Double-Edged Role of Statins in Angiogenesis Signaling

Carmen Urbich, Elisabeth Dernbach, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) exert potent vasculoprotective effects. However, the potential contribution to angiogenesis is controversial. In the present study, we demonstrate that atorvastatin dose-dependently affects endothelial cell migration and angiogenesis. In vivo relevant concentrations of 0.01 to 0.1 μmol/L atorvastatin or mevastatin promote the migration of mature endothelial cells and tube formation. Moreover, atorvastatin also increases migration and the potency to form vessel structures of circulating endothelial progenitor cells, which may contribute to vasculogenesis. In contrast, higher concentrations (>0.1 μmol/L atorvastatin) block angiogenesis and migration by inducing endothelial cell apoptosis. The dose-dependent promigratory and proangiogenic effects of atorvastatin on mature endothelial cells are correlated with the activation of the phosphatidylinositol 3-kinase–Akt pathway, as determined by the phosphorylation of Akt and endothelial NO synthase (eNOS) at Ser1177. In addition, the stimulation of migration and tube formation was blocked by phosphatidylinositol 3-kinase inhibitors. In contrast, the well-established stabilization of eNOS mRNA was achieved only at higher concentrations, suggesting that posttranscriptional activation rather than an increase in eNOS expression mediates the proangiogenic effect of atorvastatin. Taken together, these data suggest that statins exert a double-edged role in angiogenesis signaling by promoting the migration of mature endothelial cells and endothelial progenitor cells at low concentrations, whereas the antiangiogenic effects were achieved only at high concentrations. (Circ Res. 2002;90:737-744.)

Key Words: angiogenesis  ■ 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors  ■  endothelium  ■  progenitor cells

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been developed as lipid-lowering drugs. However, recent experimental and clinical trials have demonstrated that statins also exert vasculoprotective effects independent of cholesterol lowering. For example, statins protect against ischemia-reperfusion injury of the heart in normcholesterolemic animals, reduce vascular inflammation, and enhance coronary blood flow in patients. Moreover, statins have recently been shown to stimulate the growth of new blood vessels in ischemic limbs of rabbits. The improvement of blood vessel growth, which is achieved in normcholesterolemic animals, may contribute to the beneficial cholesterol-independent effects of statin treatment. The mechanisms underlying the pleiotropic effects of statins are not completely understood, but several studies have suggested that statins may improve endothelial function through NO-dependent pathways. Thus, simvastatin activates the protein kinase Akt, which leads to the posttranscriptional activation of the endothelial NO synthase (eNOS) via phosphorylation of the amino acid Ser1177. Akt-dependent phosphorylation and activation of eNOS have previously been described to mediate vascular endothelial growth factor (VEGF)-induced migration of mature endothelial cells. In contrast to these findings, a recent study has demonstrated that cerivastatin inhibits endothelial cell migration and angiogenesis. However, the antiangiogenic effects of cerivastatin were observed with concentrations that by far exceeded the serum levels achievable in patients treated with cerivastatin. Therefore, to reconcile these conflicting data with the well-established clinical benefit of statin treatment, we investigated the dose-dependent effect of atorvastatin on endothelial cell migration and angiogenesis. In addition, we further elucidated the effect of atorvastatin on the functional activity of circulating bone marrow–derived endothelial progenitor cells (EPCs), which are known to contribute to vasculogenesis.

The results of the present study demonstrate that low doses of atorvastatin (0.01 to 0.1 μmol/L) promote the migration of mature endothelial cells and EPCs and further stimulate tube formation, as assessed by a human angiogenesis assay. In contrast, higher concentrations of atorvastatin (>0.1 μmol/L) exert antiangiogenic effects by inducing endothelial cell apoptosis.

Material and Methods

Cell Culture

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems/Clonetics and cultured in endothelial basal medium (EBM, Clonetics) supplemented with hydrocortisone...
(1 μg/mL), bovine brain extract (3 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 μg/mL), and 10% FCS until the third passage. After detachment with trypsin, cells (4.0×10^6 cells) were grown on 6-cm cell culture dishes for at least 18 hours. Ly294002 (Biomol), mevalonate (Fluka), N^6-monomethyl-l-arginine (LMAA, Alexis), Z-Val-Ala-Asp(DL)-fluoromethylketone (ZVAD, Bachem), HA1077 (Calbiochem), and Y27632 (Calbiochem) were preincubated 30 minutes before stimulus. Atorvastatin was kindly donated by Goedecke/Parkin, Freiburg, Germany. Mevastatin and simvastatin were activated as previously described.22

**Scratched Wound Assay**

The migration of HUVECs was detected by using a "scratched wound assay." In vitro scratched wounds were created by scraping cell monolayers with a sterile disposable rubber policeman.23,24 Cells 10^6 mononuclear cells were plated on 24-well plates after isolation, 4°C overnight. Cells remaining in the lower chamber were counted manually in three random microscopic fields.25,26

**EPC Culture Assay**

Mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrom KG) from 20 mL of peripheral blood of healthy human volunteers according to Vasa et al.23 Immediately after isolation, 4×10^5 mononuclear cells were plated on 24-well culture dishes coated with human fibronectin and gelatin (Sigma Chemical Co) and maintained in EBM (Cell Systems) supplemented with EGFM SingleQuots (Cell Systems/Clonetics) and 20% FCS. After 3 days in culture, nonadherent cells were removed by thorough washing with PBS, and adherent cells were incubated with atorvastatin for 24 hours before investigation of EPC migration or apoptosis by fluorescence-activated cell sorter (FACS) analysis. EPCs were characterized by dual staining for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated LDL and lectin by the expression of endothelial marker proteins VEGF-R2 (kinase domain receptor [KDR]), vascular endothelial (VE)-cadherin, eNOS, and von Willebrand factor.26

**EPC Migration Assay**

Isolated EPCs were preincubated for 24 hours with atorvastatin. EPCs were detached by using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μL EBM, counted, and plated in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing EBM and human recombinant VEGF (50 ng/mL). After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4',6-diamidino-2-phenylindole. Migrating cells into the lower chamber were counted manually in three random microscopic fields.23,26

**FACS Analysis**

The adherent cells were detached with trypsin, washed in PBS, and incubated with 2.5 μL annexin-phycocythrin and 2.5 μL 7-aminoactinomycin for 15 minutes at room temperature according to the manufacturer (Annexin V-Phcycoerythrin Apoptosis Kit, Pharmingen) and analyzed by FACS with the use of a FACS SCAN flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences).

**Angiogenesis Assay**

The human angiogenesis assay was performed according to the instructions of the manufacturer (Cell Systems/Clonetics). Briefly, human endothelial cells were cocultured with human fibroblasts in a culture matrix. Medium was changed, and the cells were stimulated at days 1, 4, 7, and 9. VEGF (10 ng/mL) was used as a positive control, and suramin (20 μmol/L) was used as a negative control. Cells were fixed and stained at day 11 with an anti-CD31 antibody, and a secondary antibody conjugated with alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium was used as a substrate. Angiogenesis was quantified by measuring tube length with a computer-assisted microscope.

**Western Blot Analysis**

For determination of eNOS and the phosphorylated form of Akt, HUVECs were incubated with 200 μL lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) for 15 minutes on ice. After centrifugation for 15 minutes at 20 000g (4°C), the protein content of the samples was determined according to the Bradford method. Proteins (40 μg per lane) were loaded onto SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Western blots were performed by using antibodies directed against phospho-Akt (Ser473, 1:500, Pharmingen), phospho-eNOS (Ser1177, 1:1000), or eNOS (1:5000, Transduction Laboratories). Enhanced chemiluminescence was performed according to the instructions of the manufacturer (Amersham). Then, the blots were reprobed with total Akt (1:1000, Transduction Laboratories), eNOS, or actin (1:4000, Sigma). The autoradiographies were scanned and semiquantitatively analyzed.

**Plasmid Transfection**

Dominant-negative RasN17 was created by site-directed mutagenesis and cloned into a pcDNA3.1 vector (Invitrogen). Transient transfection of HUVECs was performed by incubation of 3.7×10^5 cells per 6-cm well with 3 μg of plasmid, as described previously,11 with the use of Superfect according to the instructions of the manufacturer (Qiagen).

**Statistical Analysis**

Data are expressed as mean±SEM from at least three independent experiments. Statistical analysis was performed by t test. ANOVA was performed for serial analyses.

**Results**

**Effect of Statins on Endothelial Cell Migration**

HUVECs were incubated with different concentrations of atorvastatin, and migration was assessed by a scratched wound assay. Concentrations of 0.0001 to 0.1 μmol/L increased endothelial cell migration, with a maximal effect at 0.01 μmol/L (Figure 1A). In contrast, increasing the concentration to 1 μmol/L significantly inhibited endothelial cell migration. Similar effects were observed with mevastatin and simvastatin, which increased cell migration at low concentrations (for 0.01 μmol/L, 1.52±0.03 and 1.51±0.03 mm, respectively, versus 1.01±0.02 mm in controls) and inhibited cell migration at high concentrations (for 10 μmol/L, 0.69±0.01 and 0.63±0.02 mm, respectively). To characterize the signaling pathways involved in the promigratory activity of atorvastatin, endothelial cells were cocultivated with the end product of HMG-CoA reductase, mevalonate, which...
Effect of Atorvastatin on Endothelial Cell Apoptosis

To determine whether the inhibitory effect of high concentrations of atorvastatin on endothelial cell migration might be due to the induction of apoptosis, endothelial cell apoptosis was determined by annexin staining. Atorvastatin and mevastatin induced respective 2.6-fold and 1.6-fold increases in the apoptosis of HUVECs (see online Figure 1). In contrast to mature endothelial cells, no inhibitory effects were observed in EPCs at high concentrations of statins (Figure 3A). In line, no apoptosis induction was shown in EPCs treated with high concentrations of atorvastatin or mevastatin (see online Figure 1). Mevalonate, the PI3K inhibitor Ly294002, or LNMA reversed the stimulatory effect of statins (Figure 3A). Furthermore, inhibition of the PI3K pathway by Ly294002 completely blocked atorvastatin-induced tube formation even below basal levels (Figures 2A and 2C), suggesting an important role of the PI3K pathway for atorvastatin-induced angiogenesis.

Effect of Statins on Migration of EPCs

Because recent studies have provided increasing evidence that EPCs contribute to postnatal vasculogenesis, we further investigated the dose-dependent effects of atorvastatin on EPC migration in a modified Boyden chamber. Atorvastatin and mevastatin induced a dose-dependent increase in EPC migration, with a maximal effect at 0.01 μmol/L (Figure 3B). Interestingly, the addition of mevalonate reversed the stimulatory effect of atorvastatin (Figure 3A and data not shown). In contrast to mature endothelial cells, no inhibitory effects were observed in EPCs at high concentrations of statins (Figure 3A). In line, no apoptosis induction was shown in EPCs treated with high concentrations of atorvastatin or mevastatin (see online Figure 1). Mevalonate, the PI3K inhibitor Ly294002, or LNMA reversed the stimulatory effect of statins (Figure 3B). Furthermore, overexpression of a dominant-negative Akt construct or pharmacological inhibition of NO synthesis by LNMA blocked the atorvastatin-induced endothelial cell migration (Figure 1B, data not shown), demonstrating the involvement of the PI3K-Akt pathway.

Effect of Atorvastatin on Activation of Akt and eNOS Expression

Statins are known to affect endothelial NO synthesis by increasing eNOS mRNA stability and posttranscriptional stimulation of eNOS via Akt-dependent phosphorylation. Therefore, we investigated the dose-dependent effects of atorvastatin on Akt phosphorylation, eNOS Ser1177 phosphorylation, and eNOS expression. As shown in Figure 5, incubation of atorvastatin for 1 hour induces phosphorylation of Akt and eNOS even at very low concentrations (0.0001 μmol/L). Coincubation with the
PI3K inhibitor Ly294002 inhibits the atorvastatin-induced phosphorylation of Akt, demonstrating the specific effect of atorvastatin on the activation of the PI3K-Akt pathway (Figure 5A). Prolonged incubation of atorvastatin for 24 hours also increased eNOS expression (Figures 5C and 5D). However, increases in eNOS protein expression were observed only at concentrations of 1 to 5 μmol/L atorvastatin, whereas doses of atorvastatin <1 μmol/L did not significantly increase eNOS expression (Figures 5C and 5D).

**Statin-Induced Akt Activation Involves the Small GTP-Binding Protein Ras**

Having demonstrated that low concentrations of statins activate the PI3K-Akt-eNOS pathway, we investigated the potential upstream signaling cascades. The small GTP-binding protein Ras in its activated GTP-bound form was shown to directly bind to the p110 subunit of PI3K and, thereby, to activate PI3K.32,33 Therefore, we investigated whether statins might activate Ras. Indeed, low concentrations of atorvastatin stimulated the activity of Ras, starting after 30 minutes of incubation (Figure 6A). The activation of Ras is followed by the stimulation of Akt phosphorylation, which is significantly increased after 1 hour of treatment (Figure 6B). Moreover, overexpression of a dominant-negative Ras inhibited atorvastatin-induced Akt phosphorylation (Figure 6C). Dominant-negative Ras but not the Rho kinase inhibitor HA1077 or Y27632 prevented atorvastatin-induced endothelial cell migration (Figures 6D and 6E). Moreover, Ly294002 did not affect statin-induced Ras activation (data not shown). These data suggest that low concentrations of atorvastatin stimulate the small GTP-binding protein Ras, which acts upstream from PI3K.

**Discussion**

The data of the present study demonstrate that atorvastatin exerts a dose-dependent effect on endothelial cell migration
and angiogenesis. Low concentrations (between 0.001 and 0.1 \( \mu \)mol/L) potently promote endothelial cell migration and angiogenesis. In contrast, increased concentrations exert an opposite effect. Thus, migration of mature endothelial cells and tube formation in the human angiogenesis assay are significantly impaired by 1 \( \mu \)mol/L atorvastatin. This is in accordance with a recent publication by Vincent et al, who demonstrated an antiangiogenic effect of cerivastatin with high concentrations (25 ng/mL). The antimigratory effect achieved by 1 \( \mu \)mol/L atorvastatin in mature endothelial cells in the present study appears to be caused by the induction of the apoptotic cell death program. Thus, apoptotic endothelial cells were detectable in endothelial cell cultures and in the human angiogenesis assay (data not shown). Furthermore, a causal role of apoptosis in mediating the antimigratory effect of high statin concentrations was suggested by the finding that inhibition of apoptosis by a caspase-blocker reverses the antimigratory effects of atorvastatin.

In addition, low concentrations of statins not only influence mature endothelial cells but also increase the migratory capacity of EPCs and promote EPC-induced tube formation. These in vitro data are in line with a recently performed clinical study that demonstrates an increase in EPC migration by statin treatment in patients with stable coronary artery diseases. The improvement of EPC function by statins may further contribute to neovascularization after ischemia. Interestingly, the dose-response curve of atorvastatin differs for EPCs and mature endothelial cells. Thus, EPCs seem to be more resistant to the inhibitory effect of statins and were not rendered apoptotic in the concentrations investigated. These findings are in accordance with a recent study by Llevadot et al, who demonstrated an antiapoptotic effect of simvastatin on EPCs. The higher Akt activation induced by high concentrations of atorvastatin in EPCs (≈2.5-fold in EPCs versus 1.8-fold in HUVECs) may contribute to the difference in apoptosis sensitivity of the two cell types. However, it is also tempting to speculate that the substrates of Akt, which include the proapoptotic transcription factors of the forkhead family, might be differentially expressed in HUVECs versus EPCs. Thus, although various control experiments confirmed...
the endothelial phenotype of the cultivated EPCs, there may be differences in apoptosis signaling of EPCs compared with mature endothelial cells. Given the concentration-dependent double-edged effect of atorvastatin on angiogenesis and endothelial cell migration, it remains to be determined which effects would be likely to be relevant in vivo. The maximal atorvastatin plasma concentrations are \(0.04 \mu mol/L\) with the use of therapeutically relevant doses of 20 mg/d, ie, in a concentration range for which proangiogenic and promigratory effects have been detected in the present study. \(0.01 \mu mol/L\) for 1 hour. Phosphorylation of Akt was detected by Western blot analysis. Akt served as a loading control. A representative blot of 3 independent experiments is shown. C, HUVECs were transfected with vector or dominant-negative RasN17 and incubated in the presence or absence of atorvastatin (0.01 \(\mu mol/L\)) for 24 hours. Cell migration was detected by using a scratched wound assay. Data are mean±SEM (n=3 to 6).

The mechanism by which statins stimulate angiogenesis requires further investigation. In accordance with the study by Kureishi et al, our data suggest that the PI3K pathway plays an essential role in migration and tube formation. The activation of the Akt pathway appears to involve the small GTP-binding protein Ras. These results are surprising and unexpected, because various studies have demonstrated that statins prevent the geranylgeranylation of small GTP-binding proteins (namely, Rho), thereby inhibiting its function. However, the inhibitory effects of Rho are achieved only when high concentrations of statins are used and are not involved in statin-induced Akt activation and cell migration. This is evidenced by the finding that two different Rho kinase inhibitors revealed no effect (Figure 6E) and that statins are effective at concentrations that do not increase the eNOS level, a process well established to be caused by Rho inhibition. How statins activate Ras and the PI3K-Akt pathway remains to be determined. Although the effects of statins are reversed by mevalonate, which implicates the involvement of HMG-CoA reductase inhibition, we cannot rule out the possibility that statins have additional effects beyond the enzymatic inactivation of HMG-CoA reductase. An HMG-CoA reductase–independent effect of statins is...
supported by the activating effects of low statin concentrations on Ras, which would be assumed to be inhibited as a consequence of HMG-CoA reductase blockade. Interestingly, a very recent report by the Walsh group (Soltys et al.17) demonstrates that fatty acids can block Akt activation. Therefore, one may speculate that statins somehow interact with lipid signaling. Given the complexity of the potential effector pathways, further studies are necessary to determine the molecular mechanisms for activation of the PI3K-Akt pathway by statins.

The downstream effector pathways of PI3K appear to include the serine/threonine kinase Akt, which is phosphorylated in response to atorvastatin. Akt may, in turn, activate endothelial NO synthesis, which is known to be essential for migration and angiogenesis.38 However, other studies also suggest an NO-independent pathway by which Akt can stimulate endothelial cell migration.14 This may be well rationalized by the effects of Akt on other substrates, eg, the glycogen synthase kinase-3 or forkhead transcription factors.39,40 In addition to the stimulation of Akt, statins have demonstrated antiapoptotic effects, suggesting that Akt phosphorylation and, thus, posttranscriptional activation of the eNOS may generate sufficient amounts of NO. Indeed, Kureishi et al.18 could not find any increase in eNOS mRNA or protein levels, although statin treatment profoundly increased neovascularization in the ischemic hindlimb model in vivo. As such, increased mRNA or protein levels of eNOS are unlikely to contribute to the improved neovascularization in mice after statin treatment. However, this does not exclude the possibility that some pathophysiological conditions in which eNOS protein levels are downregulated (eg, in aging or in the presence of proinflammatory cytokines), statins might have additional beneficial effects via increasing eNOS expression in combination with the posttranscriptional activation of the enzyme.

Strikingly, even the proapoptotic concentrations of atorvastatin lead to an increase in eNOS protein levels and Akt phosphorylation. One may speculate that this cytotoxic effect may become apparent when the Rho GTPases are completely inhibited by high statin concentrations, inasmuch as these proteins are essential mediators regulating cell growth, cytoskeletal organization, and apoptosis.36-41,42 However, the pharmacological Rho kinase inhibitor HA1077 alone did not induce apoptosis of endothelial cells (data not shown), indicating that the proapoptotic effect of statins relies not only on the inhibition of Rho but more likely involves the impairment of additional downstream signaling pathways.

Taken together, the present study demonstrates that atorvastatin exerts proangiogenic and antiangiogenic effects, depending on the concentration used. Whereas low concentrations, which resemble the plasma concentrations in patients on chronic statin therapy, improve migration and the sprouting of mature endothelial cells and EPCs, high concentrations potently prevent migration by inducing endothelial cell apoptosis.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 553, B6, and D6002/2-4). We would like to thank Melanie Nährer for technical help.

References

19. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearney M, Magner M, Isner JM. Bone marrow origin of endothelial...


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Circ Res. 2002;90:737-744; originally published online February 28, 2002;
doi: 10.1161/01.RES.0000014081.30867.F8
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/90/6/737

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Data Supplement:

Manuscript MS#3009:

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Figure 1 online: Effect of atorvastatin on apoptosis of HUVEC and EPC

HUVEC or EPC were incubated with atorvastatin for 24 h and apoptosis was measured by annexin staining as described in the Material and Method Section. Data are the mean ± SEM, n=4, *p<0.05 versus HUVEC control.