Withdrawal of Cerivastatin Induces Monocyte Chemoattractant Protein 1 and Tissue Factor Expression in Cultured Vascular Smooth Muscle Cells

R.P. Brandes, S. Beer, T. Ha, R. Busse

Objective—The withdrawal of 3-hydroxy-3-methylglutaryl–coenzyme A–reductase inhibitors (statins) deteriorates endothelial function. We determined in vascular smooth muscle cells whether statin withdrawal leads to the expression of proinflammatory genes involved in the development and progression of arteriosclerosis.

Methods and Results—The withdrawal of cerivastatin from pretreated vascular smooth muscle cells induced an increase in monocyte chemoattractant protein 1 (MCP-1) and tissue factor (TF) mRNA expression and enhanced MCP-1 secretion as well as cell surface TF activity. In the presence of cerivastatin, this effect was mimicked by geranylgeranyl pyrophosphate or mevalonate. Withdrawal-induced MCP-1 expression was sensitive to PD98059, SB203580, and diphenylene iodonium, suggesting an involvement of extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and the NADPH oxidase. Withdrawal increased the activity of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase and enhanced radical generation. Because the latter effect may result from an Rac-mediated activation of the NADPH oxidase, the effect of withdrawal on Rac translocation was studied. Statin treatment induced an increase in Rac-1 content in the cytoplasm. On withdrawal, however, an “overshoot” translocation of Rac to the plasma membrane occurred.

Conclusions—These observations suggest that statin withdrawal results in the activation of Rac and enhanced oxidative stress. The subsequent activation of redox-activated signal-transduction cascades results in the expression of MCP-1 and TF. (Arterioscler Thromb Vasc Biol. 2003;23:1794-1800.)

Key Words: statins • 3-hydroxy-3-methylglutaryl–coenzyme A–reductase inhibitors • tissue factor • arteriosclerosis • mitogen-activated protein kinase

3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA)–reductase inhibitors (statins) have multiple beneficial effects on the cardiovascular system and have been shown to improve endothelial function within a few days.1 Moreover, statins are known to inhibit the expression of monocyte-chemoattractant protein-1 (MCP-1)2,3 and tissue factor (TF).4,5 Both proteins play a central role in the development and progression of arteriosclerosis. MCP-1 contributes to the recruitment of monocytes to the vascular wall, and strategies to inhibit MCP-1 attenuate the development of arteriosclerosis in animal models.6 TF is thought to contribute to the thrombogenicity of arteriosclerotic lesions and to lesion progression, as well as vascular remodelling.7

It is a well-known phenomenon for some cardiovascular drugs that withdrawal can exert pronounced rebound symptoms. Recent studies also suggest that the withdrawal of statin therapy might be associated with a rebound phenomenon.8–10 Moreover, we reported that the withdrawal of statin therapy in mice leads to endothelial dysfunction.11 The mechanism underlying this observation was a withdrawal-induced Rho-mediated downregulation of endothelial nitric oxide (NO) synthase12 and a Rac-mediated activation of the NADPH oxidase, leading to scavenging of NO by oxygen-derived free radicals.11

The aim of the present study was to determine whether the removal of statins also induces relevant withdrawal effects in vascular smooth muscle cells (VSMCs), leading to the expression of a proarteriosclerotic phenotype in these cells.

Methods

Reagents
Cerivastatin was supplied by Bayer AG (Leverkusen, Germany). Atorvastatin was a gift from Goedecke AG (Freiburg, Germany). Lovastatin was from Merck Sharp Dohme Inc. (Haar, Germany). Unless otherwise stated, all chemicals were obtained from Sigma.

Cell Culture
Human aortic VSMCs were obtained from Clonetics. Rat VSMCs were isolated from the aortae of male Wistar rats by using the explant
technique. All experiments were performed with VSMCs from passages 3 to 11.

**Dichlorofluorescein Assay**

Human VSMCs were grown on glass cover slips, serum deprived, and incubated with cerivastatin (100 nmol/L) or atorvastatin (10 µmol/L) for 24 hours. In subgroups, statins were removed by repetitive washing of the cells. Subsequently, cells were loaded with dihydrodichlorofluorescein diacetate (10 µmol/L, Molecular Probes) and formation of dichlorofluorescein (DCF) was measured as described.\(^{13}\)

**Ethidium Assay**

Human VSMCs were grown on chamber slides, deprived of serum, and incubated with cerivastatin (100 nmol/L) for 24 hours. In subgroups, statins were removed by repetitive washing of the cells. Subsequently, cells were loaded with ethidium bromide (10 µmol/L, Molecular Probes) for 20 minutes and fluorescent images were acquired using a confocal microscope (Zeiss LSM 510 Meta, Ex 488, Em. 610).

**Apopotosis Assay**

Detection of cell death was performed by fluorescence-activated cell sorter (FACS) analysis by using annexin V-PE binding and 7-amino-actinomycin (7AAD) staining (Becton Dickinson Pharmingen) as described previously by others. Apoptotic cells were defined as annexin V-positive, 7AAD-negative cells. Necrotic cells were defined as annexin V-negative, 7AAD-positive cells.

**Northern Blot Analysis**

Cellular RNA was prepared according to standard protocols and Northern blot analysis was conducted as described.\(^{13}\) Human MCP-1 was detected using a \(^{32P}\)-labeled restriction fragment from the clone pXM-hJ3E4 (kindly provided by Dr B.J. Rollins). For rat MCP-1, a cDNA probe generated by reverse-transcription polymerase chain reaction was used.\(^{13}\) TF was detected by probes generated from human and mouse cDNA fragments (plasmids kindly provided by Dr. A. Bierhaus, University of Tubingen, Germany).

**Western Blot Analysis**

Determination of Rac localization in the cell was as follows: VSMCs were stimulated as indicated, washed with phosphate-buffered saline, lysed, and pottered in hypotonic lysis buffer (25 mmol/L Tris, 1 mmol/L EGTA and EDTA and protease inhibitors, pH 7.4, 4°C). The nuclear fraction was sedimented by centrifugation (3000 g, 10 minutes) and the membrane fraction was obtained by ultracentrifugation (100,000 g, 1 hour). The supernatant (cytoplasm) and the membrane fraction were subjected to Western blot analysis, and Rac was detected using a monoclonal antibody (Becton Dickinson Transduction). The detection of phosphorylated mitogen-activated protein (MAP) kinases was performed out using phosphospecific antibodies as reported previously.\(^{13}\)

**Procoagulant Activity**

The surface procoagulant activity of human VSMCs was determined essentially as described.\(^{15}\) Human VSMCs were treated with cerivastatin (30 mmol/L, 24 hours), thrombin (1 U/mL, 6 hours) or solvent. In subgroups, the statin was removed by repetitive washing of the cells. Three hours after withdrawal, VSMCs were washed with HEPES–Tyrode’s solution and incubated with human platelet poor plasma. The formation of thrombin was initiated by addition of CaCl\(_2\) (16.7 mmol/L). Aliquots were removed at different time points, and the formation of thrombin was determined by an activity assay with the help of a chromogenic substrate S-2238 (Hemochrom Diagnostica, Cologne).

**MCP-1 Enzyme Immunoassay**

Secretion of MCP-1 was determined from the media of rat VSMCs by using a commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, Mich). VSMCs were deprived of serum (12 hours) and stimulated with cerivastatin (1 µmol/L), atorvastatin (10 µmol/L), or lovastatin (10 µmol/L) for 12 hours after one additional exchange of the serum-free culture medium. Subsequently, cells were washed and incubated for 12 hours with serum-free medium with (treatment) and without statins (withdrawal) or thrombin (1 U/mL, positive control). Thereafter, MCP-1 release to the culture media was measured.

**Statistics**

Values are expressed as mean ± standard error of mean and were compared by Student \(t\) test or analysis of variance. A probability value less than 0.05 was considered significant. Blots shown are representative for at least 3 different independent experiments.

**Results**

**Effect of Cerivastatin Withdrawal on TF Expression**

In human and rat aortic VSMCs, basal expression of tissue factor mRNA was weak or not detectable. Treatment with either cerivastatin (0.1 to 10 µmol/L, 24 hours) or lovastatin (10 µmol/L, 24 hours) had no effect on tissue factor mRNA expression, whereas the positive control thrombin (1 U/mL) exerted a transient induction, which peaked 2 hours after stimulation.

Withdrawal of cerivastatin (0.1 µmol/L) or lovastatin (10 µmol/L) induced a transient increase in the expression of tissue factor mRNA, with a maximum after 3 hours (lovastatin: +94±31%, \(n=3\); cerivastatin: Figure 1A). Moreover, the withdrawal of statins resulted in a potentiation of the thrombin-induced expression of tissue factor (lovastatin: +161±45%, \(n=3\); cerivastatin: Figure 1A), whereas in the presence of statins, the thrombin-induced tissue factor mRNA induction was markedly attenuated (cerivastatin 0.1 µmol/L: −34%, 1 µmol/L: −77%, 10 µmol/L: −86%, lovastatin 0.1 µmol/L: −68.5%).

To determine whether the withdrawal-induced increase in tissue factor mRNA also increases tissue factor activity on the cell surface, the procoagulant activity of human SMCs was tested. In this assay, cerivastatin (30 nmol/L) had no effect on tissue factor-induced thrombin formation, whereas statin withdrawal increased the procoagulant activity (Figure 1B, control: 100%, thrombin stimulation: 181±42%, \(P<0.05\), cerivastatin 108±16%, \(P=\) ns, cerivastatin withdrawal 144±13%, \(P<0.05\), values are area under the curve for the first 10 minutes of the assay relative to the control values). The antioxidant vitamin C (100 µmol/L) and the Rac inactivating clostridium difficile lethal toxin B (20 ng/mL, 2 hours) blocked the withdrawal-induced increase in procoagulant activity (Figure 1B).

**Effect of Cerivastatin Withdrawal on MCP-1 Expression**

In human and rat aortic VSMCs, basal MCP-1 expression was detectable but unaffected by statin treatment. Withdrawal of cerivastatin induced a transient increase in MCP-1 mRNA expression, which was comparable with that achieved using a half-maximal effective dose of thrombin (1 U/mL, 3 hours). The thrombin-induced MCP-1 expression was markedly inhibited by cerivastatin (0.1 µmol/L, −87%). In contrast, cerivastatin withdrawal induced a potentiation of the MCP-1 mRNA-induction by thrombin (Figure 2A).
To demonstrate that statin withdrawal-mediated MCP-1 mRNA induction results in relevant protein expression, the MCP-1 concentration in the culture medium was determined. The positive control thrombin induced a pronounced increase in MCP-1 expression (Figure 2B). Incubation with the nuclear factor (NF)κB inhibitor pyrrolidinedithiocarbamate (10 μmol/L) also attenuated the withdrawal-induced MCP-1 expression (−34±8%, n=3, P<0.05).

Effect of Cerivastatin on MAP Kinase Phosphorylation

Because the effects of the MAP kinase inhibitors pointed toward a role of this pathway for the withdrawal-mediated gene induction, MAP kinase phosphorylation was studied. Cerivastatin per se had only a small effect on the low basal p38 MAP kinase and ERK1/2 phosphorylation and no effect on the expression of these kinases (data not shown). As reported previously,16,17 statin treatment, however, attenuated not farnesyl pyrophosphate induced a pronounced increase in MCP-1 mRNA expression (Figure 3A).

To determine further the signaling mechanism underlying withdrawal-induced MCP-1 expression, the involvement of MAP kinase pathways and of oxidative stress was studied. The extracellular signal-regulated kinase 1/2 (ERK1/2) kinase inhibitor PD98059 (50 μmol/L), the p38 MAP kinase inhibitor SB203580 (20 μmol/L), and the unselective NADPH oxidase inhibitor diphenylene iodonium (10 μmol/L) completely prevented the withdrawal-mediated MCP-1 mRNA induction under basal conditions and in cells stimulated with thrombin (Figure 3B). Incubation with the nuclear factor (NF)κB inhibitor pyrrolidinedithiocarbamate (10 μmol/L) also attenuated the withdrawal-induced MCP-1 expression (−34±8%, n=3, P<0.05).
agonist-induced p38 MAP kinase activation (data not shown). On withdrawal, a pronounced increase in the phosphorylation of both kinases was observed, peaking 2 hours after withdrawal. The same experiments were performed with subsequent stimulation of the cells with thrombin. Thrombin induced a marked increase in p38 MAP kinase phosphorylation, which was attenuated in the presence of cerivastatin. In contrast, in cells withdrawn of cerivastatin, the thrombin-induced p38 MAP kinase phosphorylation was markedly potentiated. Different effects were obtained for ERK1/2. Although thrombin induced a marked increase in ERK1/2 phosphorylation, this was completely unaffected by cerivastatin or cerivastatin withdrawal (Figure 4). Similar results were obtained using lovastatin (data not shown).

Effect of Cerivastatin on Cellular Reactive Oxygen Species (ROS) Generation
Because MAP kinases are known to be activated by H₂O₂, the effect of statin withdrawal on cellular H₂O₂ generation was studied using the DCF assay. In the presence of cerivastatin (0.1 μmol/L) and atorvastatin (10 μmol/L) H₂O₂ levels were not different to control conditions. After withdrawal, a more than 50% increase in cellular radical generation was observed (Figure 5A). To demonstrate that the increase in DCF fluorescence results from increased O₂⁻ formation, ethidium fluorescence was measured using confocal microscopy. In this assay, cerivastatin had little effect on dihydroethidium oxidation, whereas the withdrawal of the statin resulted in a marked increase in fluorescence (Figure 5B).

Effect of Cerivastatin on Apoptosis Rates
Statins have been shown to increase SMC apoptosis rates. Therefore, it is possible that the increased radical formation after withdrawal was a consequence of apoptotic cell death and that the changes in gene expression and MAP kinase phosphorylation reflect a selection bias resulting from excessive apoptosis after statin withdrawal. FACS analysis, however, revealed that statin withdrawal had no effect on apoptosis and that statin treatment per se increased apoptosis rates in the present study only from approximately 3% to 4.2% (Figure 6).

Effect of Cerivastatin on Cellular Rac Localization
One of the most important cellular sources of ROS formation is a Rac-activated NADPH oxidase, which is sensitive to diphenylene iodonium. Cerivastatin (1 μmol/L) induced a pronounced translocation of Rac from the membrane to the cytoplasm. Within 1 hour of withdrawal, Rac was translocated back to the membrane, and a significant increase in the membrane Rac content 2 and 3 hours after withdrawal was observed (Figure 7).

Discussion
In the present study, withdrawal of statins induced the expression of MCP-1 and TF in VSMCs via a pathway involving intermediates of the cholesterol de novo synthesis. Withdrawal-induced gene expression was sensitive to inhibitors of the MAP kinase pathways and an unspecific NADPH oxidase inhibitor. It was furthermore associated with an activation of MAP kinases and enhanced oxidative stress, as well as a translocation of Rac from the cytoplasm to the plasma membrane.

In several studies, statins prevented the expression of MCP-1 and TF in cell culture experiments, animal models of arteriosclerosis, and humans. Statins also have profound effects on cellular signaling, which are most likely a consequence of the statin-mediated inhibition of Rho-GTPases by preventing their anchoring in the plasma membrane. One of the best-characterized effects of statins in animal and cell culture models is the inhibition of the
phosphorylation of ERK1/2, an enzyme downstream of Ras, and the statin-mediated inhibition of the activation of p38 MAP kinase. Because ERK1/2 as well as the p38 MAP kinase pathway contribute to the formation of active AP-1 and NFκB, it is not surprising that statins also prevented the DNA binding of these transcription factors. Therefore, the inhibition of MCP-1 and TF induction, which are both under the control of NFκB and activator protein-1, indirectly occurs as a consequence of the statin-mediated inhibition of Rho-GTPases.

In this context, we focused on the Rho-GTPase Rac and confirmed that statin treatment results in a translocation of Rac from the membrane to the cytoplasm without affecting overall Rac level in the cell (data not shown). On removal of statins, Rac underwent a rapid, transient “overshoot” translocation back to the membrane, so that 2 and 3 hours after removal of the statin the membrane Rac content was higher than under control conditions.

Among many other functions, Rac is known to activate the NADPH oxidase. At least in SMCs, this enzyme is the major source of O$_2^-$ and inhibition of Rac blocked the protein phosphorylation of ERK1/2, an enzyme downstream of Ras, and the statin-mediated inhibition of the activation of p38 MAP kinase. Because ERK1/2 as well as the p38 MAP kinase pathway contribute to the formation of active AP-1 and NFκB, it is not surprising that statins also prevented the DNA binding of these transcription factors. Therefore, the inhibition of MCP-1 and TF induction, which are both under the control of NFκB and activator protein-1, indirectly occurs as a consequence of the statin-mediated inhibition of Rho-GTPases.

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kinase C as well as angiotensin II-induced ROS production.\textsuperscript{20} The observation that statins had no inhibitory effect on ROS generation in the present study is caused by the fact that quiescent, nonstimulated cells were used, and statins only prevent the agonist-induced and thus Rac-mediated ROS generation.\textsuperscript{20}

In the present study, withdrawal of cerivastatin or atorvastatin was associated with an almost doubling of cellular ROS production, suggesting that the overshoot translocation of Rac activates the NADPH oxidase. However, it cannot be excluded that statins activate other pathways of cellular ROS formation, particularly those in mitochondria, and that the Rac translocation is only a coincidental phenomenon. The NADPH oxidase inhibitor diphenylene iodonium, which attenuated withdrawal-induced gene expression in the present study, is unspecific and blocks all flavine-containing enzymes, including the respiratory chain of the mitochondria.\textsuperscript{23}

ROS alter the function of numerous enzymes involved in signal transduction pathways, including the p38 MAP kinase.\textsuperscript{24} We have previously demonstrated that the thombin-induced activation of p38 MAP kinase occurs as a consequence of the activation of the NADPH oxidase and the subsequent formation of ROS rather than by a direct activation of the kinase.\textsuperscript{13} The NADPH oxidase-mediated activation of p38 MAP kinase subsequently leads to the induction of MCP-1\textsuperscript{13} and TF.\textsuperscript{25}

In the present study, a similar pathway appears to underlie withdrawal-induced expression of MCP-1 because the inhibition of p38 MAP kinase and the NADPH oxidase inhibited the expression of these genes in response to withdrawal and because withdrawal was associated with a marked increase in p38 MAP kinase phosphorylation. The withdrawal-induced increase in p38 MAP kinase phosphorylation was observed under basal conditions, as well as under half-maximal stimulation with thrombin. Interestingly, withdrawal also increased the phosphorylation of ERK1/2, but this occurred only under basal conditions, whereas thrombin induced a pronounced increase in phosphorylation, which was unaffected by statin treatment or withdrawal. This observation was unexpected and might be restricted to high doses of statins or a specific type of cells because studies have demonstrated that statins have the potential to inhibit ERK1/2 activation.\textsuperscript{16,17} We and others have previously observed that although ERK1/2 can be activated by ROS, the angiotensin II- and thrombin-induced ERK1/2 activation is not mediated by the NADPH oxidase.\textsuperscript{13,24} Moreover, different from the thrombin-induced induction of MCP-1, which is insensitive to inhibitors of ERK1/2 activation,\textsuperscript{4,13} the withdrawal-induced expression of this gene was blocked by PD98059. This suggests that other mechanisms in addition to a direct Ras/Raf-MEK-mediated activation of ERK1/2 in response to withdrawal contribute to the induction of MCP-1. One possible explanation for this effect arises from the fact that via their inhibitory effects on NFκB and activator protein-1, statins also control the expression of numerous enzymes involved in signal transduction cascades. It has been demonstrated that lovastatin downregulates the protein phosphatase PP1. As PP1 dephosphorylates ERK1/2 kinase, the basic fibroblast growth factor-induced activation of ERK1/2 was observed to be enhanced rather than attenuated by lovastatin.\textsuperscript{26} It is conceivable that a similar phenomenon accounts for the observation made in the present study.

Little is known about the effects of statin withdrawal on cellular and vascular homeostasis. We and others have previously reported that statin withdrawal in mice attenuates NO bioavailability, an effect that occurs predominantly via 2 different but complementary pathways. An overshoot-activation of RhoA leads to the downregulation of endothelial nitric oxide synthase\textsuperscript{12} and an overshoot-activation of Rac activates the vascular NADPH oxidase, which leads to scavenging of NO by ROS.\textsuperscript{11} The observation that a withdrawal effect also occurs for the ERK1/2 pathway is indicative toward a similar overshoot activation of the other important small GTPase, Ras, in response to statin withdrawal.

Certainly, it is difficult to judge the clinical relevance of the present study because the effect of statin withdrawal was only studied in cultured cells by using high doses of statins. However, it should be mentioned that a growing body of evidence supports a relevance for the situation in humans because removal of statin therapy was associated with an increase in myocardial infarction rate, unfavorable outcome after myocardial infarction and rapid, but transient, alterations in peripheral blood flow control.\textsuperscript{8–10}

In conclusion, in the present study, we have demonstrated that withdrawal of statin treatment in VSMCs induces MCP-1 and tissue factor expression via an activation of ERK1/2, p38 MAP kinase, and NADPH oxidase.

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