Withdrawal of Statin Treatment Abrogates Stroke Protection in Mice

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Background and Purpose—Statins (3-hydroxy-3-methylglutaryl–coenzyme A [HMG-CoA] reductase inhibitors) reduce stroke damage independent of lipid lowering by upregulation of endothelial nitric oxide synthase (eNOS). Acute withdrawal of statin treatment may suppress endothelial NO production and impair vascular function.

Methods—To test this hypothesis, we treated 129/SV mice with atorvastatin (10 mg/kg) for 14 days and then withdrew treatment.

Results—Treatment with atorvastatin conferred stroke protection by 40% after filamentous occlusion of the middle cerebral artery followed by reperfusion. Withdrawal of statin treatment, however, resulted in the loss of stroke protection after 2 and 4 days. In mouse aortas and brain vasculature, statins upregulated eNOS message 2.3- and 1.7-fold, respectively, as measured by reverse transcription–polymerase chain reaction. Withdrawal of statins resulted in 5- and 2.7-fold downregulation of eNOS in aorta and brain, respectively, after 2 days. Statin treatment decreased RhoA GTPase membrane expression to 48%, while withdrawal of statins resulted in 4-fold increase of RhoA in the membrane. Moreover, platelet factor 4 and β-thromboglobulin in plasma were significantly downregulated by statin treatment, but withdrawal of statins resulted in a 2.9- and 3.1-fold upregulation after 2 days, respectively. Thrombus formation induced by ligation of the inferior vena cava was significantly reduced by statin treatment. When statin treatment was withdrawn, however, protection was lost between 2 and 4 days.

Conclusions—Acute termination of statin treatment results in a rapid loss of protection in mouse models of cerebral ischemia and thrombus formation independent of lipid lowering. In patients with acute or impending stroke, withdrawal of statins may impair outcome. (Stroke. 2003;34:551-557.)

Key Words: cerebral ischemia • HMG-CoA reductase inhibitors • nitric oxide • thrombosis

It has been shown that 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors (statins) block mevalonate synthesis and are potent cholesterol-lowering drugs. Landmarks trials have demonstrated that statins reduce the risk of myocardial infarction and stroke.1–4 Increasing evidence, however, suggests that the stroke protection conferred by statins relates not only to cholesterol lowering but rather to direct effects on endothelial function as well as antithrombotic and anti-inflammatory effects.5–9 A well-characterized pleiotropic effect of statins is the upregulation of endothelial nitric oxide synthase (eNOS).10–14 An important molecular mechanism of eNOS upregulation relates to the inhibition of geranylgeranylation of the small G-protein Rho.15 Endothelium-derived NO regulates blood pressure, augments regional blood flow, improves cerebral circulation, and inhibits platelet aggregation; therefore NO is an attractive candidate to explain the cholesterol-independent protective effects of statins.16–20 Indeed, we have demonstrated that statins upregulate eNOS in vasculature and thrombocytes, decrease platelet activation, augment cerebral blood flow, and protect from cerebral ischemia in wild-type mice.10,21–23 The reduction of stroke size after statin treatment is mediated mainly by endothelial NO because knockout mice that lack the gene for eNOS are not protected from cerebral ischemia.10

Recent evidence suggests that acute withdrawal of statin treatment may impair vascular function independent of lipid lowering, which may be of high relevance for the clinical use of these drugs. One study in patients with stable coronary heart disease showed a >3-fold increase in thrombotic vascular events after simvastatin treatment was stopped and continued with relatively lower doses of fluvastatin.24 Another study demonstrated increased event rates in patients with acute coronary syndromes after withdrawal of statins.25 Indeed, we have recently identified a rebound of impaired NO bioavailability after short-term withdrawal of statin therapy in cultured endothelial cells. The molecular mechanism by which withdrawal of statin...
treatment suppresses NO production is a negative feedback regulation of Rho GTPase gene transcription.26

In this study we tested the hypothesis that acute withdrawal of statin therapy impairs vascular function in vivo. To do so, we tested outcome in mouse models of cerebral ischemia and thrombus formation after treatment with atorvastatin and at several time points after discontinuation of treatment. We reasoned that the possible mechanism relates to a Rho-mediated suppression of eNOS expression with negative impact on endothelium function and platelet aggregation. We therefore determined expression of Rho and eNOS in aorta and brain vasculature. In addition, we measured markers of platelet activation and bleeding time. Plasma levels of β-thromboglobulin (β-TG) and platelet factor 4 (PF4), which are released from platelets via α-granules during the release reaction, were used as established and valid indices of platelet activation in vivo.31,27

Materials and Methods

Drug
Atorvastatin was a kind gift from Gödecke AG Freiburg, Germany. Stocks were prepared as 2-mg/mL solutions in PBS and 10% ethanol, pH 7.6.

Animals and Treatment
All experimental procedures that were performed on laboratory animals conformed to institutional guidelines. 129/SV wild-type mice (weight, 18 to 22 g; BgVV, Berlin, Germany) were treated with atorvastatin (10 mg/kg) or a corresponding volume of vehicle solution by daily subcutaneous injections for 14 days (statin). Serum cholesterol levels were determined by the Institut für Klinische Chemie, Universität zu Köln, Köln, Germany, and were not different between vehicle-treated and atorvastatin-treated animals (89.5±8.5 versus 87.8±15.4 mg/dL, respectively; n=4 animals; P>0.05, Student’s t test).25 In some groups of animals treatment was withheld for 2 (14+2 day group) or 4 (14+4 day group) days, respectively, before the experiment.

Model of Cerebral Ischemia and Measurement of Infarct Size
Animals were anesthetized with 1.5% halothane and maintained in 1.0% halothane in 70% N2O and 30% O2 with the use of a vaporizer. Middle cerebral artery occlusion for 1 hour was induced with a silicone-coated 8.0 nylon monofilament and was closely followed by reperfusion of 23 hours. Animals were deeply anesthetized by halothane and decapitated. Infarction areas were quantified with an image analysis system (SigmaScan Pro 4.0, Jandel Scientific) on hematoxylin and eosin–stained cryostat sections as described.10,28

Real-Time Reverse Transcription–Polymerase Chain Reaction
Brains and aortas were quickly frozen after the animals were killed. Total RNA isolation, reverse transcription (RT), and competitive polymerase chain reaction (PCR) were performed according to standard techniques. Real-time RT-PCR was performed with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems). Primers for amplification of eNOS were 5′-TTCCCGCTGCCACCTGATCTAA-3′ and 5′-AACATATGTTCC TGTCTCAAAGGCA-3′. For 18S the primers were 5′-TTG ATT AAG TCC CTG CCC TTT GT and 5′-CGA TCC GAG GGC CTA ACTA. For each run of the eNOS and 18S TaqMan RT-PCR, a separate standard curve using serial dilutions and the corresponding “takeoff points” (Ct) of a known quantity (100 ng) of the RT-cDNA from control animals was obtained. The Ct values of equal amounts (10 ng) of each RT-cDNA were referred to the standard curve of the respective run and standardized to the corresponding 18S expression. All measurements were done in triplicate. Few variations in the expression of 18S mRNA were observed.

Western Blotting
Total cell lysates and membrane and cytosolic proteins were isolated as described previously.13 Staining with Ponceau S solution (SERVA) was performed to control for protein loading. Immunoblotting was performed with the use of a RhoA polyclonal antibody (1:250 dilution; Santa Cruz Biotechnology), donkey–anti-rabbit secondary antibody (1:4000 dilution), and the enhanced chemiluminescence kit (Amersham). Selected blots were probed with β-actin (Santa Cruz Actin H-196 polyclonal antibody, 1:250 dilution) as internal control.

Blood Withdrawal, Preparation of Plasma, and Measurement of Markers of Platelet Activation
Animals were deeply anesthetized with 0.1 mL chloral hydrate (7% wt/vol in PBS) by intraperitoneal injection. Whole blood was withdrawn by puncture of the retro-orbital plexus. PF4 and β-TG measurements were performed as described (Asserachrom PF4 and β-TG-enzyme-linked immunosorbent assay [ELISA], Roche).21

Tail Bleeding Time
Bleeding times were measured as reported previously by Freedman et al29 with minor modifications. Puncture of the dorsal tail vein was performed with a lancet (Accu-Check, Roche). The bleeding times were conducted in duplicate in each animal.

Experimental Thrombosis
Animals were anesthetized with 1.0% halothane in 70% N2O and 30% O2 with the use of a vaporizer. Experimental thrombosis was induced by the ligation of the inferior vena cava for 2 hours as described elsewhere.31,30

Statistical Analysis
Data are presented as mean±SEM. Comparisons were made by ANOVA followed by Tukey post hoc test or χ2 test (thrombus formation). A probability value <0.05 was considered of statistical significance.

Results
Effects of Withdrawal of Atorvastatin Treatment on Stroke Outcome
To evaluate the effects of statin treatment on stroke outcome, we treated normocholesterolemic 129/SV mice with daily subcutaneous injections of atorvastatin (10 mg/kg) for 14 days. Serum cholesterol levels did not change significantly after 14 days of treatment. Mice were subjected to focal cerebral ischemia by 1-hour filamentous occlusion of the middle cerebral artery followed by reperfusion of 23 hours. Lesion volume was determined after 24 hours on 2-mm coronal brain sections by computer-assisted volumetry. Animals that were treated with atorvastatin had 40±10% smaller stroke volumes than vehicle-treated mice (Figure 1). Significantly smaller lesions were evident in 2 (ie, coronal sections 3 and 4) of the 5 standardized coronal brain sections. When infarct volume was corrected for brain swelling (ie, calculated by an indirect method), infarcts were still significantly smaller by 36±10% (50.0±7.7 versus 80.0±5.7 mm3 in
atorvastatin- versus vehicle-treated animals; *P<0.05, ANOVA plus Tukey post hoc test).

To determine the effects of withdrawal of statin treatment, mice were treated with 10 mg/kg for 14 days. Thereafter, treatment was stopped, and animals were subjected to focal cerebral ischemia after 2 and 4 days. Both groups in which treatment was stopped for 2 and for 4 days were not significantly protected from cerebral ischemia compared with controls (*P<0.05, ANOVA plus Tukey post hoc test) (Figure 1). While animals in the 14+2 day group tended to have smaller (27±12%) infarcts compared with controls, this trend was lost in the 14+4 day group (12±10% smaller infarcts) (Figure 1). Determination of indirect infarct size yielded similar results (data not shown). Therefore, chronic atorvastatin treatment significantly protects from cerebral ischemia/reperfusion; this effect, however, is quickly abrogated after acute withdrawal of treatment.

Effects of Withdrawal of Atorvastatin Treatment on eNOS Expression in Mouse Brain and Aorta
Next we evaluated the effects of atorvastatin treatment on eNOS expression in brain and aorta in vivo. Levels of eNOS mRNA in brain were 1.7-fold higher after treatment with 10 mg/kg atorvastatin compared with control mice (Figure 2A). We had previously determined that treatment with different doses of atorvastatin (0.5, 1, and 10 mg/kg) dose-dependently upregulated eNOS mRNA by 1.1-, 1.7-, and 2.3-fold in mouse aortas (Figure 2B).

To determine the effects of withdrawal of statin treatment, mice were treated with 10 mg/kg for 14 days. Thereafter, treatment was stopped and brains and aortas were harvested after 2 and 4 days. Two days after withdrawal of statin treatment, eNOS mRNA expression decreased 2.3-fold in brain (to 69±8% of control; *P<0.05 versus 14-day treatment, ANOVA plus Tukey post hoc test) and 5-fold in aorta (to 46±7% of control; Figure 2B). Four days after the withdrawal of treatment, eNOS mRNA returned to control levels in brain and aorta (Figure 2A and 2B). These data are in agreement with a rebound phenomenon with suppressed eNOS expression in peripheral and brain vasculature after withdrawal of statin treatment.

Effects of Withdrawal of Atorvastatin Treatment on Rho Expression in Aorta
RhoA GTPase is a negative regulator of eNOS mRNA stability. Upregulation of eNOS expression by statin treatment is mediated by inhibition of isoprenoid-dependent translocation of RhoA GTPase to the cell membrane.15 To determine the effect of termination of statin treatment on RhoA membrane expression, Western blot analysis of mem-
brane and cytosolic proteins of aortas from mice treated with atorvastatin was performed (Figure 2C). Statin-treated mice showed accumulation of RhoA in the cytosol (209±23% of control; P<0.05). In the membrane, atorvastatin decreased Rho protein expression to 48±10% (P<0.05). Two days after termination of statin treatment, RhoA membrane expression increased 4-fold (223±15% of control; P<0.05), but cytosolic Rho expression was downregulated by 21±9% (P=NS). Four days after statin treatment was stopped, cytosolic as well as membrane expression of RhoA returned to baseline levels. These data suggest isoprenoid-dependent regulation of RhoA membrane expression as an important underlying mechanism for the effects of statin withdrawal on eNOS expression.

Effects of Withdrawal of Atorvastatin Treatment on Markers of Thrombocyte Activation and Tail Bleeding Time

We have previously determined that statins suppress platelet activation by an eNOS-dependent mechanism. Therefore, we wanted to evaluate whether discontinuation of statin treatment affects platelet activation and bleeding time in 129/SV mice. Two markers of platelet activation, i.e., PF4 and β-TG, which are released via α-granules from platelets on activation, were quantified in plasma by means of ELISA. Compared with vehicle-treated mice, we found that 14 days of atorvastatin treatment significantly downregulated PF4 plasma levels by 57±6% and β-TG by 31±8% (Figure 3A and 3B). Downregulation of PF4 and β-TG by atorvastatin was dose dependent (downregulation by 15%, 24%, and 57% for PF4 and by 8%, 20%, and 31% for β-TG with atorvastatin at 0.5, 1, and 10 mg/kg, respectively). Two days after withdrawal of statin treatment, however, PF4 increased 2.9-fold (to 125±5% of control; Figure 3A), and β-TG increased 3.1-fold (to 199±27% of control; Figure 2B). Four days after the withdrawal of treatment, both PF4 and β-TG returned to control (ie, baseline) levels (Figure 3A and 3B). Hence, our data are indicative of a rebound phenomenon with increased markers of platelet activation after withdrawal of statin treatment.

Because both treatment with atorvastatin as well as termination of treatment had effects on markers of platelet activation, we determined tail bleeding times in animals treated with 10 mg/kg atorvastatin for 14 days. Tail bleeding times tended to be longer (153±37%) in atorvastatin-treated animals, although this did not reach statistical significance (Figure 3C). On the other hand, both 2 and 4 days after termination of treatment, tail bleeding times were similar to control levels (110±21% and 113±13%, respectively) (Figure 3C).

Effects of Withdrawal of Atorvastatin Treatment on Thrombus Formation

Next we evaluated the effects of atorvastatin withdrawal on thrombus formation. To do so, we used a model of experimental thrombosis induced by ligation of the inferior vena cava, which we had adapted from a model in rat. Atorvastatin treatment significantly reduced thrombus formation, extending previous findings. While all 18 animals (100%) developed thrombi in the control group (vehicle-treated mice), only 33% (5 of 15 mice) developed thrombi in the atorvastatin-treated group (10 mg/kg for 14 days; Figure 4). Mean thrombus weight was 0.48±0.11 versus 0.37±0.05 mg in controls versus atorvastatin-treated mice, respectively. Two days after withdrawal of statin treatment, thrombus formation was still significantly inhibited compared with vehicle-treated mice: only 14% (1 of 7) of the mice developed thrombi (Figure 4). Four days after the withdrawal of treatment, however, 70% of animals (7 of 10; mean thrombus weight, 0.57±0.17 mg) developed thrombi, which was not statistically different from controls (χ² test) (Figure 4). In conclusion, atorvastatin treatment protects from thrombus formation; this effect, however, is lost between 2 and 4 days after withdrawal of treatment.

Discussion

This study demonstrates that the stroke-protective effects of chronic statin treatment are rapidly and completely abrogated after acute withdrawal of treatment. While 14 days of atorvastatin treatment reduced lesion size after focal cerebral ischemia by as much as 40%, protection (ie, statistical significance) was lost only 2 days after withdrawal of
treatment, and lesion size reached control levels 4 days after treatment was stopped. Cholesterol levels were not significantly affected by 14 days of atorvastatin treatment, and therefore both the stroke-protective effects of chronic treatment as well as the rapid abrogation of protection after withdrawal of treatment are cholesterol independent.

In the present study we demonstrate that while 14 days of atorvastatin treatment upregulated eNOS expression and activity, which is an important mechanism of stroke protection.\textsuperscript{10,21–23} In the present study we demonstrate that while 14 days of atorvastatin treatment upregulated eNOS message in brain and aorta, eNOS was significantly suppressed 2 days after withdrawal of treatment. This downregulation of eNOS message below levels in vehicle-treated animals complies with a rebound mechanism. eNOS upregulation by statins is mediated by the inhibition of geranylgeranylation of the small G-protein Rho.\textsuperscript{14} In its isoprenylated (ie, geranylgeranylated) form, Rho is membrane bound and has high GTPase activity; when isoprenylation is inhibited, Rho accumulates in the cytoplasm and is inactive. After 14 days of atorvastatin treatment Rho is mostly found in the cytoplasmic fraction, while 2 days after withdrawal of treatment the majority of protein is membrane bound and therefore active. These results are compatible with our previous findings in which we identified a negative feedback regulation of Rho GTPase gene transcription as the molecular mechanism of the profound suppression of NO production in endothelial cells after statin withdrawal.\textsuperscript{26}

Endothelium-derived NO mediates vasodilation and inhibits leukocyte adhesion and platelet aggregation. In fact, we have demonstrated that the stroke-protective effects of (statin-induced) eNOS upregulation are mediated by decreased platelet activation in addition to augmentation of cerebral blood flow.\textsuperscript{10,21,22} For example, markers of platelet activation, such as PF4 and β-TG, are downregulated by statin treatment by an eNOS-dependent mechanism. In the present study we investigated whether the suppression of eNOS after withdrawal of treatment correlates with a reciprocal increase of these platelet activation markers. Indeed, similar to eNOS downregulation, there is a rebound of increased platelet activation after withdrawal of statin treatment. Although we did not measure eNOS expression in platelets in this study, our data suggest that suppression of eNOS expression and NO production after statin withdrawal directly mediates the upregulation of the platelet release reaction and platelet activation. Therefore, we propose that the observed changes in eNOS regulation are, at least in part, responsible for the rapid effects of atorvastatin withdrawal on stroke outcome (ie, the complete abrogation of protection). The effects of statins on outcome after filamentous middle cerebral artery occlusion depend mainly on NO-mediated effects on cerebral blood flow, while the impact of antithrombotic effects is less clear.\textsuperscript{10,21} Therefore, we additionally tested the effects of statin treatment and withdrawal of treatment in a model of experimental thrombosis. Similar to the cerebral ischemia model, we observed a loss of protection after termination of treatment but not quite as quickly as in the stroke model (ie, 4 rather than 2 days after termination of treatment). It would be interesting to test the effects of atorvastatin treatment and withdrawal in a thrombotic stroke model. Indeed, preliminary evidence from the laboratory of Moskowitz et al\textsuperscript{31} suggests that statins are protective in a clot-occlusion model of cerebral ischemia. Recently, it was demonstrated in a model of vascular reactivity using aortic rings that withdrawal of statin treatment induces endothelial dysfunction in mice.\textsuperscript{32}

These findings may be of clinical relevance. Large clinical trials have demonstrated that statins reduce the incidence of stroke and myocardial infarction.\textsuperscript{1–4} Moreover, statins may be protective not only when given chronically over months and years: there is evidence from prospective trials like the Effects of Atorvastatin on Early Recurrent Ischemic Events in Acute Coronary Syndromes (MIRACL) Trial or the Effects of Fluvastatin Administered Immediately After an Acute MI on Myocardial Ischemia (FLORIDA) Trial that statins have protective effects on short-term outcome when given directly after an acute coronary syndrome.\textsuperscript{33} In fact, in the MIRACL Trial stroke incidence was 50% lower in atorvastatin-treated patients (ie, 12 versus 24 events) after 16 weeks, suggesting rapid and cholesterol-independent protective effects.\textsuperscript{24,25,34} On the other hand, there is compelling clinical evidence that withdrawal of statin medication acutely impairs vascular function and negatively affects outcome.\textsuperscript{24,25,34} A subgroup analysis of the Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study demonstrated that statin pretreatment in patients with acute coronary syndromes is associated with improved clinical outcome, while discontinuation of statins after onset of symptoms completely abrogates this beneficial effect.\textsuperscript{25} This was observed despite a lack of change in the cholesterol levels during the initial 72 hours, indicative of a cholesterol-independent mechanism. Indeed, 80 mg atorvastatin improved endothelial function in humans,
as measured by volume plethysmography within 24 hours, while withdrawal of treatment acutely impaired vascular function independent of cholesterol levels and the inflammatory state.14

Similar to the findings on event rate by Heeschen et al,25 we found a rapid abrogation of protection rather than an increase in damage compared with controls after withdrawal of statin treatment in the stroke model. While there was a true rebound phenomenon with regard to expression of Rho, eNOS, and markers of platelet activation, this apparently did not completely translate into outcome in in vivo models of vascular injury. Longer-lasting pleiotropic effects of statins other than eNOS upregulation may partly compensate for the suppression of NO production and attenuate the adverse effects of statin withdrawal. Alternatively, since increased NO bioavailability is the main mechanism of stroke protection by statins in our model,10 it could be speculated that NO itself induces enduring protective effects (eg, antithrombotic and anti-inflammatory effects, transcriptional regulation of genes)15 that outlast the rapid suppression of eNOS expression and NO production. Of note, atorvastatin has a plasma half-life of approximately 14 hours (and even longer for active metabolites), which is considerably longer than that of many other statins such as lovastatin, simvastatin, or pravastatin. Hence, effects after abrupt termination of treatment might be exaggerated with the latter compounds.

In conclusion, withdrawal of atorvastatin treatment leads to a rapid abrogation of statin-mediated stroke-protective effects. The molecular mechanism relates to a rebound effect on eNOS expression mediated by a feedback mechanism on Rho expression. Although we did not observe increases in brain damage but rather a rapid abrogation of protection in the animal model, withdrawal of statin treatment in patients with acute or impending stroke may impair their outcome and therefore should be avoided. Further clinical evidence is needed to support these observations, but prospective clinical trials are difficult to perform because of ethical considerations.

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