Suppression of Endothelial Nitric Oxide Production After Withdrawal of Statin Treatment Is Mediated by Negative Feedback Regulation of Rho GTPase Gene Transcription

Ulrich Laufs, MD; Matthias Endres, MD; Florian Custodis; Karen Gertz; Georg Nickenig, MD; James K. Liao, MD; Michael Böhm, MD

Background—Statins improve endothelial function by upregulating endothelial nitric oxide (NO) production that is mediated by inhibiting the isoprenylation of rho GTPase. Withdrawal of statin treatment could suppress endothelial NO production and may impair vascular function.

Methods and Results—To test this hypothesis, mice were treated for 14 days with 10 mg/kg atorvastatin per day; this led to the upregulation of endothelial NO synthase expression and activity by 2.3- and 3-fold, respectively. Withdrawal of statins resulted in a dramatic, 90% decrease of NO production after 2 days. In mouse aortas and cultured endothelial cells, statins upregulated the expression of rho GTPase in the cytosol, but statins blocked isoprenoid-dependent rho membrane translocation and GTP-binding activity. Inhibiting the downstream targets of rho showed that rho expression is controlled by a negative feedback mechanism mediated by the actin cytoskeleton. Measuring rho mRNA half-life and nuclear run-on assays demonstrated that statins or disruption of actin stress fibers increased rho gene transcription but not rho mRNA stability. Therefore, treatment with statins leads to the accumulation of nonisoprenylated rho in the cytosol. Withdrawing statin treatment restored the availability of isoprenoids and resulted in a massive membrane translocation and activation of rho, causing downregulation of endothelial NO production.

Conclusions—Withdrawal of statin therapy in normocholesterolemic mice results in a transient increase of rho activity, causing a suppression of endothelial NO production. The underlying molecular mechanism is a negative feedback regulation of rho gene transcription mediated by the actin cytoskeleton. (Circulation. 2000;102:3104-3110.)

Key Words: nitric oxide • endothelium • statins • rho GTPase

Large clinical trials have demonstrated that hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reduce the risk of myocardial infarction and stroke. Increasing evidence suggests that statins may exert effects on the vasculature beyond the lowering of serum cholesterol levels. Indeed, it has been shown that HMG-CoA reductase inhibitors upregulate the expression and function of endothelial nitric oxide synthase (eNOS) independent of cholesterol levels. Impaired release of endothelial nitric oxide (NO) causes endothelial dysfunction. Endothelium-derived NO mediates vasodilatation and inhibits leukocyte adhesion, platelet aggregation, and smooth muscle cell proliferation. The activation of endothelial NO by superoxide anion leads to nitrate tolerance and vasoconstriction. In animal models, the upregulation of eNOS by statin treatment improved cardiac function and decreased stroke size during ischemia. The reduction of stroke size after statin treatment is mainly mediated by endothelial NO, because mutant mice lacking the gene for eNOS were not protected from cerebral ischemia. However, despite the clinical effects of statins, the effects of withdrawing statin treatment are not known. Interestingly, a recent study noted a 3-fold increase in thrombotic vascular events after the treatment of patients with simvastatin was stopped and then continued with relatively lower doses of fluvastatin because of reference pricing for statins in New Zealand. We hypothesized that terminating statin treatment may suppress endothelial NO production and impair vascular function.

Upregulation of endothelial NO by statins is mediated by decreased levels of geranylgeranylpyrophosphate (GGPP), an isoprenoid intermediate of the cholesterol synthesis pathway. Isoprenoids are important for the post-translational modification of proteins, such as the small GTP-binding proteins ras and rho. Isoprenylation of small GTPases is necessary for intracellular trafficking and membrane association. We identified the small G-protein rho as a negative regulator of endothelial NO release. Hence, statins increase endothelial NO production by inhibiting the geranylgerany-
lation of rho. Other cholesterol-independent effects of statins may also be mediated by the inhibition of rho GTPase, such as the release of tissue plasminogen activator or the inhibition of cell-cycle progression of vascular smooth muscle cells. Indeed, rho GTPases play a key role in the regulation of actin stress-fiber formation, the organization of the actin cytoskeleton, cell growth, and cell proliferation. However, despite the importance of rho for cellular function, little is known about the regulation of rho expression and activity in endothelial cells.

We reasoned that statin-induced reduction of rho isoprenylation inhibits rho activity rather than rho production. Therefore, we hypothesized that an early withdrawal of statin therapy could lead to a profound rebound phenomenon, because the rapid isoprenylation of cytosolic rho could induce massive membrane translocation and activation of rho, leading to the suppression of eNOS activity and expression.

**Methods**

**Materials**

Cytoschlarasin D (cytoD), mevastatin, farnesylpyrophosphate, GGPP, L-mevalonate, and 5,6-dichlorobenzimidazole riboside (DRB) were purchased from Sigma. H-7, ML-7, 2,3-butanedione monoxime, and nocodazole were from Calbiochem. Clostridium botulinum C3 transferase was purchased from List Biological Laboratories. [32P]-dCTP, [35S]GTP, L-[2,3,4,5-3H]arginine-monohydrochloride, Hybond N-nylon membranes, and x-ray film were obtained from Amersham. [3H]arginine to [3H]citrulline conversion assay. Treatment of bovine aortic endothelial cells with 1, 5, and 10 μmol/L atorvastatin for 24 hours increased eNOS activity in a concentration-dependent way to 130±7%, 170±12%, and 219±8.3%, respectively, compared with control (n=5 separate experiments; P<0.05). To study the effects of terminating statin treatment, endothelial cells were treated with atorvastatin (10 μmol/L). After 24 hours, the drug was completely removed by replacing the medium, and eNOS activity was measured at the indicated time points (Figure 1). At 8, 12, and 16 hours after stopping statin treatment, eNOS activity decreased 142%, 43%, and 37%, respectively, compared with untreated cells. After 20 hours, eNOS activity decreased as described previously.

**Cell Culture**

Endothelial cells were harvested from bovine aortas. Confluent endothelial cells from <4 passages cultured with 10% fetal calf serum were used for all treatment conditions. In some experiments, endothelial cells were treated with DRB for 30 minutes before the addition of atorvastatin and cytoD. Cellular viability was determined by cell count, morphology, and trypan blue exclusion.

**Animal Treatment**

All animal experiments were conducted in accordance with the guidelines of the National Institutes of Health and the authors’ institutions. Mice (strain 129/SV; weighing 18 to 22 g) were injected subcutaneously with 0.1 mL of atorvastatin (1 or 10 mg/kg) or PBS once daily for 14 days. Animals were treated and killed under identical external conditions (time of day, temperature, light, etc).

**eNOS Activity Assay**

Endothelial cells and mouse aortas were homogenized in 250 mmol/L Tris-HCl (pH 7.4), 10 mmol/L EDTA, and 10 mmol/L EGTA. Lysates were pelleted (10 minutes at 13000 rpm and 4°C), and supernatants were used for the assay. The total protein concentration in the supernatants was quantitated, and 10 μg of protein per sample was used. eNOS activity was determined by measuring the conversion of [14C]arginine to [14C]citrulline using the NOS assay kit from Calbiochem. Rat cerebellum served as positive control. Lysates incubated with the eNOS inhibitor nitro-l-arginine methyl ester L (1 mmol/L) served as blanks. Time-course experiments showed that basal NO production in confluent endothelial cells did not change between 0 and 24 hours.

**Western Blotting**

Total cell lysates and membrane and cytosolic proteins were isolated as described previously. Immunoblotting was performed using a rhoA monoclonal antibody (1:250 dilution; Santa Cruz Biotechnology), donkey-anti–rabbit secondary antibody (1:4000 dilution), and the enhanced chemiluminescence kit (Amersham).

**Northern Blotting**

Northern blotting using [32P]-labeled full-length rhoA cDNA was performed as described previously.

**Assay for Rho GTP-Binding Activity**

Rho GTP-binding activity was determined by specific immunoprecipitation of [35S]GTPγS–labeled rho, as described previously.

**eNOS Reverse Transcription–Polymerase Chain Reaction**

RNA isolation, reverse transcription, and competitive polymerase chain reaction (PCR) were performed according to standard techniques. The sense (5'-TTCCGGGCTGCACTTGATCTAA-3') and antisense (5'-AACATAGTCCC TTGCTCAAGGCA-3') primers were used to amplify a 340-bp murine eNOS cDNA fragment and a 1052-bp mutated eNOS cDNA fragment, which served as an internal standard. GAPDH was amplified as the external standard. Each PCR cycle consisted of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elobtaining at 72°C for 60 seconds. The linear exponential phases for eNOS and GAPDH PCR were 35 and 22 cycles, respectively. Equal amounts of corresponding NO9S and GAPDH reverse transcription-PCR products were loaded on agarose gels, and optical densities of ethidium bromide–stained DNA bands were expressed as the ratio of murine eNOS to eNOS mutant PCR signal.

**Nuclear Run-On Assays**

Nuclei of endothelial cells were isolated, and in vitro transcription was performed in the presence of [32P]-UTP. Purified, denatured, full-length rhoA-, GAPDH- and linearized pcDNA3 cDNA were vacuum-transferred onto nitrocellulose membranes. Hybridization of radiolabeled mRNA transcripts to the membranes was performed at 45°C for 24 hours.

**Data Analysis**

Band intensities were analyzed by densitometry. All values are expressed as mean±SEM compared with controls. Paired and unpaired Student’s t tests and ANOVA for multiple comparisons were employed. Differences were considered significant at P<0.05.

**Results**

**Withdrawal of Statin Treatment Transiently Decreases eNOS Activity**

The effect of atorvastatin on eNOS activity was assessed by [3H]arginine to [3H]citrulline conversion assay. Treatment of bovine aortic endothelial cells with 1, 5, and 10 μmol/L atorvastatin for 24 hours increased eNOS activity in a concentration-dependent way to 130±7%, 170±12%, and 219±8.3%, respectively, compared with control (n=5 separate experiments; P<0.05). To study the effects of terminating statin treatment, endothelial cells were treated with atorvastatin (10 μmol/L). After 24 hours, the drug was completely removed by replacing the medium, and eNOS activity was measured at the indicated time points (Figure 1). At 8, 12, and 16 hours after stopping statin treatment, eNOS activity decreased 142%, 43%, and 37%, respectively, compared with untreated cells. After 20 hours, eNOS activity...
increased 61% and, after 24 hours, eNOS activity returned to control levels.

**Inhibition of Isoprenoid Synthesis Upregulates Rho Expression**
Rho GTPase negatively regulates eNOS expression and function.\(^1\)\(^5\) Therefore, the effects of atorvastatin on rho protein expression in endothelial cells were characterized. Atorvastatin (1 \(\mu\)mol/L) time-dependently (Figure 2A) and concentration-dependently (data not shown) upregulated rho protein expression. Upregulation of rho expression by atorvastatin was a class effect of HMG-CoA reductase inhibitors: mevastatin, lovastatin, and simvastatin also upregulated rho expression (data not shown). Upregulation of rho was mediated by the inhibition of mevalonate synthesis; the addition of L-mevalonate completely reversed the effects of the statins (Figure 2B). Similarly, adding the isoprenoid GGPP reversed the upregulation of rho by statins. In contrast, adding farnesylpyrophosphate had no effect. These findings indicate that rho expression is negatively regulated by geranylgeranylation.

**Rho Is Negatively Regulated by the Actin Cytoskeleton**
How rho expression in endothelial cells is regulated is not known. Downstream targets of rho GTPase include the regulation of myosin light chain (MLC) phosphorylation and actin stress fiber formation.\(^1\)\(^2\),\(^18\),\(^26\) To test the hypothesis that rho might be negatively regulated by its downstream effectors, we inhibited several levels of the signal transduction cascade downstream of rho. Direct inactivation of rho GTPase activity by ADP-ribosylation with *Clostridium botulinum* C3 transferase (50 \(\mu\)g/mL, 24 hours)\(^1\)\(^7\) resulted in the upregulation of rho protein expression (Figure 3A). Inhibiting MLC kinase with H-7 (10 \(\mu\)mol/L) or 2,3-butanedione 2-monoxime time-dependently increased rho expression, with a maximum effect after 8 hours (data not shown). Inhibiting MLC phosphorylation and MLC ATPase prevents the formation of actin stress fibers.\(^26\) Therefore, we directly disrupted actin stress fibers with cytoD. Treating endothelial cells with cytoD for 8 hours showed a dose-dependent increase of rho protein (Figure 3B) and mRNA expression (data not shown).
Upregulation of rho was specific to changes in microfilaments rather than the microtubule cytoskeleton because treatment with nocodazole slightly decreased rho expression (Figure 3C). As a negative control, the expression of rac GTPase was studied (Figure 3D). Interestingly, rac was upregulated by atorvastatin in a manner similar to that of rho, but although cytoD upregulated rho, it had no effect on rac expression. In summary, these data indicate that actin stress fiber formation negatively regulates rho expression.

Rho function depends on its membrane-associated GTP binding activity rather than rho expression in total cell lysates. Therefore, the effect of disrupting actin stress fibers with cytoD (1 μmol/L, 24 hours) on rho expression in isolated cell membranes and the cytosol was studied. Treatment with atorvastatin and simvastatin (10 μmol/L, 24 hours) upregulated rho expression in the cytosol of endothelial cells (Figures 4A and 4B). Similarly, cytoD increased cytosolic rho. Conversely, inhibiting rho isoprenylation by statins decreased the translocation of rho to the cell membrane, but disrupting actin stress fibers downstream of rho upregulated rho expression in the cell membrane.

To determine whether rho function is regulated by statins and the actin cytoskeleton, we immunoprecipitated [35S]GTPγS-labeled rho from cell membranes. Treatment with atorvastatin decreased rho GTP-binding activity by 50% (Figure 4C). In contrast, cytoD upregulated rho function by 25%. Taken together, these findings show that statins upregulate rho expression but inhibit rho function, and they identify the actin cytoskeleton as a negative regulator of rho expression and function.

Rho Gene Transcription Is Regulated by the Actin Cytoskeleton

To characterize the molecular regulation of rho expression, rho mRNA half-life was determined using the RNA polymerase inhibitor DRB. There was no significant difference in post-transcriptional regulation of rho mRNA levels after 0, 4, 8, and 12 hours. Band intensities of 3 separate experiments were normalized to corresponding ethidium-stained 28S and 18S ribosomal RNA and plotted semilogarithmically as a function of time (relative intensity). C, Nuclear run-on assay showing effects of atorvastatin (Ator; 10 μmol/L) and cytoD (CyD; 1 μmol/L) on rho gene transcription. GAPDH and pcDNA3 gene transcription served as controls for standardization and nonspecific binding, respectively.

Figure 4. Effects of treatment with atorvastatin (Ator; 10 μmol/L), simvastatin (Sim; 10 μmol/L), and cytoD (CyD; 1 μmol/L) for 24 hours on rho membrane translocation and activity in endothelial cells compared with control (C). A, Representative Western blot of rho expression in membrane and cytosol. B, Quantification of 3 separate experiments. C, [35S]GTPγS-binding activity in endothelial cell membranes. n=3 separate experiments each; *P<0.05 vs control.

Figure 5. Representative northern blot (A) and analysis of time-dependent effects of DRB (50 μmol/L; B) alone (control) or in combination with cytoD (CyD; 1 μmol/L) on rho steady-state mRNA levels after 0, 4, 8, and 12 hours. Band intensities of 3 separate experiments were normalized to corresponding ethidium-stained 28S and 18S ribosomal RNA and plotted semilogarithmically as a function of time (relative intensity). C, Nuclear run-on assay showing effects of atorvastatin (Ator; 10 μmol/L) and cytoD (CyD; 1 μmol/L) on rho gene transcription. GAPDH and pcDNA3 gene transcription served as controls for standardization and nonspecific binding, respectively.

GAPDH and pcDNA3 gene transcription served as controls for standardization and nonspecific binding, respectively.
Termination of Statin Treatment Transiently Increases Rho Membrane Translocation

To study the effects of stopping statin treatment on rho expression, cytosolic and membrane proteins of endothelial cells were analyzed. In the presence of atorvastatin, rho protein expression decreased in the membrane but increased in the cytosol (Figure 6). Three hours after the withdrawal of atorvastatin, rho expression started to increase in the membrane and, after 6 hours, rho protein expression exceeded control levels. Rho membrane expression remained upregulated after 8 hours and then gradually returned to baseline levels after 16 hours. In contrast, rho expression in the cytosol was downregulated after 3 and 6 hours, suggesting a massive translocation of rho from the cytosol to the cell membrane. Rho expression in the cytosol increased to control levels after 16 hours.

Withdrawal of Statin Treatment Transiently Decreases Endothelial NO Production In Vivo

To determine the effects of atorvastatin on eNOS in vivo, 129/SV wild-type mice were subcutaneously injected daily for 14 days with 1 and 10 mg/kg atorvastatin. As observed previously with simvastatin and lovastatin, serum cholesterol levels did not change significantly when treated with 10 mg/kg atorvastatin (data not shown). Compared with control mice, treatment with 1 and 10 mg/kg atorvastatin concentration-dependently upregulated eNOS mRNA (165 ± 15% and 230 ± 29%, respectively; n = 3 separate experiments; *P < 0.05 vs control).

To determine the effects of stopping statin treatment in vivo, mice were treated with 10 mg/kg atorvastatin. After 14 days, treatment was stopped and aortas were harvested after 2 and 4 days. Two days after terminating statin treatment, eNOS mRNA expression decreased 5-fold (46 ± 7%; Figures 7A and 7B). Four days after the discontinuation of statin treatment, eNOS mRNA expression returned to control levels.

Figure 6. Representative Western blots (A) and analysis of time course of rho protein expression in endothelial cell membrane (B) and cytosol (C) after treatment of cells with atorvastatin (10 μmol/L, 24 hours) and withdrawal of statin treatment for 0, 6, 8, 16, and 24 hours compared with control (C). n = 3 separate experiments; *P < 0.05 vs control.

Figure 7. 129/SV Mice were treated with 10 mg/kg atorvastatin or vehicle (control, C) per day for 14 days. Aortas were harvested on day 14 (statin) and 2 (statin +2 ) and 4 days (statin +4 ) after withdrawal of treatment. A, Calcium-dependent NOS activity as determined by [3H]arginine to [3H]citrulline conversion assays. Contr indicates control. B, Representative competitive PCR showing expression of murine eNOS mRNA compared with eNOS mutant mRNA (mutant) and GAPDH. C, Quantification of band intensities of eNOS PCR expressed as mean ratio of murine aortic eNOS to eNOS mutant mRNA compared with control (C). n = 4; *P < 0.05 and **P < 0.01 vs control.

Termination of Statin Treatment Increases Rho Membrane Translocation in Vivo

To determine whether statins regulate rho membrane expression in vivo, Western analysis of membrane and cytosolic proteins of aortas from mice treated with atorvastatin were performed. Statin-treated mice showed upregulation of rho in the cytosol (Figure 8). In the membrane, atorvastatin de-
increased rho protein expression. Two days after terminating statin treatment, rho membrane expression increased 3-fold but cytosolic rho expression was downregulated. Four days after stopping statin treatment, rho expression returned to baseline levels. These data show, for the first time, that statins regulate vascular rho expression and localization in vivo.

Discussion

We showed that 2 days after the withdrawal of statin treatment, endothelial NO production decreased by 90%. The molecular mechanism of this finding was identified as the regulation of the small GTP-binding protein rho. Statin treatment inhibits rho isoprenylation and function, and rho is a negative regulator of eNOS expression. Statins upregulate eNOS by inhibiting rho function. Surprisingly, the inhibition of endothelial isoprenoid synthesis by statins increased rho gene transcription. This effect was mediated by a negative feedback signal of the actin cytoskeleton. In the presence of statins, however, the increased amount of transcribed rho cannot be geranylgeranylated and it accumulates in the cytosol of the endothelial cell; therefore, it is functionally inactive. Withdrawing statin treatment restores the availability of GGPP. The inactive (GDP-bound) cytosolic rho is isoprenylated, translocates to the cell membrane, and becomes activated (GTP-bound). This transient increase of rho GTP-binding activity causes the decrease of endothelial NO production.

Treatment of normocholesterolemic mice with atorvastatin for 14 days increased endothelial NO production but did not alter serum cholesterol levels. Upregulation of endothelial NO has been observed with different statins in a variety of species, including humans. NO is a key mediator of vascular homeostasis and blood flow. Decreased amounts of endothelial NO impair vascular function by promoting vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion. Our experiments show that terminating statin treatment reverses the upregulation of eNOS and transiently decreases NO production below baseline levels.

Rho GTPases are important mediators of vascular function. For example, in addition to negatively regulating eNOS expression, rho controls the release of tissue plasminogen activator and the adhesion of monocytes to the endothelium. Rho mediates the proliferation and migration of vascular smooth muscle cells and is a mediator of mechanotransduction in the vascular wall. In cardiac myocytes, rho serves as a regulator of the signaling pathway leading to hypertrophy. Yet, the available information about the regulation of rho expression is limited. In the present study, we show that statin treatment transcriptionally upregulates rho expression but post-translationally inhibits rho membrane–associated function in the vascular wall. Rho geranylgeranylation, membrane translocation, and GTP-binding activity are inhibited in the presence of statins. Recently, several cholesterol-independent effects of statins have been reported. Inhibiting the isoprenylation of small GTP-binding proteins is an important, cholesterol-independent effect of statin treatment. Our study demonstrates that statins can be used as tools to modify rho function in vivo.

Interestingly, the direct inhibition of rho GTPase activity by C3 transferase upregulates rho expression. Similarly, inhibiting MLC phosphorylation and directly disrupting actin stress fibers lead to increased rho expression. These experiments identify the actin cytoskeleton as a downstream sensor of rho function in the endothelium. The molecular mechanism of this feedback regulation of rho expression by the actin cytoskeleton was identified as an increase in gene transcription but not rho mRNA stability. These findings agree with a recent study showing that rho activation in Swiss 3T3 cells is negatively regulated by cytoskeletal structures during adhesion to the extracellular matrix. Further experiments are needed to characterize the signaling from the actin cytoskeleton to the rho promoter.

Recent evidence suggests that statins exert beneficial vascular effects in addition to lowering serum cholesterol. Indeed, recent and ongoing clinical trials are comparing statin therapy with angioplasty and testing the effects of statins on acute coronary syndromes. In the present study, we show that terminating statin treatment suppresses endothelial NO production. Therefore, withdrawing statin therapy may acutely impair vascular function and precipitate acute coronary syndromes (eg, when statin therapy is discontinued during the early postoperative period after aortocoronary bypass surgery or when patients are switched from one statin drug to another). Clearly, clinical studies are needed to characterize the time course and significance of the clinical events after the withdrawal of statin therapy and their correlation with the downregulation of eNOS activity.
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References

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