastatin 7–14 group). The HI-simvastatin 7–14 group received the first dose of the drug directly after the period of hypoxia. Vehicle-treated sham-operated control (control group) and HI-injured animals received a corresponding volume of PBS from postnatal day 4 to day 11. We had previously reported that simvastatin administration to control animals from postnatal day 1 to day 7 did not result in long-lasting behavioral deficits and that the decrease in body weight observed during treatment and at weaning was completely recovered in adulthood.[12] Thus, in our experimental protocol we did not include a control group of animals treated with simvastatin.

Two ischemic animals, 1 from group HI-simvastatin 1–7 and 1 from group HI-simvastatin 7–14, died during the period of hypoxia. To avoid variability in the growth rate among treatments, the number of animals in every experimental group was reduced from 8 to 7.

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

Reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blot analyses were performed in additional groups of sham-operated (control), HI, and HI-simvastatin 1–7 rats (n=5 per group). Control and HI animals were treated from postnatal day 1 to day 7 with vehicle. Behavioral and histological measurements were performed by investigators blinded to the experimental conditions.

Left and Right Choices in a T-Maze

At postnatal day 28, each animal was given 3 trials in a T-maze, and the number of left or right choices was recorded.[13] The test was repeated for 3 consecutive days.

Circular Water Maze

Animals were trained in a circular maze[12] and evaluated in a training-to-criterion test. 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. 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. The performance criterion was 3 trials in a row with an average time before escape of $<$20 seconds. When the animal reached the criterion, it was transferred to the next location on the next day. Each animal received a maximum of 32 trials for each of the 2 positions of the pedestal. Data are expressed as the number of sessions needed to reach the criterion.

Histology

Histological analysis was performed at the end of the behavioral experiments, as previously described.[12] Measurements included only intact tissue determined on the basis of the intensity and uniformity of the staining.

RNA Extraction and RT-PCR Analysis

Total RNA was extracted from rat cortex according to the acid guanidinium-phenol-chloroform method.[14] For transcription, total RNA (0.8 μg) was used as a substrate for single-stranded cDNA synthesis with the use of the Gene Amp RNA PCR Kit (Perkin Elmer), according to the manufacturer's instructions. An aliquot (10 μL) of cDNA synthesis mix was used for the PCR reaction. Gene-specific oligonucleotide primers were as follows (forward and reverse, 5′-3′): CCAGGATTCATGTGCCAGG and CCACTCAGTCTTGGCAGTGC (product size: 191) for cyclophilin; CTCCATGAGCTTTGTACAAGG and TGCTGATGTACCAGTTGGGG (product size: 245) for IL-1β; TCAGCCTCTTCTCATTCCTGC and TTGGTGGTTTGCTACGACGTG (product size: 203) for TNF-α; and GAAAACAGAGCTTCAGCATGCTTGG and TTTGAGTGTCACGTAGGCTTCTATGC (product size: 543) for interleukin-10 (IL-10). PCRs were conducted in a Perkin Elmer Gene Amp 9700 System. After an initial denaturation at 95°C for 5 minutes, amplification was conducted through 22 to 36 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C (60°C for IL-10) for 30 seconds, and extension at 72°C for 30 seconds. Final extension was at 72°C for 7 minutes. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers and the linear phase of the product amplification (data not shown). In each PCR (RNA extraction, cDNA synthesis, and PCR), negative and positive controls were included. A portion of the PCR mixture (7 μL) was electrophoresed in a 1.5% (wt/vol) agarose gel, stained with ethidium bromide. The relative intensity of the bands was measured by an Image Quant Analyzer (Molecular Dynamics) The level of gene expression of each transcript was normalized to that of the reporter gene cyclophilin.

Western Blot Analysis

This analysis was performed on ischemic and contralateral cortices of rats in the HI and HI-simvastatin 1–7 groups or the whole cortex of controls. Samples (50 μg protein; Bradford dye-binding procedure, Bio-Rad Laboratories) were separated onto 6% sodium dodecyl sulfate–polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane (40 mA, overnight, 4°C). Blots were probed with monoclonal eNOS primary antibody (1:2000; Transduction Laboratories), processed with horseradish peroxidase–conjugated anti-mouse IgG (1:4000), and detected with the ECL system (Amersham Pharmacia Biotech). A monoclonal antibody against β-actin (1:4000; Sigma) was used as a control for protein gel loading. Western blot were analyzed with the use of NIH Image software. Data were normalized to those of β-actin and expressed as percentage of control.

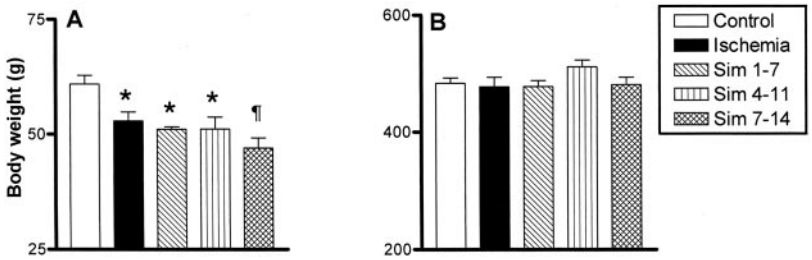

Figure 1. Effect of different schedules of simvastatin (Sim) treatment and neonatal HI on body weight measured at 21 (A) and 80 days (B) of age. Data are mean±SE. *$P<0.05$, ¶$P<0.001$ compared with control group.

Cholesterol Measurement

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

Data Analysis

Data are presented as mean±SE. Statistical analyses were performed with the Prism computer program (GraphPad Software Inc). Body weight data were analyzed by 1-way ANOVA followed by the Newman-Keuls multiple comparison test. T-maze, circular water maze, Western blot, and RT-PCR data were analyzed with the Kruskal-Wallis test followed by the Dunn multiple comparison test.

The Mann-Whitney *U* test was used to analyze data from brain volume. Percent reduction in whole hemisphere or in selected brain regions was calculated by the following formula: 100×(left-sided volume−right-sided volume)/left-sided volume. Statistical analysis was performed by the Kruskal-Wallis test followed by the Dunn multiple comparison test.

Results

Body weights for the different treatment groups were measured at weaning (21 days) and in the adult animals (80 days). At weaning, the body weights of HI- and HI-simvastatin–treated animals were significantly lower than those of the control group (Figure 1A; $P<0.05$). A progressive recovery of body weight was observed after weaning, and at 80 days of age no differences were found among groups (Figure 1B).

In agreement with our previous studies,[12,13] ischemic rats, when tested in the T-maze, preferentially chose the arm ipsilateral to the damaged side (Figure 2; right/left ratio: control, 1.29±0.21; HI, 4.14±0.69; $P<0.05$). The right/left ratio of all groups of HI-simvastatin–treated animals did not differ from that of controls. In addition, the right/left ratio of the HI-simvastatin 1–7 and HI-simvastatin 4–11 groups differed significantly from that of the ischemic group ($P<0.05$).

Learning abilities were tested in a circular water maze (Figure 3). Groups differed significantly in the number of sessions required to find the submerged pedestal in both the first and the second positions ($P=0.0027$ and $P=0.042$, respectively). The post hoc test revealed that only the HI and the HI-simvastatin 7–14 groups differed from controls ($P<0.01$ and $P<0.05$, respectively, for the pedestal in position 1 and $P<0.05$ for the pedestal in position 2).

At the end of the behavioral tests, animals were killed and evaluated for histological damage. The Table shows the results of a quantitative evaluation of brain damage. Brain injury was significantly attenuated in the HI-simvastatin 1–7 and HI-simvastatin 4–11 groups but not in the HI-simvastatin 7–14 group. This pattern was observed when histological damage was measured with consideration of the whole hemisphere, the cerebral cortex, or the hippocampus.

In adult animals statins upregulate eNOS expression, with a subsequent amelioration of cerebral blood flow.[5,15] We therefore investigated whether a similar effect could be observed after a prophylactic treatment with simvastatin in brain tissue of HI immature animals. The pattern of eNOS expression assessed by Western blotting in the cerebral cortex at different times after the ischemic insult is shown in Figure 4. Expression of eNOS was significantly higher in the ischemic side 24 hours after HI. However, the expression of the protein in the HI-simvastatin 1–7 group did not differ from that observed in HI animals.

Since statins modulate cytokine production,[2,8] we evaluated whether a prophylactic treatment with simvastatin modulates mRNA expression of IL-1β and TNF-α, 2 proinflammatory cytokines that appear to contribute to neuronal damage after stroke, and of the anti-inflammatory cytokine IL-10. A marked activation of IL-1β and TNF-α mRNA

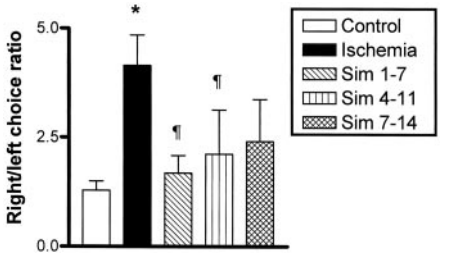

Figure 2. Effect of different schedules of simvastatin (Sim) treatment and neonatal HI on left and right choices in a T-maze. Animals were tested at 28 to 30 days of age. Results represent the right/left choice ratio and are expressed as mean±SE. *$P<0.05$ compared with control group; ¶$P<0.05$ compared with HI group.

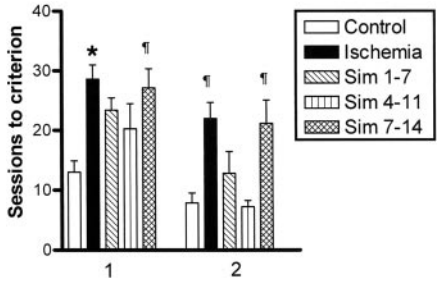

Figure 3. Effect of different schedules of simvastatin (Sim) treatment and neonatal HI on circular water maze performances. Animals were assessed with a training-to-criterion test, as described in Materials and Methods. Results represent the number of sessions needed to reach the criterion and are expressed as mean±SE. Results for first (1) and second (s) positions of the pedestal are shown ($P<0.0028$ and $P<0.0042$, respectively; Kruskal-Wallis test). *$P<0.01$, ¶$P<0.05$ compared with control group (Dunn multiple comparison test).

Regional Volume Measurements of 80-Day-Old Ischemic and Simvastatin-Treated Ischemic Animals

	n	Whole Hemisphere$^{Y'}$	Cortex$^{Y'}$	Hippocampus$^{Y'}$
Ischemic animals				
Left	7	494.2±30.2	121.4±10.5	41.9±4.7
Right	7	270.5±19.9¶	50.7±6.2¶	18.5±2.5¶
% ipsilateral damage$^{y'}$		45.3±1.9	57.7±4.2	55.24±3.3
HI-simvastatin 1–7 group				
Left	7	462.2±15.6	119.1±10.4	39.3±2.4
Right	7	364.8±33.4§	84.6±12.1§	28.1±3.0§
% ipsilateral damage$^{y'}$		21.1±7.1**	29.5±5.9**	27.9±7.6**
HI-simvastatin 4–11 group				
Left	7	472.6±31.1	116.7±15.3	38.0±3.3
Right	7	383.6±36.2	89.7±17.4	30.4±4.9
% ipsilateral damage$^{y'}$		18.8±5.6**	21.7±8.0**	23.3±5.6**
HI-simvastatin 7–14 group				
Left	7	438.1±25.4	111.6±8.8	34.9±3.2
Right	7	259.1±28.9¶	48.07±12.9¶	20.3±2.6¶
% ipsilateral damage$^{y'}$		38.4±9.3	53.8±13.4	39.1±10.2

Simvastatin (20 mg/kg) was administered from postnatal day 1 to day 7. $^{Y'}$, measured in cubic millimeters; $^{y'}$, percent ipsilateral damage was calculated from bilateral regional volumes, using the formula: 100×(L−R)/L, where L=left-sided volume and R=right-sided volume.

§$P<0.05$ and ¶$P<0.001$, Mann-Whitney U test comparing lesioned side to the nonlesioned side.

**$P<0.05$ comparing the % ipsilateral damage of ischemic animals with simvastatin-treated ischemic animals (Kruskal-Wallis test followed by the Dunn multiple comparison test).

expression was induced in neonatal rat cortex 4 hours after HI (Figure 5). Simvastatin treatment partially prevented this activation. In the cortex of animals in the HI-simvastatin 1–7 group, IL-1β and TNF-α mRNA expression was not significantly increased. IL-1β and TNF-α activation was no longer present 24 hours after HI in any experimental groups (data not shown). In the same animals IL-10 mRNA was also measured, and no significant changes were found in any experimental group (data not shown).

No differences in cholesterol levels were found in 7-day-old animals (116.0±6 and 96.0±10 mg/dL in control and HI-simvastatin 1–7 groups, respectively) or in adult animals (63.2±13 and 63.0±18 mg/dL in control and HI-simvastatin 1–7 groups, respectively).

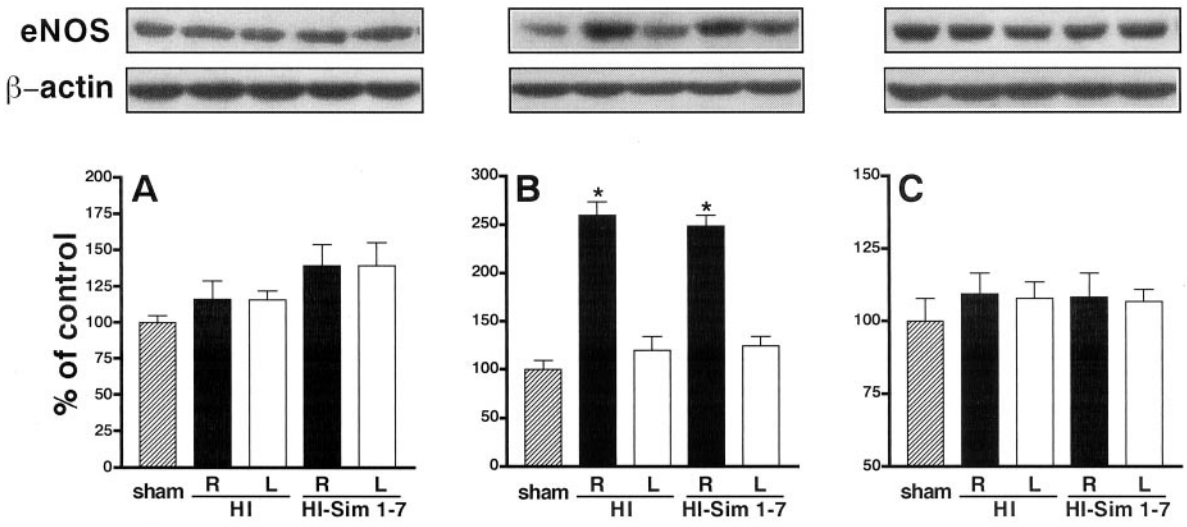

Figure 4. Western blot analysis of eNOS expression in cerebral cortex of rats in control, HI, and HI-simvastatin (Sim) 1–7 groups evaluated at 6 hours (A), 24 hours (B), and 5 days (C) after the end of the HI insult. Data are expressed as percentage of optical density detected for the respective group of control animals and are mean±SE of 5 animals. The immunoblots for eNOS and β-actin (as an internal standard) reported above each graph are representative of 3 independent experiments. Exposure times of the blots were not identical. R indicates damaged side; L, contralateral side. *$P<0.05$ compared with control group.

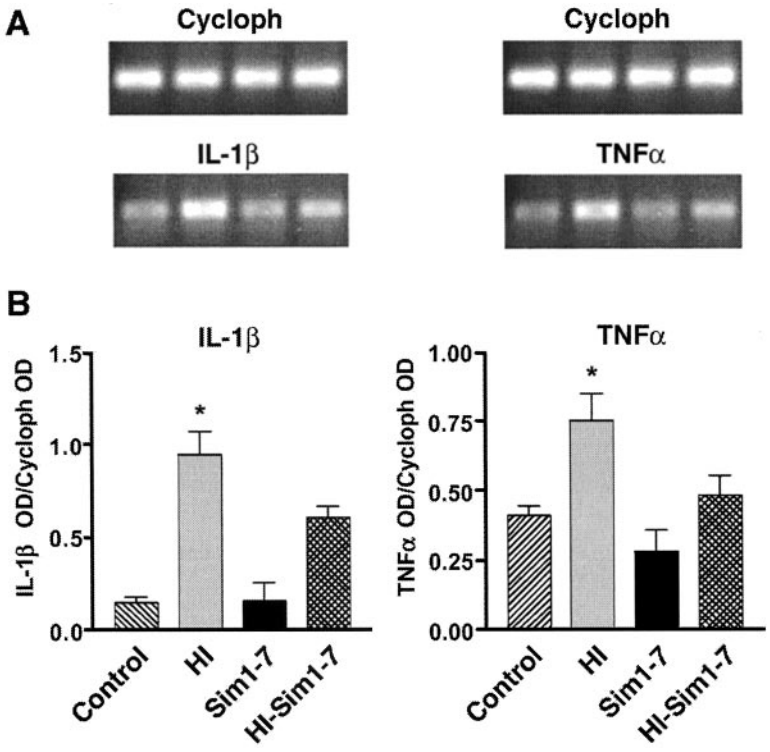

Figure 5. IL-1β and TNF-α mRNA expression in cerebral cortex 4 hours after HI. A, Representative gels obtained for cyclophilin (Cycloph), IL-1β, and TNF-α. B, Amount of cytokine mRNA is expressed as ratio of densitometric measurements (optical density [OD]) of samples to corresponding reporter gene. Data are mean±SE of 5 rats. Sim indicates simvastatin. *$P<0.05$ compared with control group.

Discussion

In the present study we have shown that prophylactic but not delayed administration of simvastatin, a statin that crosses the blood-brain barrier, significantly protects from brain damage and its behavioral consequences in a neonatal model of HI. In agreement with our previous work,[12] the neuroprotective effect of simvastatin was found to be long-lasting and to occur under conditions that resulted in severe tissue injury in most ischemic animals. Simvastatin did not induce any significant reduction in body weight since it did not differ from that observed in HI vehicle-treated animals and was completely recovered in the adult animals. Furthermore, neuroprotection occurred without a significant alteration in blood cholesterol levels measured both at the end of the treatment and at the adult age, indicating that neuroprotection is independent of the lipid effects of the drug.

The behavioral asymmetry and learning deficits observed in adult ischemic animals were significantly attenuated by 1-week pretreatment or 3-day pretreatment followed by a 4-day posttreatment with simvastatin but not when simvastatin was administered for 1 week immediately after the ischemic insult. These results are consistent with the finding that animals in the HI-simvastatin 1–7 and HI-simvastatin 4–11 groups, but not those in the HI-simvastatin 7–14 group, developed smaller infarctions and indicate that a prophylactic treatment is necessary to obtain neuroprotection. Our findings are in agreement with those previously reported in adult mice after middle cerebral artery occlusion and reperfusion showing that statins are effective only when administered before the ischemic insult.[5,15] In a recent study, however, using MRI techniques, we confirmed the preventive neuroprotective effect of statins after permanent middle cerebral artery occlusion in adult rats and reported that simvastatin was also protective when administered immediately after the ischemic insult.[16] The fact that the neuroprotective effect of simvastatin can be observed with different schedules of drug administration may be due to the different experimental models of HI used in which different mechanisms may be involved in the evolution of ischemic injury (mouse versus rat and transient versus permanent middle cerebral artery occlusion). This may be particularly important when adult and immature animals are compared because the temporal progression of the injury appears to be faster in the newborn than in the adult.[17] A question that still remains open and that deserves further investigation is the neuroprotective effect of a shorter schedule or a single prophylactic administration of the drug. Indeed, shortening the length of the treatment and decreasing the total amount of the drug would reduce the risk of toxic effects and make clinical use possible under conditions in which the risk of stroke is foreseeable and particularly high.

Several studies performed in adult animals reported that statins may exert their neuroprotective effect by increasing eNOS expression and activity in endothelial cells.[5,6,15] NO produced by eNOS, indeed, might orchestrate paracrine homeostatic functions of the endothelium such as inhibition of leukocyte and platelet adhesion and control of vascular tone. Herein, we have shown that in the cerebral cortex of immature rats, eNOS expression was increased in the damaged side of HI animals 24 hours after the insult and returned to control values after 5 days, suggesting that eNOS induction may represent a reactive response that probably plays a role in protecting tissue from additional ischemic damage. Treatment with simvastatin, however, was not able to further increase the expression of the protein, suggesting that, at least in immature animals, the neuroprotective effect may result from additional actions of the drug. Indeed, statins produce

pleiotropic effects, which are also mediated by NO production,[3,4,18] and it is possible that the relative contribution of these actions on their neuroprotective effect may change during brain maturation. The anti-inflammatory and immunomodulatory effects of statins, in particular, may play an important role in the neuroprotective activity in immature animals. There is evidence, indeed, that statins inhibit both acute and chronic inflammation probably by interfering with endothelial adhesion and transendothelial migration of leukocytes to sites of inflammation[2]. In primary astrocytes, microglia, and macrophages, statins were found to reduce the induction of inflammatory mediators such as TNF-α, IL-1β, and interleukin-6.[8] The expression of TNF-α and IL-1β, on the other hand, is increased after focal cerebral ischemia in the neonatal rat,[19] and pharmacological strategies aimed at reducing the activity of these pro-inflammatory cytokines attenuate tissue damage.[20] In the present study we found that the expression of mRNA for the 2 proinflammatory cytokines IL-1β and TNF-α was significantly increased in HI but not in HI-simvastatin 1–7 animals. Thus, in immature animals the anti-inflammatory properties of simvastatin could play an important role in its neuroprotective effect, resulting in reduction of infiltrating leukocytes and production of proinflammatory cytokines in the degenerating tissue. In this regard, it has recently been demonstrated that simvastatin attenuates leukocyte–endothelial cell interactions only after pretreatment with the drug,[21] and this may explain why, in our study, pharmacological treatment after HI was not neuroprotective.

In conclusion, the studies described in this report indicate that prophylactic but not delayed administration of simvastatin leads to an improvement in functional outcome in neonatal stroke. The reduced induction of IL-1β and TNF-α mRNA suggests that the neuroprotective effect of statins during brain development may be related to a dampening of the inflammatory response.

Most perinatal strokes are unforeseeable events. The finding that the effect of simvastatin can be detected only if the drug is administered before the ischemic insult suggests that statins may be helpful in conditions in which the risk is particularly high, such as in children undergoing cardiac surgery for congenital heart diseases. Experiments are in progress to assess the lowest dosage and the optimal schedule of simvastatin administration resulting in neuroprotection in immature rats.

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