Rosuvastatin, but not Simvastatin, Provides End-Organ Protection in Stroke-Prone Rats by Antiinflammatory Effects

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Objective—Brain abnormalities, preceded by a systemic inflammation, develop in spontaneously hypertensive stroke-prone rats (SHRSP). In this model, we investigated whether the hydrophilic statin, rosuvastatin, influences the development of inflammation associated with brain abnormalities. Because differences in hydrophilicity/hydrophobicity contribute to the differences in statin pharmacology, we also evaluated the effects of simvastatin, a lipophilic molecule

Methods and Results—SHRSP, fed a high-salt diet, were treated long-term with vehicle or rosuvastatin (1 and 10 mg/kg per day). Brain abnormalities developed after 40±5 days and after 60±5 days of salt loading, in vehicle-treated and in rosuvastatin-treated (1 mg/kg per day) SHRSP, respectively. After 100 days of treatment, no damage was detectable in 30% of the rats treated with the highest dose of the drug. In comparison with vehicle-treated SHRSP, rosuvastatin treatment attenuated the transcription of monocyte chemoattractant protein-1, transforming growth factor-β1, IL-1β, and tumor necrosis factor-α in the kidney, and of P-selectin in brain vessels and increased the transcription of endothelial nitric oxide synthase mRNA in the aorta. Urinary excretion of acute-phase proteins increased with time in vehicle-treated animals but remained negligible in drug-treated animals. These effects are independent of changes in physiological parameters. Treatment of SHRSP with simvastatin (2 to 20 mg/kg per day) did not exert any protective effect.


Key Words: inflammation ■ statins ■ rats ■ brain ischemia ■ proteome ■ rosuvastatin ■ simvastatin ■ stroke-prone rats

Inflammatory processes accompany tissue injury no matter what organ is involved. Data suggest that inflammation predisposes to ischemic vascular disease in general and to stroke in particular, because patients with chronic inflammatory disorders or with chronic or acute infection are at high risk for stroke.1 High blood levels of inflammatory markers are associated with increased cardiovascular risk in healthy populations and in patients with coronary heart disease and ischemic stroke.2 Moreover, in patients with an acute ischemic neurological event, a strong inflammatory response at the time of admission is associated with a less favorable outcome.3 The inflammatory reaction therefore presents an attractive pharmacological opportunity for novel approaches in stroke. The spontaneously hypertensive stroke-prone rat (SHRSP) provides a useful tool to evaluate the contribution of inflammation to brain injury and to answer the question of whether antiinflammatory strategies may affect the genesis, progression, and outcome of brain damage.4 We previously reported that SHRSP subjected to salt loading have an inflammatory condition characterized, before the appearance of brain damage, by the accumulation in plasma and urine of several acute-phase proteins (APP), including thiostatin, the most typical marker of an inflammatory response in the rat.4 Serum and urine levels of this protein were found to be predictive of the appearance of brain abnormalities in SHRSP.4 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, or statins, the most widely used cholesterol-lowering drugs, significantly reduce the incidence of ischemic stroke in patients with and without high-serum cholesterol levels.5 In addition to delaying atherosclerosis, statins inhibit a number of inflammatory processes known to be important during brain damage.6 These drugs reduce the in vivo induction of inflammatory mediators, such as monocyte chemoattractant protein (MCP-1), IL-1β, and tumor necrosis...
factor (TNF)-α, inhibit endothelial surface expression of cell adhesion molecules and decrease oxidative stress.7–10 These effects are independent of changes in cholesterol levels and are reversible by simultaneous treatment with mevalonate or geranylgeranyl pyrophosphate.6,10 These observations support the hypothesis that statins represent a novel means of attenuating inflammatory processes associated with cerebrovascular damage. Moreover, new evidence points to differences among statins in lipid-unrelated effects on the inflammatory cascade.10,11 In the present study, we have evaluated the effects of rosuvastatin, a hydrophilic statin, and of simvastatin, a lipophilic statin, on the inflammatory processes in SHRS on occurring during the onset and evolution of spontaneous brain damage. Differences in the hydrophilic/hydrophobic characteristics of statins contribute to the differences in their pharmacokinetics and pharmacodynamics, as well as possible beneficial or harmful pleiotropic effects.12 Whereas the neuroprotective effects of lipophilic statins have been extensively investigated, only limited data are available on these effects for hydrophilic statins in vivo.

Materials and Methods

Animals and Protocol
Male SHRS (n = 95), from different litters born in a period of 3 to 4 days and randomly pooled at the age of 4 weeks were obtained from Charles River, Italy (Calco, Lecco). Procedures involving animals and their care were conducted in conformity with this department’s guidelines, which comply with national and international rules and policies. Rosuvastatin was supplied by AstraZeneca, UK. Baseline measurements were performed in all rats at 6 weeks of age. All animals were then immediately switched to a specific permissive diet low in potassium and protein and high in sodium (Japanese permissive diet [JPD]; Laboratorio Dr Piccioni, Gessate, Italy; 18.7% protein, 0.63% potassium, 0.37% sodium) and received 1% NaCl in drinking water. Simultaneously with the start of salt loading, 40 SHRS were divided into 4 groups and treated by gavage, once per day, with vehicle (0.5% deionized water), simvastatin (10 mg/kg per day), or rosuvastatin (10 mg/kg per day) after proteinuria exceeded 40 mg/d. The selected doses of rosuvastatin were dissolved in 4 groups and treated by gavage, once per day, with vehicle (0.5% sodium carboxymethylcellulose, n = 10) or with 1 of 3 doses of simvastatin (2, 10, and 20 mg/kg per day; n = 10 per group). The vehicle and method of administration differed from rosuvastatin because of the inability to solubilize the lipophilic simvastatin. Because the median effective doses (ED50s) of hepatic cholesterol synthesis are 0.8 mg/kg and 1.2 mg/kg for rosuvastatin13 and simvastatin,13 respectively, the doses of the 2 statins we used in this study are comparable. Every week, all the rats were weighed and their systolic arterial blood pressure was measured by means of tail-cuff plethysmography (PB Recorder 8006; Ugo Basile); they were then housed individually in metabolic cages for 24 hours for measurement of food and liquid intake and collection of urine. Blood was drawn every week from the tail vein; serum was obtained and stored at −20°C until analyzed. The 24-hour urine protein concentrations were measured according to Bradford, with bovine albumin as a standard. The SHRS underwent magnetic resonance imaging (MRI) every week until 24-hour proteinuria exceeded 40 mg/d, then every other day until brain damage was manifest, and then every day for the next 3 days. After the last MRI session, the animals were euthanized, and the brains, aortas, and kidneys were collected for subsequent analysis; at the average time-point (6 weeks of dietary treatment) of the appearance of brain damage in controls, a group of rats treated with rosuvastatin (10 mg/kg per day; n = 5) were euthanized for tissue collection. Survival was evaluated by following-up some animals (≥3) for each group until their spontaneous death.

Proteome Studies
Urine proteins were concentrated by trichloroacetic acid–acetone precipitation. One-dimensional electrophoresis was performed on urine proteins in the presence of SDS, without sample reduction, in a discontinuous buffer system on polyacrylamide gradients 4% to 20% T. The sample load was 3.75 μg per lane. Two-dimensional electrophoresis maps were obtained by the immobilized pH gradient Dalt method.14 Sample proteins, reduced with 2% 2-mercaptoethanol, were first resolved according to charge on a nonlinear pH 4 to 10 immobilized pH gradient in the presence of 8 mol/L urea and 0.5% carrier ampholytes, with an anode-to-cathode distance of 8 cm. The focused proteins were then fractionated according to size by SDS-PAGE on 7.5% to 17.5% polyacrylamide gradients, with 2 immobilized pH gradient strips mounted on each 160 × 140-mm2 SDS slab. Sample load was 2 μL of serum. Proteins were stained with 0.3% weight/volume Coomassie. The protein patterns were digitalized with a scanner. Spot volumes in 2-DE maps were quantified with the software PDQUEST version 5.1 (PDI, Huntington Station, NY).

Evaluation of Brain Damage by MRI
Rats were anesthetized with 2% isoflurane in 70% N2/30% O2, and placed in the magnet (4.7 T, vertical 15-cm bore) of a Bruker spectrometer (AMX3 with microimaging accessory). A 6.4-cm-diameter birdcage coil was used to acquire a T2-weighted multislice image. Sixteen contiguous 128 × 128-point 1-mm-thick slices were analyzed caudally to the olfactory bulb; field of view was 4 × 4 cm2. Turbo spin-echo sequence was used with 16 echoes per excitation, 10 ms interecho time, 85 ms equivalent echo time, and 4-second repetition time. Lesions were identified as hyperintense areas on T2-weighted MRI. The damage, visualized with MRI, was confirmed by light microscopy analysis on the brains stained with hematoxylin/eosin.

Histology and Immunohistochemistry
For histological examination, the brains were fixed in Carnoy reagent (Merck, Darmstadt, Germany) and embedded in Paraplast (Sigma, St. Louis, Mo), and coronal sections (5 μm) were stained with hematoxylin/eosin and examined by light microscopy. For immunohistochemical studies, paraffin-embedded brain coronal sections were dewaxed in xylene and dehydrated, heated in 10 mmol/L sodium citrate buffer (pH 6.0) at 95°C for 5 minutes, and cooled in deionized water. The sections were incubated overnight at 4°C with the primary antibodies (anti–P-selectin CD62P; DAKO, Glostrup, Denmark), then with biotinylated secondary antibodies and streptavidin peroxidase (LSAB2 kit; DAKO, Glostrup, Denmark). Horseradish peroxidase was detected with H2O2 and diaminobenzidine (Sigma). Quantitative evaluation of vessels positive for P-selectin immunoreactivity was performed by computer-assisted image analysis (OPTIMAS 6.2; Media Cybernetics, Silver Spring, Md) on representative microphotographs taken with a Nikon digital camera (Coolpix 990) of 20 coronal brain sections from 5 control and 5 rosuvastatin-treated (10 mg/kg per day) rats. Measurements were performed blind, by 3 independent observers, on 3 fields (each area equaling 250 × 250 μm) in the different coronal brain slices; the number of P-selectin–positive vessels, expressed as percentage of total vessels counted per field, was evaluated.

Analysis of mRNA for MCP-1, Transforming Growth Factor-β1, IL-1 β, TNF-α, and Endothelial Nitric Oxide Synthase
Total RNA was prepared by guanidium thiocyanate denaturation from frozen kidneys and aortas collected from vehicle-treated rats (n = 5) euthanized when MRI analysis first detected brain abnormal-
ities and from rosuvastatin-treated rats (10 mg/kg per day; n=5) euthanized at the same time point. The transcription of mRNA for MCP-1, transforming growth factor-β1 (TGF-β1), IL-1β (IL-1β), and TNF-α was evaluated by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) as previously described.15,16 Transcription of endothelial nitric oxide synthase (eNOS) mRNA was evaluated as previously described,17 with minor modifications (our cycle conditions: 38 cycles, 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute). The RT-PCR products were separated on 1.5% agarose gel and visualized by staining with ethidium bromide. The intensity of each band was quantified using the corresponding GAPDH signal amplified as a standard.

### Cholesterol and Triglyceride Assay

Total serum cholesterol and triglycerides were quantified by enzymatic reaction with a commercially available kit (Sigma).

### Statistical Analysis

Differences between groups were computed by ANOVA for repeated measurements, followed by Bonferroni post-hoc test. Differences among intensities of RT-PCR products were analyzed using a 2-tailed Student t test and ANOVA followed by Tukey test. The overall differences in survival rate of vehicle- and drug-treated rats were determined by survival analysis, and the probability values were determined by the log-rank (Mantel–Cox) test. Data are expressed as mean±SD and significant differences were assumed at P<0.05.

### Results

#### Effects of Rosuvastatin on Physiological Parameters of SHRSP

SHRSP fed JPD and exposed to 1% NaCl had severe hypertension that was not affected by treatment with 1 or 10 mg/kg per day rosuvastatin (Figure 1, available online at http://atvb.ahajournals.org). In control rats, body mass decreased immediately before the appearance of brain abnormalities, whereas in rats treated with rosuvastatin, body weight increased until the end of the experimental period (Figure 1B). The treatment did not affect either plasma cholesterol or triglyceride levels (86±5 and 54.64±5 mg/dL, n=8, in rats treated with vehicle, and 81±2 mg/dL and 51.05±2, n=8, in rats treated with 10 mg/kg per day rosuvastatin, respectively).

#### Effects of Rosuvastatin on Proteinuria

Control animals showed a progressive increase of 24-hour proteinuria, which increased sharply and linearly after 5 weeks of salt loading, averaging 272±22 mg/d at week 7 of diet. In rats treated with 1 mg/kg per day rosuvastatin, proteinuria increased later, with a peak maximum of 220±21 mg/d just before the appearance of brain abnormalities (Figure 1A). At 10 mg/kg per day rosuvastatin, proteinuria increased smoothly, reaching only 130±33 mg/d after 14 weeks of treatment (Figure 1A). The identity/assortment of proteins excreted by untreated SHRSP (Figure 2, left) and by those given 1 mg/kg per day rosuvastatin (Figure 2, right) was compared by running the urine on 1-dimensional gels. In both cases, high-molecular-weight proteins, mainly albumin and transferrin, were first evident in detectable amounts between 3 and 4 weeks before the development of brain abnormalities, although this damage was delayed by rosuvastatin treatment; major urinary protein, the main component in control urine, was drastically decreased at the last sampling time, when brain damage had occurred.

#### Rosuvastatin Delays the Appearance of Brain Abnormalities

SHRSP in the control group (group 1) had cerebral lesions 40±5 days after JPD was started. Treatment with rosuvastatin significantly delayed the appearance of brain dam-

![Figure 1. Daily proteinuria (A) and event-free survival (B) as a function of the duration of JPD, for SHRSP not receiving medication n, SHRSP treated with 1 mg/kg per day rosuvastatin, and SHRSP treated with 10 mg/kg per day rosuvastatin (P<0.05; **P<0.01; vehicle-treated rats versus 1 mg/kg per day; §P<0.05; §§P<0.01; vehicle-treated rats versus 1 mg/kg per day). Representative MRI images (C) showing comparable coronal slices from SHRSP at the start of salt-loading (1) from vehicle (2) and rosuvastatin-treated rat (1 mg/kg per day) 3 days after the first appearance of brain abnormalities, and from rosuvastatin-treated rat, (10 mg/kg per day) after 14 weeks of dietary treatment. The arrows point to the site of brain lesions.

![Figure 2. One-dimensional electrophoresis of urinary proteins, collected weekly, from an SHRSP not receiving medication (left) and from an SHRSP receiving 1 mg/kg per day rosuvastatin (right). Aliquots of 3.75 µg from a 24-hour collection in metabolites were loaded per lane. MUP indicates major urinary protein; SA, albumin; Tf, transferrin. The patterns and the trends are representative of all the animals undergoing treatment.](http://atvb.ahajournals.org)
The appearance of brain damage invariably preceded the death of the animal by 10±4 days.

**Effects of Rosuvastatin on Inflammatory Proteins**

High levels of thiostatin (α1-major acute phase protein) were observed in the serum of untreated rats after 4 weeks of JPD (Figure 3), whereas much smaller amounts of this inflammatory marker were found in the serum of SHRSP treated with 1 mg/kg per day rosuvastatin, 4 and 7 weeks after the start of the dietary treatment (Figure 3). The spot volumes of thiostatin were 267±83, 1918±162, 184±38, and 501±109 arbitrary units in the 4 samples. To address the question of whether the statin was able to reverse the increase in proteinuria, SHRSP subjected to JPD (n=20) were treated with rosuvastatin (10 mg/kg per day) in the drinking water after proteinuria was >40 mg/d. In this latter group of animals, rosuvastatin failed to affect the increase in proteinuria induced by salt loading or the animals’ survival (39±7 days and 42±5 days for control and rosuvastatin-treated rats, respectively; data not shown).

**Transcription of mRNA for MCP-1, TGF-β1, IL-1β, TNF-α, and eNOS**

In vehicle-treated rats, a consistent renal accumulation of mRNA for MCP-1, TGF-β1, IL-1β, and TNFα was detected by RT-PCR. The treatment with rosuvastatin significantly attenuated the transcription of the 4 mRNA investigated. In particular, in the drug-treated rats, the transcription of mRNA for MCP-1 and TGF-β1 was completely inhibited whereas for IL-1β and TNF-α mRNA, a reduction of 58% (P<0.001, n=5) and 81% (P<0.01, n=5) were recorded (Figure 4). At the level of the aorta, the bands corresponding to eNOS mRNA were only barely visible in the vehicle-treated rats, whereas a strong signal was visible in the statin-treated animals. In particular, in the drug-treated rats, the transcription increased by 240% (P<0.001, n=5) in comparison with the vehicle-treated rats (Figure 4).

**P-Selectin Expression**

Because previous studies have suggested that rosuvastatin exerts antiinflammatory effects via inhibition of P-selectin expression on endothelial cells, we compared P-selectin immunoreactivity in the brain of rats treated with vehicle or rosuvastatin and euthanized after the first detection of brain abnormalities in vehicle-treated animals. Figure 5B shows that brain slices from vehicle-treated rats were immunonegative for P-selectin around brain vessels throughout the brain. Rosuvastatin treatment (10 mg/kg per day; Figure 5A) fully prevented the expression of P-selectin in salt-loaded SHRSP, as reported in Figure 5C.

**Effects of Simvastatin in Salt-Loaded SHRSP**

The severe hypertension and the proteinuria typical of SHRSP subjected to salt loading were not influenced by the chronic treatment with simvastatin in a dose range of 2 to 20 mg/kg per day (Figure IIA, available online at http://atvb.ahajournals.org). All the rats treated with vehicle had cerebral lesions 44±8 days after salt loading was started. Treatment with simvastatin failed to delay the appearance of brain damage: on 2, 10, and 20 mg/kg per day the rats had brain damage after 42, 41±7, and 37±9 days, respectively (Figure IIB). The appearance of brain damage invariably preceded the death of the animal by 7±4 days, regardless of treatment regimen. Furthermore, simvastatin has no effect on the inflammatory mediators mRNA synthesis at the level of the kidney (Figure IIC).

**Discussion**

We show here that rosuvastatin, at the doses that have been shown to exert vascular and cardioprotective effects in vivo, delays the appearance of brain damage and prolongs survival in salt-loaded SHRSP. These effects are independent of changes in cholesterol levels or other physiological parameters, such as blood pressure, but are linked to the attenuation of the inflammatory condition.
occuring in these animals both at local and systemic levels. In particular, rosuvastatin prevents the accumulation of inflammatory mediators, and of thiostatin in particular, in the urine and serum of salt-loaded SHRSP, as detected by 1-dimensional and 2-dimensional electrophoresis. The accumulation of APP in the serum of these animals represents the body’s overall response to a local inflammation. Epidemiological and experimental studies indicate that the synthesis of APP is rapidly upregulated in hepatocytes under the control of inflammatory mediators, mainly cytokines, originating at the site of persistent inflammation. Although no definitive evidence has been collected on the tissues/organ where the inflammatory status first develops in SHRSP, data demonstrate that soon after the start of salt-loading, kidney damage develops in these animals both at local and systemic levels. In particular, rosuvastatin prevents the accumulation of inflammatory mediators, and of thiostatin in particular, in the urine and serum of salt-loaded SHRSP, as detected by 1-dimensional and 2-dimensional electrophoresis. The accumulation of APP in the serum of these animals represents the body’s overall response to a local inflammation. Epidemiological and experimental studies indicate that the synthesis of APP is rapidly upregulated in hepatocytes under the control of inflammatory mediators, mainly cytokines, originating at the site of persistent inflammation. Although no definitive evidence has been collected on the tissues/organ where the inflammatory status first develops in SHRSP, data demonstrate that soon after the start of salt-loading, kidney damage develops in these animals, paralleled by the initiation of an inflammatory process. These phenomena may represent the initial insult triggering the synthesis of APP that, in fact, appear in the serum of SHRSP a few days after the start of salt loading. The data reported in this article show that rosuvastatin treatment prevents the inflammatory process at the level of the kidney, as indicated by the attenuation of mRNA synthesis for inflammatory mediators (TNF-α, TGF-β1, IL-1β, and MCP-1), a mechanism that may be related to the reduced APP accumulation in the body fluids of SHRSP. In humans, the most sensitive marker of inflammatory conditions is C-reactive protein (CRP), the level of which increases 10-fold within a few hours of a noxious stimulus; CRP has been shown to be a strong predictor of myocardial infarction and stroke. A number of clinical trials have shown the ability of statins to lower CRP levels after short and long periods of treatment. However, the set of acute-phase proteins differs between species. In the rat, CRP is only marginally affected during early inflammation, but there is a dramatic increase in thiostatin (also called α1-major acute-phase protein), a species-specific thiol protease inhibitor, as well as in α2-macroglobulin. Inflammation may act together with hypertension and the abnormal structure of the vessel wall in SHRSP to effect vessel, specifically endothelium, impairment. Much evidence indicates that the normally tight blood–brain barrier is weakened by pathological conditions such as infection, stroke, and multiple sclerosis. This alteration results in passage of blood proteins and even inflammatory cells into the brain, with detrimental consequences for the tissues. The chronic treatment of SHRSP with rosuvastatin increases transcription of eNOS mRNA. The enzyme is able to promote multiple protective mechanisms by preventing endothelial dysfunction, by reducing production of reactive oxygen species, and by inhibiting leukocyte–endothelial adhesion. Furthermore, we report here that rosuvastatin inhibits endothelial cell surface expression of P-selectin, an adhesion molecule that plays a pivotal role in leukocyte–endothelium interactions. Taken together, these data indicate that benefits obtained in SHRSP with rosuvastatin are mediated by vascular-protective effects without the need for the drug to cross the blood–brain barrier. Failure of rosuvastatin to protect the rats when given after proteinuria has increased is probably because of the fact that the disease is too advanced and that the inflammatory cascade has been initiated and is difficult to control, but this aspect needs further investigation. Interestingly, the chronic treatment of SHRSP with simvastatin, a lipophilic statin, failed to exert any protective effect on the appearance of brain damage. These data, however, agree with previous observations of the differences among statins in their ability to interfere with the inflammatory cascade. In vivo and in vitro studies indicate that lipophilic statins may induce proinflammatory responses at the level of endothelial cells, monocytes, and leukocytes and sensitize the cells to a subsequent challenge with inflammatory agents. Recent data show that simvastatin increases the lipopolysaccharide-induced proinflammatory response in macrophages via a c-Fos–based and c-Jun–based mechanism. Together, these data strongly suggest that the potential of the various statins to affect the inflammatory process varies among the members of this class of drugs. Given the large use of statin therapy, it may be critical to explore the differences in proinflammatory or antiinflammatory properties of the different molecules.
In summary, our data show that in an animal model of brain ischemia, the treatment with rosuvastatin, but not with simvastatin, exerts a beneficial effect by modulating the inflammatory condition that precedes the development of cerebral damage in these animals. Knowledge of these differences, still barely investigated, may assist in the selection of the more appropriate statin for each individual pathological situation and for each individual patient.

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Figure I. Blood pressure (A) and body weight (B), as a function of the duration of dietary treatment (Japanese Permissive Diet, JPD), for SHRSP not receiving medication ■, SHRSP treated with 1 mg/kg/day rosuvastatin ● and SHRSP treated with 10 mg/kg/day rosuvastatin ▲ (* and §p<0.05 vs 10 and 1 mg/kg/day treated rats, respectively).

Figure II. Daily proteinuria (A) and event-free survival (B), as a function of the duration of JPD, for SHRSP treated with vehicle ■ or 2 ●, 10 ▲ and 20 ◆ mg/kg/day of simvastatin. RT-PCR analysis (C) for transcription of selected inflammatory mediators mRNA in kidney from rats treated with vehicle (white column) or simvastatin (20 mg/kg/day, black column) and sacrificed at the first MRI visualization of brain damage in vehicle-treated rats. The bar reports the densitometric analysis of PCR bands, from four separate experiments, normalized to the corresponding GAPDH signals.