Intravenous Rosuvastatin for Acute Stroke Treatment
An Animal Study

Vincent Prinz, MD; Ulrich Laufs, MD; Karen Gertz, MD; Golo Kronenberg, MD; Mustafa Balkaya, MSc; Christoph Leithner, MD; Ute Lindauer, DVM; Matthias Endres, MD

Background and Purpose—Statins exert rapid cholesterol-independent vasoprotective effects. Here, we tested whether postevent treatment with intravenously (i.v.) administered rosuvastatin improves acute stroke outcome in mice.

Methods—129/SV wild-type mice were subjected to 1-hour filamentous middle cerebral artery occlusion (MCAo), followed by reperfusion, and were postevent treated with i.v. or intraperitoneal (i.p.) rosuvastatin given up to 6 hours after MCAo (dose range 0.02 to 20 mg kg⁻¹ body weight).

Results—Rosuvastatin, when administered i.v., significantly reduced lesion size when given up to 4 hours after MCAo and in doses as low as 0.2 mg kg⁻¹. In contrast, i.p. administration provided protection only when given directly on reperfusion at a dose of 20 mg kg⁻¹ but not at lower doses or later time points. Lesion protection was evident as late as 5 days after brain ischemia and was associated with functional improvements in the pole-test and wire-hanging test (2.0 mg kg⁻¹ dose). Neuroprotection with i.v. rosuvastatin was achieved with peak plasma concentrations <0.5 ng ml⁻¹ (ie, with 0.2 mg kg⁻¹) and was associated with increased levels of phosphorylated Akt kinase and endothelial nitric oxide synthase in the vasculature.

Conclusions—Rosuvastatin, given intravenously at pharmacologically relevant concentrations, protects from focal brain ischemia up to 4 hours after an event. In our opinion, the development of an intravenous statin formulation is warranted for acute stroke trials with statins in humans. (Stroke. 2008;39:433-438.)

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

Statins, inhibitors of HMG-CoA reductase, are widely used cholesterol-lowering drugs and reduce the incidence of myocardial infarctions and stroke. Experimental and clinical evidence suggests that chronic statin treatment may also decrease stroke severity and improve outcome.¹ Statins may also provide stroke-protection when given after ischemia onset.² For rapid administration such as for acute stroke treatment, however, we propose the development of an intravenous formulation. Therefore, we tested the effects of intravenous versus intraperitoneal administration of rosuvastatin in a mouse model of focal cerebral ischemia/reperfusion.

Methods
Mice, Treatment, and Measurements of Rosuvastatin Plasma Levels
All experimental procedures conformed to institutional guidelines and were approved by an official committee (G0206/06, LaGeSo, Berlin, Germany). 129/SV wild-type mice (BfR, Berlin, Germany) aged 6 to 8 weeks were used for this study. Rosuvastatin (AstraZeneca) was dissolved in normal saline and administered intravenously (i.v.) or intraperitoneally (i.p.) in a total volume of 150 µL. In a subset of animals, blood was withdrawn for plasma preparation at 1, 3, or 5 hours after rosuvastatin administration and plasma samples were analyzed for rosuvastatin levels by an LC/MS/MS method.

Model of Cerebral Ischemia
Mice were anesthetized by 1.0% (vol/vol) isoflurane in 69% nitrous oxide (N₂O) and 30% oxygen (O₂) administered via a facemask. Focal cerebral ischemia was induced by 60-minute filamentous middle cerebral artery occlusion (MCAo) and reperfusion as described previously.³ Rectal temperature was controlled and kept constant at 36.5±0.5°C. Regional cerebral blood flow (rCBF) was measured by means of a flexible probe and laser-Doppler monitoring (Perimed). There was an equivalent drop in rCBF to less than 20% of baseline at filament insertion and an equivalent rise in rCBF at filament withdrawal. In randomly assigned animals, the left femoral artery was cannulated for arterial blood pressure monitoring and blood withdrawal. Arterial blood samples were analyzed for pH, arterial oxygen pressures, and partial pressure of carbon dioxide as described.³ After 24 hours or 5 days, animals were euthanized by a pentobarbital overdose, and brains were quickly removed from the skull and snap-frozen in isopentane on dry ice for cryostat sectioning. Direct and indirect lesion areas were quantified by computer-assisted volumetry on 20 µm hematoxylin-stained cryostat sections as described.³

Western Blot Analysis
Immunoblotting for endothelial nitric oxide synthase (eNOS, BD 610296), phosphorylated eNOS (BD 612706), phosphorylated Akt

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when lesion size was calculated with an indirect method to
3.1, 73.5
5.8 mm$^3$ (0.02, 0.2, and 2.0
4.5, and 69.9
79.5
82.4
3.1 mm$^3$ (vehicle) versus 68.3
2B). Infarct sizes measured with the indirect method were
provided protection up to 4 hours postevent (Figure 2A and
Rosuvastatin plasma levels at different time points after
injection of a single dose of rosuvastatin via the
intravenous injection of rosvastatin given up to 6 hours after MCAo (dose
Sensory-motor scores were determined as described (0
no deficit, 1
mild deficit, 2
moderate deficit, 3
severe deficit).3 Endurance
loading using the enhanced chemiluminescence kit (Amersham) was
in the wire-hanging test was measured on a horizontal steel wire
(1 mm) stretched horizontally 50 cm above a foam pad. For the pole
test the mouse was placed head upward on the top of a vertical
wooden rough-surfaced pole (diameter 1 cm, height 50 cm). Each
mouse was habituated on the day before testing then allowed to
descend 5 times on a single session. The total time needed to turn
completely head downward (“time-to-turn”) and the time until the mouse
reached the floor with its 4 paws (“time-to-come-down”) was
recorded. Results were expressed as the mean of the 5 trials.

Statistical Analysis
Data are presented as mean±SEM. Comparisons were made by
ANOVA followed by Tukey test or by Student t test as applicable.

Results
Rosuvastatin Plasma Levels
Rosuvastatin plasma levels at different time points after intravenous injection of a single dose of rosuvastatin via the
tail vein of male 129/SV mice are presented in Table 1.

Effect of Rosuvastatin Treatment on Cerebral Lesion Size After MCAo
Mice were subjected to 1-hour filamentous MCAo/reperfusion and were postoevent treated with i.v. or intraperitoneal
(i.p.) rosuvastatin given up to 6 hours after MCAo (dose range 0.02 to 20 mg kg$^{-1}$ body weight). When administered at 1 hour after ischemia onset, i.v. rosuvastatin provided protection in concentrations of 0.2 and 2.0 mg kg$^{-1}$ but not 0.02 mg kg$^{-1}$ (Figure 1A). Similar results were observed when lesion size was calculated with an indirect method to correct for brain swelling: ie, 90.8±4.3 mm$^3$ (vehicle) versus 79.5±3.1, 73.5±4.5, and 69.9±5.8 mm$^3$ (0.02, 0.2, and 2.0 mg kg$^{-1}$ rosvastatin, respectively). When administered intraperitoneally (i.p.), rosuvastatin provided protection only when administered at 20 mg kg$^{-1}$ but not with lower doses (ie, 2.0 or 0.2 mg kg$^{-1}$; Figure 1B).

To determine the time-window of protection, rosvastatin was administered at 3, 4, and 6 hours after MCAo. When administered intravenously at 0.2 or 2.0 mg kg$^{-1}$, rosuvastatin provided protection up to 4 hours postevent (Figure 2A and 2B). Infarct sizes measured with the indirect method were 82.4±3.1 mm$^3$ (vehicle) versus 68.3±6.6, 63±5.4, 75.6±6.7, and 80.6±3.8 mm$^3$ (0.2 mg kg$^{-1}$ given at 1, 3, 4, and 6 hours, respectively) versus 68.4±6.7, 65.8±8.3, and 72.1±4.0 mm$^3$ (2.0 mg kg$^{-1}$ rosuvastatin given at 1, 4, and 6 hours, respectively). When given at 6 hours, 0.2 mg kg$^{-1}$ was ineffective whereas 2.0 mg kg$^{-1}$ provided significant protection when calculated by the indirect method ($P<0.05$).

In addition, we compared whether the treatment window was different between the i.p. and i.v. formulations. Therefore, groups of animals received 2.0 or 20 mg kg$^{-1}$ rosvastatin given i.p. or 2.0 mg kg$^{-1}$ given i.v. at 3 hours after MCAo. As demonstrated in Figure 3, high-dose i.p. rosvastatin did not provide protection at 3 hours as did the low-dose i.v. formulation. Similar results were obtained with the indirect method: 92.8±3.4 mm$^3$ (vehicle) versus 99.6±7.0 mm$^3$ (2.0 mg kg$^{-1}$ i.p.) versus 91.4±5.1 mm$^3$ (20.0 mg kg$^{-1}$ i.p.) versus 72.2±5.2 mm$^3$ (2.0 mg kg$^{-1}$ i.v.).

Physiological Parameters
We carefully measured physiological parameters after 1 hour in selected mice subjected to MCAo and treated with vehicle
versus 2.0 mg kg$^{-1}$ rosvastatin. Overall, there were no differences in mean arterial blood pressure, heart rate (not shown), $P_{O_2}$, $P_{CO_2}$, pH, or rectal temperatures after rosuvastatin administration compared with vehicle-treated animals (Table 2).

Effect of Rosuvastatin Treatment on Functional Deficits and Lesion Size at Day 5
In addition, we tested whether i.v. statin treatment had an effect on functional deficits. Mice were treated with rosvu-
astatin 2 mg kg⁻¹ or vehicle given i.v. at 3 hours after a 1-hour MCAo (n=10 per group), and treatment was continued over 5 days (daily i.p. injections; 2 mg kg⁻¹). Although all animals had a moderate (ie, grade 2) deficit in the Bederson score directly after reperfusion, rosuvastatin-treated mice had less severe functional deficits on day 5 with a median score of 1.0 (1.0 and 2.0 for 25% and 75% confidence intervals) corresponding to a mild deficit compared with vehicle-treated animals with median scores of 2.0 (1.75 and 2.0 for 25% and 75% confidence intervals) corresponding to a moderate deficit. In addition, rosuvastatin-treated animals had significantly better performance in the pole test (Figure 4A and 4B) and also longer endurance in the wire-hanging test (Figure 4C). Moreover, lesion size at day 5 was also significantly reduced in rosuvastatin treated animals with median scores of 2.0 (1.75 and 2.0 for 25% and 75% confidence intervals) corresponding to a mild deficit compared with vehicle-treated animals with severe functional deficits on day 5 with a median score of 1.0 (1.0 and 2.0 for 25% and 75% confidence intervals) corresponding to a moderate deficit.

In addition, rosuvastatin-treated animals had significantly better performance in the pole test (Figure 4A and 4B) and also longer endurance in the wire-hanging test (Figure 4C). Moreover, lesion size at day 5 was also significantly reduced in rosuvastatin treated animals with median scores of 2.0 (1.75 and 2.0 for 25% and 75% confidence intervals) corresponding to a mild deficit compared with vehicle-treated animals with severe functional deficits on day 5 with a median score of 1.0 (1.0 and 2.0 for 25% and 75% confidence intervals) corresponding to a moderate deficit.

**Discussion**

This study has the following major findings: rosuvastatin when given as an intravenous formulation provides protection phosphorylated, ie, activated, Akt-kinase (phospho-Akt) were quantified by immunoblotting in the aorta at different time points after administration of i.v. rosuvastatin. Significant increases in eNOS and phospho-eNOS were observed with 2.0 mg kg⁻¹ rosuvastatin and of phospho-Akt with 0.2 and 2.0 mg kg⁻¹ rosuvastatin as early as 1 hour after administration (Figure 5). In addition, rosuvastatin i.v. also increased eNOS, phospho-eNOS, and phospho-Akt in cerebral vascular beds: immunoblots from brain tissue lysates revealed a significant (P<0.05) increase of eNOS (to 160% after 3 hours and 17% after 5 hour) in mice treated with 2.0 mg kg⁻¹ rosuvastatin i.v. versus vehicle (n=4 animals per group).

**Table 2. Physiologic Parameters in 129/SV Mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>Rosuvastatin [2.0 mg kg⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td>140±4</td>
<td>134±8 (ns)</td>
</tr>
<tr>
<td>pH</td>
<td>7.28±0.03</td>
<td>7.28±0.02 (ns)</td>
</tr>
<tr>
<td>P_{CO₂}, mm Hg</td>
<td>47±2</td>
<td>49±2 (ns)</td>
</tr>
<tr>
<td>P_{O₂}, mm Hg</td>
<td>131±9</td>
<td>134±10 (ns)</td>
</tr>
<tr>
<td>CT, °C</td>
<td>36.7±0.2</td>
<td>36.9±0.3 (ns)</td>
</tr>
</tbody>
</table>

*Animals were subjected to 60 minutes filamentous middle cerebral artery occlusion (MCAo) followed by reperfusion. Rosuvastatin was administered i.v. 1 hour after ischemia onset. MABP (mean arterial blood pressure in mm Hg) was measured until 2 hours after treatment. Fifty microliters of blood were withdrawn during cerebral ischemia for blood gas determination (pH, P_{CO₂}, P_{O₂}). Rectal (core) temperature (CT in °C) was controlled and kept constant by means of a feedback temperature-control unit; ns=not significant; n=9 animals per group.*
from focal brain ischemia/reperfusion in the mouse when given as late as 4 hour after ischemia in doses as low as 0.2 mg kg\(^{-1}\). The stroke protective effects of i.v. rosuvastatin extended to 5 days after ischemia and were accompanied by functional improvements as determined by a number of functional tests (Bederson score, wire hanging test, pole test). Neuroprotection with i.v. rosuvastatin was achieved with peak plasma concentrations lower than 0.5 ng ml\(^{-1}\) (ie, with the 0.2 mg kg\(^{-1}\) dose), and was associated with increased levels of phosphorylated Akt kinase and phosphorylated eNOS in the vasculature. In contrast, intraperitoneal administration provided protection only when given as early as 1 hour after ischemia onset and at a dose of 20 mg kg\(^{-1}\).

Statins exert pleiotropic effects on the vascular wall that may occur rapidly before any effect on cholesterol levels.\(^1\) We have previously demonstrated that the stroke protective effects of chronic statin treatment are—at least in part—mediated by upregulation of eNOS in the vascular wall.\(^1,3\) In addition to more delayed upregulation of eNOS expression, statins may directly activate eNOS via protein kinase Akt.\(^6\) Activation of phosphatidylinositol 3 (PI3)-kinase mediates the phosphorylation and activation of Akt, and PI3 inhibitors block the effects of statins on Akt.\(^5\) Increased bioavailability of endothelial-derived NO improves endothelium function, increases cerebral blood flow, inhibits platelet aggregation, and exerts antiinflammatory effects, which may all contribute to the protective effects of rosuvastatin.\(^1\) We have previously demonstrated that stroke-protective effects after chronic statin treatment are completely absent in animals deficient in eNOS expression (ie, eNOS knockout mice).\(^3\) It would be interesting to determine whether acute protective effects after intravenous statin treatment are also absent in eNOS knockout mice. Recently, it has also been demonstrated that statins activate AMP-activated protein kinase which may additionally contribute to eNOS activation.\(^6\) In this study, intravenous administration of rosuvastatin conferred increased phosphorylation of Akt-kinase within 60 minutes at doses of 0.2 mg

Figure 4. Functional deficits and lesion size at day 5. Mice were subjected to 1-hour MCAo/reperfusion and treated with rosuvastatin (2.0 mg kg\(^{-1}\)) or vehicle intravenously at 3 hours. On day 5, animals were exposed to functional analysis in the pole test (A and B) and wire-hanging test (C). Thereafter, animals were euthanized and direct ischemic lesion volumes determined on coronal brain cryostat sections by computer-assisted volumetry. n=10 animals per group. Mean±SEM; *P<0.05 vs vehicle.

Figure 5. Expression of aortic eNOS, phospho-eNOS, and phospho-Akt after rosuvastatin treatment. Expression of endothelial nitric oxide synthase (eNOS; A), phosphorylated (phospho)-eNOS (B), phosphorylated (phospho)-Akt (C) in aortas of 129/SV wild-type mice standardized to β-actin determined by immunoblots. Animals were euthanized and aortas quickly snap-frozen at 1, 3, or 5 hours after a single intravenous injection of rosuvastatin (dose range 0.02 to 2.0 mg kg\(^{-1}\)). n=5 animals per group. Mean±SEM; *P<0.05 vs vehicle.
kg\(^{-1}\) and higher. Interestingly, it should be noted that the 0.2 mg kg\(^{-1}\) dose of rosuvastatin provided neuroprotection and increased phospho-Akt but not phospho-eNOS or eNOS. Moreover, the 0.02 mg kg\(^{-1}\) dose, which failed to increase phospho-Akt, also did not confer protection, supporting the concept that Akt activation correlates with acute protection in the stroke model. Importantly, the effects of rosuvastatin on eNOS and Akt-kinase were not limited to peripheral arteries but were also evident in the cerebrovasculature.

Chronic statin treatment provides protection in animal models of stroke particularly when given at high systemic concentrations.\(^1\)\(^-\)\(^7\) Also, postevent treatment may be effective, however only when administered at high doses soon after MCAo.\(^8\)\(^-\)\(^10\) For example, Kilic and coworkers demonstrated that i.p. rosuvastatin was only effective at 20 mg kg\(^{-1}\) and not lower doses and only when administered directly after reperfusion after MCAo.\(^8\) Moreover, postevent treatment with atorvastatin (administered subcutaneously) exerts neuroprotective effects when administered 4 hours after stroke but only in combination with tissue-type plasminogen activator and not alone.\(^10\) In this study, we demonstrate that the therapeutic window for acute stroke treatment with rosuvastatin can be extended significantly (ie, by at least 3 hours) and the effective dose significantly reduced (ie, by 2 orders of magnitude) with an intravenous formulation.

Statins are ordinarily metabolized extensively in the liver through the first-pass mechanism after oral administration. Data in the rat and human indicate that rosuvastatin is not extensively metabolized and in the main is fecally excreted predominantly as active parent compound.\(^11\) What little metabolism there is yields less potent metabolites. Cerebral exposure for rosuvastatin is low because of its hydrophilicity, and any direct protective effects are likely via effects on the cerebral vasculature (see above). It is presently not entirely clear how i.v. administration bypasses this and whether direct effects on the brain are more important for neuroprotection than effects mediated through liver metabolism. Neuroprotection with i.v. rosuvastatin was achieved with very low peak plasma concentrations smaller than 0.5 mg ml\(^{-1}\). Also, there appeared to be a nonlinear relationship of drug levels with increasing doses. This, however, is most likely attributable to the limit of detection. It has been demonstrated that liver-derived mevalonate levels fall dramatically after administration of statins.\(^1\)\(^1\) Thus, if peripheral cells, including cerebral vascular endothelial cells, are dependent on circulating mevalonate for production of isoprenoids, this might explain some, if not all, of the pleiotropic effects seen at very low circulating drug concentrations. The liver is a sink for statins, particularly rosuvastatin, which is both an intrinsically potent statin and actively removed by the liver by an active transport mechanism.

In our study, we did not measure plasma levels after i.p. administration; however, these measurements were performed in the Kilic study using the same detection method.\(^8\) Indeed, rosuvastatin plasma levels after i.p. administration were much higher than after i.v. administration and found to have linear pharmacokinetics: after administration of 0.5, 5, and 20 mg kg\(^{-1}\) plasma drug levels were 263.5, 1559.6, and 2231.8 ng ml\(^{-1}\) at 30 minutes (equating to 0.527, 1.56, and 4.46 \(\mu\)mol/L, respectively). The differences in the plasma drug levels may reflect differences in drug handling after i.v. or i.p. dosing, and the drug may be cleared and concentrated in the liver much more rapidly after i.v. dosing compared with i.p. Therefore, for i.p. dosing there remains a significant plasma drug level, whereas for i.v. the drug is in the effect compartment (ie, liver). This might also fit the hypothesis of hepatic-mediated peripheral effects (see above).

A number of retrospective studies indicate that statin medication before ischemic stroke is associated with a favorable outcome.\(^10\) Recently, a secondary analysis of data from the Stroke Prevention With Aggressive Reduction in Cholesterol Levels (SPARCL) trial showed that patients had a less severe recurrent stroke if they were receiving treatment with 80 mg atorvastatin within the month before the event, compared with those not on treatment.\(^12\) Also, there is preliminary evidence that acute administration of statins in stroke patients may be beneficial: the Markers of Inflammation after Simvastatin in Ischemic Cortical Stroke (MISTICs) trial demonstrated improved neurologic outcome at 90 days in patients treated with simvastatin 40 mg orally within 3 to 12 hours after stroke onset.\(^13\)

In conclusion, we propose the development of an intravenous statin formulation for clinical use. Intravenous formulations of hydrophilic statins such as rosuvastatin that are water soluble are pharmacologically feasible. Indeed, 8 mg rosuvastatin was safely administered intravenously in healthy volunteers, and this dose directly corresponds to the dose used in this study to reduce ischemic brain damage in mice.\(^14\)

There is evidence that in patients with acute vascular syndromes or those undergoing cardiovascular surgery discontinuation of statin medication may be associated with impaired vascular function and worse outcome.\(^15\) Therefore, in addition to treatment trials for acute ischemic stroke, intravenous statins could be used in patients previously treated with statins who cannot be treated with statins per os or even via nasogastric tube (eg, unable to swallow, intensive care patients, before major surgery).

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References


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