Withdrawal of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Elicits Oxidative Stress and Induces Endothelial Dysfunction in Mice

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Abstract—3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) improve endothelial function. We determined whether withdrawal of statin therapy affects endothelium-dependent relaxation in mice and studied the underlying mechanism. Mice were treated with daily injections of cerivastatin (2 mg/kg per day SC), atorvastatin (1 and 10 mg/kg per day SC), or placebo. Vascular reactivity was studied in aortic rings from these mice after 10 days of treatment and after cessation of therapy for several days. Both statins improved endothelium-dependent relaxation to acetylcholine. Compared with control, withdrawal of statin treatment transiently (from day 4 to 7) attenuated endothelium-dependent relaxation. In vessels from animals subjected to atorvastatin withdrawal, the antioxidant tiron restored relaxations. Vascular superoxide anion generation was unaffected by statin therapy but was increased during withdrawal. In mice lacking the gp91phox subunit of the NADPH oxidase, no attenuation of acetylcholine-induced relaxation and no increase in superoxide generation were observed after withdrawal of atorvastatin. In human umbilical vein endothelial cells, statins, which decrease the membrane association of NADPH oxidase–activating Rac-1, increased the activity of this GTPase in whole-cell lysates. Withdrawal of statins induced a translocation of Rac-1 from the cytosol to the membrane and transiently increased NADPH-induced lucigenin chemiluminescence in membrane preparations. Rac-1 inactivation by Clostridium difficile tox in B inhibited the cerivastatin-induced oxygen radical production in human umbilical vein endothelial cells. These observations indicate that the withdrawal of statins induces endothelial dysfunction. The underlying mechanism involves activation of a gp91phox-containing NADPH oxidase by Rac-1 and the subsequent scavenging of endothelium-derived NO by superoxide anions generated from this enzyme. (Circ Res. 2002;91:173-179.)

Key Words: endothelium ■ Rac-1 ■ NADPH oxidase ■ 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ■ nitric oxide

Materials and Methods

Study Design
Mice lacking the gp91phox subunit of the NADPH oxidase (gp91phox−/− mice, cybb Tm1; genetic background: c57 black b6, backcrossed for 7 generations) were purchased from Jackson Laboratories (Bar Harbor, Maine). Healthy male wild-type (WT) mice (C57 black b6, 12 weeks of age) were purchased from Charles Rivers (Sulzbach, Germany). Mice were injected daily (100 μL SC interscapularly) with cerivastatin (2 mg/kg per day, dispersed in sterile PBS [GIBCO-BRL]), atorvastatin (1 or 10 mg/kg per day, dispersed in PBS), or PBS placebo control for 10 days. In subgroups, statin therapy was followed by a daily injection of PBS for different time periods. Experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23) and were approved by the local government (approval No. II25.3-19c20/15-F28/02).
Drugs
Cerivastatin was supplied by Bayer AG (Leverkusen, Germany). Atorvastatin was a gift from Gödecke AG (Freiburg, Germany).

Organ-Chamber Experiments
Organ-chamber experiments were performed as described\textsuperscript{15} using phenylephrine-preconstricted mouse aortic rings from gp91phox\textsuperscript{−/−} and WT mice. Endothelium-dependent relaxations to acetylcholine (ACh) and endothelium-independent relaxations to nitroglycerin were recorded in the presence or absence of the antioxidant tiron (1 mmol/L). NO bioavailability was estimated from the constrictor response to the NO synthase inhibitor \textsuperscript{N} \textsuperscript{-} nitro-L-arginine (L-NA, 300 \mu mol/L) in aortic rings preconstricted to 10\% of the maximal KCl constriction with the use of phenylephrine.

Vascular Superoxide Anion Generation
Measurements were performed by using a chemiluminescence assay in intact mouse aortic rings as described previously,\textsuperscript{18} with lucigenin (5 \mu mol/L) used as an enhancer.

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in medium 199 (GIBCO-BRL) supplemented with antibiotics and FCS (20\%) as described.\textsuperscript{16}

Immunoblotting
Western blot analysis from Triton X-100 (1\%)-soluble aortic protein or aortic membrane protein was performed as described previously.\textsuperscript{15,17} Human leukocytes were used as positive controls. Antibody against chicken anti-p22phox was generously provided by Dr J. Kreuzer (Medizinische Klinik, Universitätsklinikum Heidelberg, Heidelberg, Germany). Mouse anti-gp91phox (AB48) was a kind gift from Dr D. Roos (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, the Netherlands), and the rabbit anti–manganese superoxide dismutase (anti-MnSOD) antibody was from Dr W. Gwinner (Medizinische Hochschule, Hannover, Germany). Rabbit anti–extracellular superoxide dismutase (anti-ecSOD) was kindly supplied by Dr T. Oury, (University of Pittsburg, Pittsburg, Pa). The following commercially available antibodies were used: mouse anti-endothelial NO synthase (anti-eNOS) from BD Transduction, mouse anti–Rac-1 from BD Transduction, sheep anti–copper/zinc superoxide dismutase (anti-CuZnSOD) from Calbiochem), rabbit anti-catalase from Calbiochem, and mouse anti–β-actin from Sigma.

Rac-1 Immunohistochemistry
Primary cultured HUVECs were seeded in chamber slides (Falcon) and grown to confluence. During serum starvation, cells were treated with atorvastatin (10 \mu mol/L) or cerivastatin (100 \mu mol/L) for 24 hours. Subsequently, statins were removed in some cases by repeated washing, and cells were fixed in formaldehyde solution (4\%). Measurements were performed by using a chemiluminescence assay in intact mouse aortic rings as described previously, with lucigenin (5 \mu mol/L) used as an enhancer.

Rac-1 Activity Assay
Rac-1 activity was determined using a commercially available kit (Upstate Biotechnology, distributed by Biomol). Briefly, HUVECs (P1) were grown in 6-cm dishes and serum-deprived for 24 hours. After exposure to cerivastatin (100 \mu mol/L) or atorvastatin (10 \mu mol/L) and subsequent withdrawal of the statins in subgroups, cells were lysed in a buffer containing NP-40. A PAK-GSH fusion protein bound to agarose beads was added, and active Rac, binding PAK-GSH, was separated by repetitive centrifugation and washing. After some experiments, cell lysates were stimulated with GTPyS (100 \mu mol/L) to obtain maximal activation of Rac.

Dichlorofluorescein Fluorescence
Primary cultures of HUVECs were grown on glass coverslips. At confluence, cells were serum-deprived and incubated with cerivastatin (100 \mu mol/L) for 24 hours. In subgroups, cerivastatin was removed by repetitive washing of the cells. Subsequently, cells were loaded with dihydrodichlorofluorescein (DCHF) diacetate (10 \mu mol/L, dissolved in dimethyl sulfoxide; Molecular Probes) for 15 minutes. The assay was carried out in Hanks’ buffer containing L-NA (100 \mu mol/L) to inhibit NO synthase activity and peroxynitrite formation. Fluorescence images were obtained every 20 seconds by use of a fluorescence microscope (Zeiss, Axiovert; excitation 488 nm, emission 512 nm) and an imaging system (OpenLab, Improvision). Changes in intensity over time were calculated from the mean intensities of individual images.

NADPH Lucigenin Assay
First passages of HUVECs were grown in 6-cm dishes. At confluence, cells were serum-deprived and incubated with cerivastatin (100 \mu mol/L) for 24 hours. In subgroups, cerivastatin was removed by repetitive washing of the cells. Subsequently, cells were washed with ice-cold PBS and scraped in hypotonic lysis buffer (in mmol/L: Tris-HCl 25, EDTA 1, and EGTA 1, along with protease inhibitors, pH 7.4) and allowed to swell for 10 minutes. Subsequently, samples were homogenized (10 strokes) and centrifuged (1000 g, 4\°C, 15 minutes). The supernatant was centrifuged at 100 000 g (4\°C, 60 minutes). The pellet, containing the membrane fraction, was dissolved in suspension buffer (in mmol/L: triethanolamine 50, NaCl 150, MgCl\textsubscript{2} 2, and EGTA 0.1, along with protease inhibitors), and protein content was determined by a commercial assay (Rotiquant, Roth). Protein (1.5 \mu g per sample) was added to the assay buffer (PBS, 1 mmol/L EGTA, 1 mmol/L EDTA, and protease inhibitors) containing lucigenin (5 \mu mol/L), NADPH (100 \mu mol/L) was added, and NADPH-induced chemiluminescence was determined in a Berthold Biolumat LB7505 in a final volume of 500 \mu L.

Statistical Analysis
All values are mean±SEM. Maximal relaxation and half-maximal effective dose were calculated from individual dose-response curves. Statistical analysis was carried out by use of ANOVA for repeated measurements, followed by the Fisher least significant difference test.

Results
Endothelium-Dependent Relaxation
In vivo treatment with cerivastatin as well as with atorvastatin significantly improved the ACh-induced relaxation of isolated mouse aortic rings. Two days after discontinuing statin therapy, endothelium-dependent relaxation was identical or better than that observed in vessels from the control group (Table). However, endothelium-dependent relaxation was significantly impaired compared with control at ≥4 days after cessation of statin treatment (Figures 1A through 1C, Table). This impairment of endothelial function was transient; compared with control, no difference in the endothelium-dependent relaxation was detected 9 days after the withdrawal of cerivastatin (Table). As reported previously,\textsuperscript{18} the antioxidant tiron (1 mmol/L) significantly enhanced ACh-induced relaxations in aortic rings from control mice. In rings from mice subjected to withdrawal of atorvastatin, this effect was even more pronounced, so that in the presence of tiron, no difference in the relaxation of rings from control animals and statin-withdrawn animals was observed (Figure 1D).
Relaxations to nitroglycerin were unaffected by statin treatment or withdrawal (Figure 2).

The effect of a 5-day withdrawal of atorvastatin treatment was also studied in gp91phox−/− mice. This strain lacks the activity of the endothelial NADPH oxidase and, as a consequence of a low agonist-induced superoxide anion generation, exhibits enhanced endothelium-dependent vasodilatation to ACh. During treatment with atorvastatin, there was a nonsignificant trend toward a further improvement of endothelium-dependent relaxation (n=8, P=0.067) in aortas from gp91phox−/− mice. When treatment was discontinued for 5 days, no attenuation of endothelium-dependent relaxation was observed, and even the trend toward an improved relaxation was preserved (n=10, P=0.073) (Figure 3A).

Similar results were obtained when basal NO bioavailability was estimated as the increase in tone in response to L-NA in rings preconstricted to 10% of maximal constriction. In WT mice, treatment with atorvastatin increased aortic constriction to L-NA compared with that observed in vessels from control mice (n=10 each group, P<0.01). In contrast, after withdrawal of statin therapy for 4 days, constriction was less than that observed in control rings (n=10 each group, P<0.01). In gp91phox−/− mice, withdrawal of atorvastatin was not associated with an attenuation of L-NA–induced constriction (n=8, P=NS) (Figure 3B).
Vascular Superoxide Anion Generation

Treatment of WT mice with atorvastatin had no effect on aortic superoxide anion generation, as measured with the use of lucigenin chemiluminescence (n=9, P=NS). However, when atorvastatin treatment was withdrawn for 4 days, lucigenin chemiluminescence was increased by 2-fold compared with the signal observed in control rings (n=9, P<0.05) (Figure 3C). Compared with aortic rings from WT animals, O$_2^-$ generation under basal conditions as well as after atorvastatin treatment was not different in rings from gp91phox$^{-/-}$ mice. In contrast to the data obtained in aortic rings from WT mice, no increase in O$_2^-$ generation on statin withdrawal was observed in aortic segments of gp91phox$^{-/-}$ mice (n=8, P<0.05) (Figure 3D).

Protein Expression in Aortic Rings

As determined in the Triton X-100–soluble protein fraction of homogenates of mice aortas, treatment with atorvastatin (10 mg/kg per day) or withdrawal for 5 days had no effect on the expression of Rac-1, eNOS, and the antioxidative enzymes catalase, ecSOD, and CuZnSOD. However, MnSOD expression was significantly attenuated by atorvastatin (P<0.05, n=5 each lane) (Figure 4).

Rac-1 localization and p22phox and gp91phox protein expression were studied in membrane preparations of mice aortas. For each lane, membrane protein of two complete aortas was pooled. Atorvastatin withdrawal had no effect of gp91phox and p22phox protein expression. Rac-1 protein appeared to be increased on withdrawal, although this did not reach statistical significance (n=3 lanes for each group, P=NS) (Figure 5).

Rac-1 Translocation in Cultured HUVECs

To further study the effect of statin withdrawal on Rac-1 translocation, HUVECs were treated with cerivastatin (100 nmol/L) or atorvastatin (10 μmol/L) for 24 hours. Thereafter, cells were repeatedly washed to remove the drugs, and Rac-1 localization was analyzed after 90 minutes. Whereas statin treatment had no overt effect on Rac-1 localization in HUVECs, statin withdrawal was associated with a translocation of Rac-1 to the plasma membrane (Figure 6).
Figure 7. Mechanisms of statin-induced NADPH oxidase activation. A, Effect of cerivastatin (Ceri, 100 nmol/L) and Ceri withdrawal on NADPH (100 μmol/L)-induced lucigenin (5 μmol/L) chemiluminescence in membrane preparation of HUVECs. Data are expressed relative to control values of the individual experiment (n=4). *P<0.05 vs control. B, Effect of Ceri (100 nmol/L) withdrawal (2 hours) and Rac-inactivating C difficile ToxB on DCHF oxidation in HUVECs. Values shown indicate the increase in the dichlorofluorescein (DCF) fluorescence per minute in arbitrary units (n=4). *P<0.05 vs control. C, Effect of Ceri (100 nmol/L), atorvastatin (Ator, 10 μmol/L), and GTPγS (100 μmol/L) on Rac expression and activity in HUVECs, as determined by Western blot analysis and PAK-GSH agarose absorption pull-down assay, as described in Materials and Methods. Blots shown are representative of 3 additional experiments. Pos denotes positive control (human leukocytes); CTL, control. D, Effect of Ceri (100 nmol/L) withdrawal on Rac activity in HUVECs. WB indicates Western blot.

NADPH-Induced Lucigenin Chemiluminescence in Cultured HUVECs

A 24-hour exposure of HUVECs to cerivastatin (100 nmol/L) induced a significant decrease in NADPH-induced lucigenin chemiluminescence in HUVEC membrane preparations. After statin withdrawal, the lucigenin signal transiently increased and was significantly higher than that obtained from control 2 hours after withdrawal (Figure 7A).

Dichlorofluorescein Fluorescence in Cultured HUVECs

To determine whether Rac is involved in the withdrawal-induced increase in oxygen radical generation formation, the effect of Clostridium difficile toxin B (ToxB) was tested. We and others have previously shown that this compound inhibits Rac-mediated activation of NADPH oxidase.18–20 In the present study, withdrawal of cerivastatin induced an ~2-fold increase in DCHF oxidation compared with control cells. A 2-hour preincubation with ToxB (2 μg/mL) completely inhibited this effect without affecting DCHF oxidation in control cells (Figure 7B).

Rac Activity Assay

To study the effect of statins on Rac activity, the interaction of Rac with PAK, which occurs only with active Rac, was tested. Statin treatment per se induced a pronounced increase in Rac activity, without affecting Rac expression (Figure 7C). After withdrawal of cerivastatin (Figure 7D) or atorvastatin (data not shown), Rac activity declined to control levels within 4 to 6 hours.

Discussion

In the present study, in vivo treatment of mice with statins improved, and the withdrawal of statins attenuated endothelium-dependent relaxation. Withdrawal of atorvastatin attenuated NO bioavailability and increased vascular superoxide anion generation in WT mice, whereas in gp91phox−/− mice, withdrawal was not associated with attenuation of NO bioavailability and endothelium-dependent relaxation or increased vascular superoxide anion generation. Statins increased Rac activity in cultured human endothelial cells, and statin withdrawal was associated with a translocation of Rac to the membrane. Moreover, inhibition of Rac prevented the statin-induced increases in oxygen radical generation in endothelial cells.

It is generally accepted that statins have beneficial cardiovascular effects that exceed what could be expected from the lowering of cholesterol plasma levels21 and that arise from the statin-mediated inhibition of de novo synthesis of farnesyl pyrophosphate and geranylgeranyl pyrophosphate.22 These compounds are required for the function of the small GTPases Ras, Rho, and Rac-1,23 which play a central role in cellular homeostasis and which functions are reported to be affected in cardiovascular diseases and inflammation.24

Little is known about the effects of statin withdrawal. It has been reported previously that withdrawal of atorvastatin results in a downregulation of eNOS expression and eNOS activity in homogenates of the aorta of SV129 mice, although the functional consequences of withdrawal remained unclear.25 In the present study, we did not observe an effect of statins on eNOS expression, a fact that may be related to the use of different mouse strains (C57 black b6). However, endothelium-dependent relaxation in mice was markedly attenuated after withdrawal of atorvastatin and cerivastatin, a phenomenon that can be attributed to an enhanced scavenging of NO by superoxide anions. Indeed, the antioxidant tiron completely restored endothelium-dependent relaxation in vessels from animals subjected to withdrawal. The fact that superoxide anion generation and the impairment of endothelium-dependent relaxation were not observed in gp91phox−/− mice after statin withdrawal indicates that the gp91phox-containing NADPH oxidase, which is present in endothelial cells15,26 and fibroblasts,26 plays a central role in this rebound phenomenon.
Two requirements have to be met for Rac-induced activation of target enzymes such as NADPH oxidase in the plasma membrane. First, active Rac has to be anchored in the membrane via its geranylgeranyl tail, a process that is inhibited by the statin-mediated depletion of geranylgeranyl pyrophosphate. Second, on activation, Rac has to bind GTP and dissociate from the Rac/Rho-GDI complex to allow interaction with its effector in the plasma membrane.19,23,27,28

It has previously been shown that statin treatment prevents anchoring of Rac and Ras in the plasma membrane.29,30 Because Rac-1 is critically involved in the activation of NADPH oxidase,29,30 it is tempting to speculate that statin withdrawal activated the NADPH oxidase via an overshoot activation or translocation of Rac-1. The observations of the present study support this assumption, inasmuch as withdrawal of statin treatment in cultured endothelial cells and also in the mouse aorta was indeed associated with such an enhanced translocation of Rac-1 to the plasma membrane. Moreover, whereas pretreatment with statins decreased the NADPH-dependent lucigenin chemiluminescence in the membrane fraction of endothelial cells, withdrawal was associated with a transient increase of the lucigenin signal in response to NADPH.

It is generally accepted that Rac activates the NADPH oxidase in many cells, including vascular tissue.18–20,27 The assumption that the withdrawal-induced radical generation can be attributed to a Rac-mediated activation of the oxidase is further supported by the observation that ToxB, which inhibits Rac, completely blocked the withdrawal-induced oxygen radical generation in the present study. It has previously been demonstrated that ToxB is as effective as dominant-negative Rac in inhibiting Rac-mediated effects but that it is certainly not as specific, inasmuch as ToxB also inhibits Ras and Rho.31 However, in this context, it should be noted that to our knowledge, the NADPH oxidase is the only oxygen radical–generating enzyme that is activated by one of these GTPases.

To understand the mechanism underlying the overshoot activation of Rac after statin withdrawal, Rac activity and expression in cell lysates were determined. Although statin treatment had no effect on Rac expression, atorvastatin, as well as cerivastatin, markedly increased Rac activity, an effect that slowly disappeared after statin withdrawal. We can only speculate about the mechanism underlying enhanced Rac activity during statin treatment. However, it has been reported that prenylation of Rac, which is also prevented by statin treatment, attenuates Rac binding to Rho-GDI,32 which usually keeps Rac in its inactive state. Likewise, it is conceivable that by reducing the membrane cholesterol content, statins alter membrane properties affecting caveolar integrity and membrane fluidity with consequences for receptor functions or function of membrane-bound Rac-GT-Pase–activating proteins. Despite the observed high Rac-PAK interaction in cell lysates, Rac-dependent signaling is inhibited by statins.30 However, this is a consequence of the failure to anchor Rac in the membrane, which is due to the lack of geranylgeranylation. After withdrawal, geranylgeranyl pyrophosphate becomes available, and the active Rac is anchored in the membrane and activates NADPH oxidase.

Statin treatment, per se, and deletion of gp91phox did not suppress oxygen radical generation in vascular segments and cultured cells, which is in line with previous studies from our group15 and others29 and most likely indicates that the NADPH oxidase is not active under resting conditions. Accordingly, after agonist stimulation,29 in hypertensive animals,30 or in vascular segments from hypercholesterolemic patients,33 statin treatment attenuated vascular superoxide anion generation. Different from the radical measurements in live tissue and cells, NADPH-induced lucigenin chemiluminescence in membrane fractions was significantly attenuated by statin pretreatment. Once again, changes in membrane composition may contribute to this phenomenon; alternatively, the expression of other oxidases that elicit lucigenin chemiluminescence in response to NADPH might be changed.

One drawback of the present study is that we were able to study the mechanism of Rac-dependent NADPH oxidase activation and the mechanism of Rac activation in cultured cells only because of the very low protein yield from mouse aortic tissue. Unfortunately, withdrawal-induced effects were much more rapid and transient in cultured cells compared with the effects in live animals. Although this might be attributed to the different pharmacokinetics of the two models, the possibility cannot be excluded that compared with the culture model, additional mechanisms may contribute to the activation of NADPH oxidase in vivo.

One finding of the present study was that atorvastatin downregulated MnSOD in the mouse aorta without affecting the expression of other antioxidative enzymes. Previously, it has been reported that Rac-1 and also the redox environment modulate MnSOD expression.34–36 suggesting that in the present study, statin-mediated inhibition of Rac-1 signaling is responsible for the downregulation of MnSOD expression. The observation that aortic MnSOD levels were not higher during withdrawal than during placebo treatment might be related to the early time point studied after withdrawal.

It is unlikely that modulation of MnSOD expression contributes to the withdrawal-mediated superoxide anion generation and inhibition of endothelium-dependent relaxation. Indeed, a role for MnSOD in the control of endothelium-dependent vasodilatation has not been demonstrated, and during atorvastatin treatment, when there was a clear improvement of endothelial function, MnSOD expression was markedly attenuated.

In conclusion, in the present study, we have demonstrated that withdrawal of statin treatment in mice impairs endothelium-dependent relaxation by increasing vascular superoxide anion generation via a pathway involving the Rac-dependent activation of the gp91phox-containing vascular NADPH oxidase. As a consequence of the different metabolism and, most notably, the statin-induced effects on the cholesterol plasma levels in humans, caution should be exerted when our findings are extrapolated to the situations in humans. However, the data presented clearly highlight the essential role of NADPH oxidase in controlling vascular function and NO bioavailability.
Acknowledgments
This study was supported by grants from the Deutsche Forschungsgemeinschaft (BR1839/1-1) and the Schaufller-Stiftung to Dr Brandes and a grant from the Italian Society of Hypertension to Dr Vecchione. We are indebted to the team of the animal care facility of Frankfurt University Hospital for breeding and maintaining the gp91phox−/− colony and to Sina Bätz, Tanja Mareczek, and Isabel Winter for excellent technical assistance.

References
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Circ Res. 2002;91:173-179; originally published online June 27, 2002;
doi: 10.1161/01.RES.0000028004.76218.B8

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