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Simvastatin Improves Disturbed Endothelial Barrier Function

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**Background**—Recent clinical trials have established that inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (statins) reduce the risk of acute coronary events. These effects of statins cannot be fully explained by their lipid-lowering potential. Improved endothelial function may contribute to the positive effects of statin treatment.

**Methods and Results**—In the present study, we report that simvastatin reduces endothelial barrier dysfunction, which is associated with the development of atherosclerosis. Treatment of human umbilical vein endothelial cells for 24 hours with 5 μmol/L simvastatin reduced the thrombin-induced endothelial barrier dysfunction in vitro by 55±3%, as assessed by the passage of peroxidase through human umbilical vein endothelial cell monolayers. Similar effects were found on the thrombin-induced passage of 125I-LDL through human aortic endothelial cell monolayers. This reduction in barrier dysfunction by simvastatin was both dose and time dependent and was accompanied by a reduction in the thrombin-induced formation of stress fibers and focal adhesions and membrane association of RhoA. Simvastatin treatment had no effect on intracellular cAMP levels. In Watanabe heritable hyperlipidemic rabbits, treatment for 1 month with 15 mg/kg simvastatin reduced vascular leakage in both the thoracic and abdominal part of the aorta, as evidenced by the Evans blue dye exclusion test. The decreased permeability was not accompanied by a reduction of oil red O–stainable atherosclerotic lesions.

**Conclusions**—These data show that simvastatin, in a relatively high concentration, improves disturbed endothelial barrier function both in vitro and in vivo. The data also support the beneficial effects of simvastatin in acute coronary events by mechanisms other than its lipid-lowering effect. (Circulation. 2000;102:2803-2809.)

**Key Words:** cells ■ statins ■ endothelium ■ thrombin

**Recent clinical trials have shown that statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis in the liver, are effective in the prevention of acute coronary events. Further analysis of these studies suggests that the benefits of statin therapy cannot be fully explained on the basis of reductions in plasma cholesterol levels.**

**Experimental atherosclerosis in hypercholesterolemia models is associated with changes in endothelial integrity (see review).** Additionally, during the development of atherosclerosis, the permeability of the vessel wall for LDL increases (see review). Because gross endothelial loss probably only occurs very late in the development of atherosclerosis, dysfunction of the intact endothelial layer provides a more likely explanation for the increase in permeability.

The endothelial actin cytoskeleton is important in maintaining the structural integrity of the endothelium. The junction-associated actin filament system forms a dense peripheral band of F-actin and is the most prominent assembly in the majority of endothelial cells. Activation of the endothelium by inflammatory mediators results in a contraction process at the margins of the cell. Small gaps between neighboring cells are formed, increasing the permeability of the endothelium. In vitro, these changes in permeability are accompanied by the formation of long F-actin filaments or stress fibers. In vivo, endothelial stress fibers are found in areas of altered flow. The function of these stress fibers is not precisely known at the moment, but they could have contractile properties.

In the present study, we investigated whether simvastatin could improve disturbed endothelial barrier function, in both in vitro and in vivo models of endothelial perturbation.

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Methods

Materials
Tissue culture plastics and Transwells (diameter 0.65 cm, pore size 3 μm) were obtained from Corning Costar; cell culture reagents were as described previously.2 Bovine thrombin was from Leo Pharma-ceutical Products. Horseradish peroxidase (HRP), Evans blue, oil red O, and anti-vinculin immunoglobulin were obtained from Sigma Chemical Co. Rhodamine-phalloidin was from Molecular Probes. Rabbit anti-mouse IgG-FITC was from Dakopatts. Simvastatin-lactone used in the in vitro studies was from Merck, Sharp & Dohme, and simvastatin-lactone used in the in vivo studies was from Sankyo.12,13 I was purchased from Amersham. Pyrogen-free human serum albumin (HSA) was from the Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands.

Cell Culture and Evaluation of Barrier Function In Vitro
Human endothelial cells were isolated and cultured as described previously.13 Cells were seeded at high density and grown on fibronectin-coated polycarbonate filters of the Transwell system for 4 to 6 days until highly confluent monolayers were obtained. Culture medium was renewed every other day (medium 199, 10% human serum, 10% newborn calf serum, 150 μg/mL crude endothelial cell growth factor, 5 U/mL heparin, 100 IU/mL penicillin, and 100 μg/mL streptomycin). Subsequently, cells were treated with simvastatin (0.1 to 5.0 μmol/L) for the indicated time points (usually 24 hours) in culture medium. After treatment with simvastatin, cells were washed with medium 199/1% HSA to remove serum, incubated for 1 hour in medium 199/1% HSA, and subsequently stimulated with thrombin. Barrier function was evaluated by the transfer of HRP for 1 hour in medium 199/1% HSA, and subsequently stimulated were washed with medium 199/1% HSA to remove serum, incubated 24 hours) in culture medium. After treatment with simvastatin, cells

Preparation and Iodination of LDLs
LDL was isolated from fresh serum prepared from the blood of healthy volunteers by gradient ultracentrifugation13 and labeled with 125I as previously described.12

Extraction and Assay of Intracellular cAMP Levels
Intracellular cAMP levels were determined by radioimmunoassay from Amersham as described previously.8,11

Immunocytochemistry
The presence of vinculin and F-actin was visualized by indirect immunofluorescence with mouse anti-vinculin antibody and by direct staining with rhodamine-phalloidin with use of a confocal laser scan microscope (type TCS 4D, Leica Heidelberg). Overlaying of pictures was accomplished with Photo-Paint software (version 6.00, 1995, Corel Co).

Detection of Cytosolic and Membrane-Bound RhoA
Confluent endothelial monolayers were preincubated for 24 hours with 5 μmol/L simvastatin in normal culture medium. One hour before lysis, the cell culture medium was replaced by medium 199/1% HSA. After they were washed with PBS, the cells were lysed in 300 μL/10 cm2 ice-cold hypotonic lysis buffer (5 mmol/L Tris-HCl, pH 7.0, 5 mmol/L NaCl, 1 mmol/L CaCl2, 2 mmol/L EGTA, 1 mmol/L MgCl2, 2 mmol/L dithiothreitol, and freshly added protease inhibitors [Complete, Boehringer]) and incubated for 30 minutes on ice. Cells were scraped and sucked 5 times through a 25-gauge needle. Membrane and cytosolic fractions were separated by centrifugation at 100,000g for 1 hour. The pellet was dissolved in an equal volume of lysis buffer+1% Triton X-100. Proteins were separated on SDS-PAGE and transferred to polyvinylidine difluoride, and immunoblotting was performed with the use of an antibody to RhoA (Santa Cruz Biotechnology Inc). Immunodetection was accomplished with the use of a goat anti-rabbit secondary antibody and an enhanced chemoluminescence kit (ECL kit, Amersham Corp).

Animals
Sixteen-month-old Watanabe heritable hyperlipidemic rabbits were obtained from the Oriental Yeast Co, Tokyo, Japan. They were housed individually and fed standard rabbit chow ad libitum, with water supplied ad libitum. For 4 weeks before euthanasia, the animals received simvastatin-lactone (15.0 mg/kg body wt) daily, in addition to the standard rabbit chow.

Cholesterol Measurement
Total serum cholesterol was determined by using a commercially available enzymatic kit (Roche Diagnostics).

Evaluation of Vascular Leakage and Atherosclerosis
Animals were sedated with Hypnorm (0.4 mL/kg IM; Janssen Pharmaceutical), mildly anticoagulated with heparin (500 U/rabbit IV), and euthanized with pentobarbital sodium (2.5 mL/kg IV). Aortas were rapidly removed and cleaned from the adjacent tissue. They were perfused with medium 199 buffered with Hanks’ salts, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% HSA to remove blood cells; subsequently, with Evans blue in medium 199 (0.3% [wt/vol]) for 5 minutes; and finally, with medium 199 to remove unbound Evans blue. They were then fixed with 3.7% buffered formaldehyde. Aortas were opened longitudinally and photographed en face with use of a digital Nikon Coolpix 900 camera. Evans blue staining was quantified 4-fold with Tina image analysis software (Raytest), and optical density was expressed in arbitrary units per square millimeter. Subsequently, the extent of atherosclerosis was assessed by staining the Evans blue–treated aortic segments with oil red O and evaluated.

Statistical Analysis
Data are reported as mean±SEM. Comparisons between >2 groups were made by 1-way ANOVA, followed by a Bonferroni-adjusted χ2 test. Comparisons of time curves of 2 groups were made by repeated-measures MANOVA, and individual group comparisons were performed by a Student t test for post hoc comparisons of the means. Differences were considered significant at the P<0.05 level.

Results
Effects of Simvastatin on In Vitro Endothelial Barrier Function
Confluent HUVEC and human aortic EC monolayers were incubated for 24 hours in medium 199 containing 5 μmol/L simvastatin in the presence of serum to evaluate the effect on cell morphology (Figure 1). Simvastatin-treated ECs remained tightly confluent, but cells were slightly elongated (which was most easily visible in aortic monolayers) with a condensed cytoplasm around the nucleus. To test whether simvastatin influences endothelial barrier function, HUVECs were grown to confluence on porous filters, and the passage of a marker protein (HRP, 42 to 44 kDa) was measured. Pretreatment with 5 μmol/L simvastatin...
Simvastatin reduces endothelial permeability

Effect of Simvastatin on Morphology of HUVECs

For 24 hours slightly increased HRP passage. However, the thrombin-induced HRP passage was significantly decreased (Figure 2A). This simvastatin pretreatment inhibited thrombin-induced HRP passage by 55±3% during a 1-hour incubation period (mean±SEM, 5 different cultures in triplicate). The reduction was concentration dependent (Figure 2B) and was maximal at 5 μmol/L simvastatin. Higher concentrations were not used because they affected basal EC barrier function. Lower concentrations of simvastatin required longer incubation periods. Preincubation with 0.5 μmol/L simvastatin for 96 hours reduced the thrombin-enhanced HRP passage by 35±5% (mean±SEM; 6 filter cultures of 2 different donors, Figure 2C). Coincubation with mevalonate fully abolished the effect of simvastatin on cell morphology (not shown) and thrombin-induced HRP passage (Figure 2B), indicating that simvastatin reduced the thrombin-induced HRP passage by inhibition of HMG-CoA reductase. Simvastatin inhibited the thrombin-induced HRP passage in a time-dependent manner (Figure 2D). This inhibition was maximal after 8 hours of simvastatin pretreatment and continued for at least 24 hours. Because atherosclerosis is associated with an enhanced passage of LDL across the aortic endothelium and an increase in the accumulation of LDL in the aortic wall,7 we preincubated human aortic EC monolayers with simvastatin and measured its effect on (thrombin-induced) 125I-LDL passage. Simvastatin slightly increased basal aortic endothelial permeability but attenuated thrombin-enhanced LDL passage and was maximally effective at 2 μmol/L (Figure 2E).

Effect of Simvastatin on Permeability Does Not Involve cAMP or Endothelial NO Synthase

Elevation of intracellular cAMP levels is known to improve endothelial barrier function.8 When HUVECs were preincubated with 5 μmol/L simvastatin for 24 hours, cAMP concentrations remained unaltered both under basal conditions (2.6±0.2 and 1.9±0.4 pmol per 3.5×10⁵ cells in control and simvastatin-pretreated cells, respectively) and after a 2-minute stimulation of the cells with 1 U/mL thrombin (2.5±0.8 and 2.1±0.4 pmol per 3.5×10⁵ cells, respectively) or after a 30-minute stimulation (2.6±0.1 and 2.1±0.2 pmol per 3.5×10⁵ cells, respectively) (3 experiments). As a positive control, cells were pretreated for 15 minutes with 10 μmol/L forskolin, which raised intracellular cAMP concent-

Figure 1. Effect of simvastatin on morphology of HUVECs of passage number 1 (A and B) and human aortic ECs of passage number 4 (C and D). Phase-contrast micrographs of control cells (A and C) and of cells preincubated for 24 hours with 5 μmol/L simvastatin (B and D).

Figure 2. Effect of simvastatin (Simva) on human endothelial barrier function. A, HUVEC monolayers were preincubated for 24 hours with 5 μmol/L Simva (∆, ▲) or without Simva (○, ●). HRP passage was measured under basal conditions (○, △) and after exposure to 1 U/mL thrombin (∆, ▲) as described in Methods. Values are mean±SEM of 2 different cultures in triplicate. Interaction between time and Simva treatment was significant (P=0.000) for thrombin-stimulated cells. *P<0.05 for Simva-pretreated vs nonpretreated cells that were stimulated with thrombin. B, HUVEC monolayers were pretreated for 24 hours with various concentrations of Simva (0.1, 0.5, 1.0, 2.5, or 5.0 μmol/L) in presence or absence of 100 μmol/L mevalonic acid (+Meva). HRP passage was measured 1 hour after exposure to 1 U/mL thrombin. Values are mean±SEM of 2 different cultures in triplicate. There was a significant difference between HRP passage of various thrombin-stimulated groups (P=0.000). C, Prolonged preincubation (96 hours) with 0.5 μmol/L Simva enhances effect of Simva on thrombin-induced HRP passage through HUVEC monolayers. HRP passage was measured 1 hour after exposure to 1 U/mL thrombin (hatched bars) or without stimulation (stippled bars). Values are mean±SEM of 6 filter cultures of 2 donors. D, HUVEC monolayers were pretreated for indicated time periods with 5 μmol/L Simva, and HRP passage was subsequently measured under basal conditions (stippled bars) or 1 hour after stimulation with 1 U/mL thrombin (hatched bars). Values are mean±SD of 1 representative experiment of 3 experiments. E, Human aortic endothelial monolayers were pretreated for 24 hours with 2 μmol/L Simva. 125I-LDL passage was measured 1 hour after exposure to 1 U/mL thrombin (hatched bars) or sham treatment (stippled bars). Values are mean±SEM of 3 independent experiments in triplicate. *P<0.05 Simva-pretreated cells vs nonpretreated cells (B, C, and E).
Statins can increase the cellular amount of endothelial NO synthase (eNOS). NO counteracts the thrombin-induced permeability in our model. Although prolonged incubation (>24 hours) of HUVECs and aortic ECs with simvastatin indeed increased the level of eNOS protein, simvastatin had no effect on eNOS protein expression within the 24-hour incubation period in our experimental conditions (Western blotting, data not shown).

Effect of Simvastatin on Localization of Vinculin and F-Actin of Basal and Thrombin-Stimulated Monolayers

The actin cytoskeleton of ECs is important in maintaining the structural integrity of the endothelium. HUVEC monolayers were stained for vinculin, a marker for focal adhesion formation, and for F-actin. Control cells showed hardly any vinculin staining and were characterized by cortical F-actin microfilaments (Figure 3A to 3C), indicating the resting state of the monolayers. Thrombin induced a dramatic increase in the formation of focal adhesions and stress fibers (Figure 3G to 3I). Many small gaps between the cells were observed. Pretreatment with simvastatin decreased basal F-actin filaments, and only a thin peripheral rim of F-actin remained (Figure 3D to 3F). Formation of focal adhesions by thrombin was reduced by simvastatin pretreatment, as was the formation of stress fibers and of gaps (Figure 3J to 3L). Thus, simvastatin to a large extent prevented the thrombin-induced changes of the EC cytoskeleton.

Simvastatin Affects Membrane Translocation of RhoA

It has been shown previously that the small GTPase RhoA is involved in the thrombin-induced endothelial hyperpermeability and accompanying cytoskeletal rearrangements. Isoprenylation of small GTPases, which is essential for their translocation to the plasma membrane, is inhibited by statins. Under resting conditions, a small fraction of the cellular RhoA content was present in the plasma membrane (Figure 4). Treatment with simvastatin (5 μmol/L for 24 hours) reduced the membrane localization of RhoA. Stimulation with thrombin for 30 minutes induced the translocation of RhoA to the membrane fraction, which was completely prevented by preincubation with simvastatin. Mevalonate reversed the effects of simvastatin (data not shown).

Simvastatin Treatment Reduces Vascular Leakage In Vivo

To test whether simvastatin could improve endothelial barrier function in vivo, Watanabe rabbits were treated with 15
mg/kg body wt simvastatin over 4 weeks. Simvastatin induced a slight nonsignificant reduction in plasma cholesterol (from 10.2±2.7 mmol/L before to 8.5±2.1 mmol/L after simvastatin treatment, P=0.30; n=3), but cholesterol levels remained elevated after this relatively short treatment period. After the animals were euthanized, endothelial barrier function of the thoracic and abdominal aorta was assessed ex vivo by determining the penetration of the Evans blue–albumin complex in the vessel wall. In the thoracic aortas of control animals, an intense blue staining was observed (Figure 5A). Treatment with simvastatin reduced Evans blue staining (Figures 5A and 6). Similar results were obtained in the abdominal aorta of the same rabbits (Figure 6).

To exclude the possibility that the decrease in endothelial permeability by simvastatin treatment was due to a reduction in the extent of atherosclerotic plaques, the same aortic segments were subsequently stained with oil red O. Aortas either from control or simvastatin-treated animals were severely atherosclerotic, with no visible changes in lipid accumulation after simvastatin treatment for 4 weeks (Figure 5B). As a control, umbilical veins, which showed no oil red O staining, were used (data not shown). It is of interest to note that in the control animals, a strong blue staining was observed in the areas of low lipid accumulation (Figure 5A, white arrow). This extravasation is not likely to be caused by endothelial damage, because in aortic areas of simvastatin-treated animals with a comparable low degree of lipid accumulation (Figure 5B, yellow arrow), a relatively small amount of Evans blue–albumin complex was observed (Figure 5A, yellow arrow). This exclusion of Evans blue dye indicates the presence of an intact endothelial barrier. It is likely that the accumulated lipids interfered with maximal accumulation of Evans blue.
Thus, simvastatin had already improved vascular barrier function in Watanabe rabbits at a time point at which no obvious changes in the extent of atherosclerotic plaques were observed.

**Discussion**

The major finding of the present study is that simvastatin treatment reduced endothelial barrier dysfunction both in an established in vitro model of endothelial permeability consisting of human ECs grown on porous filters and in ex vivo aortic segments of Watanabe rabbits, which have an increased endothelial permeability.

Accumulation of LDL in the arterial wall is one of the hallmarks of the development of atherosclerosis. The focal nature of plaque formation is remarkably similar to the focal occurrence of vascular leakage sites. In laboratory animals, the regional variation in the arterial wall permeability predicts the pattern of experimental atherosclerosis. It is not known at the moment whether this association reflects a causal relationship. Our finding, ie, that simvastatin treatment, which decreases the progression of atherosclerosis, reduces enhanced permeability, supports the idea that an increased permeability to LDL increases the risk of atherosclerosis and that a reduction in endothelial permeability, in addition to a lowering in plasma LDL concentration, may assist in the prevention of the progression of atherosclerosis.

We have previously shown that NO improves the endothelial barrier in vitro and acts as a negative feedback regulator of the thrombin-induced barrier dysfunction. Statins can increase eNOS expression on the posttranscriptional level in ECs exposed to oxidizing conditions. However, during the relatively short period of our in vitro experiments, the cellular eNOS antigen concentration did not change. This excluded the possibility that simvastatin improves barrier function by increasing NO production. Nor did simvastatin have any effect on intracellular cAMP concentration, which also improves endothelial barrier function.

Simvastatin reduced F-actin staining in control HUVEC monolayers, in accordance with the decrease in F-actin content by lovastatin treatment in NIH 3T3 cells. Furthermore, simvastatin largely reduced the formation of stress fibers induced by thrombin. This finding is of interest because prominent stress fibers are found in endothelial cells in vivo in areas prone to the development of atherosclerosis.

Several authors have shown that statins may affect the F-actin cytoskeleton through inactivation of Rho proteins. Lovastatin prevented the lysophosphatidic acid–induced translocation of RhoA and cell contraction in neuronal cells by inhibition of isoprenylation of RhoA. An effect of statins on Rho proteins in vascular smooth muscle and ECs has also recently been indicated. We and others have previously shown that RhoA is involved in the thrombin-induced endothelial barrier dysfunction in vitro, which involves Rho kinase–dependent inhibition of myosin light chain phosphorylation. In accordance, we now demonstrate that simvastatin prevents both the basal and the thrombin-induced translocation of RhoA to the plasma membrane. In these experiments, compared with other studies, a low fraction of RhoA was present in the plasma membrane under resting conditions. This explains that no accompanying increase in cytosolic RhoA was observed after treatment with simvastatin. To our knowledge, no in vivo data are yet available on the role of activation of RhoA in vascular leakage.

Improvement of endothelial integrity by simvastatin in vivo was probably not due to a reduction of atherosclerotic lesions. A short regimen of 4 weeks of simvastatin treatment was chosen, which had only a moderate effect on plasma cholesterol levels (17% reduction). No visible plaque regression was detectable in the rabbit aortas after oil red O staining. We cannot exclude the possibility that circulating LDL in the control animals contains an endothelium-activating fraction, which is reduced in parallel with the drop in plasma cholesterol. However, we feel more likely that the prevention of RhoA activation explains the improved barrier function.

Highly confluent cells were required to demonstrate the positive effect of simvastatin on disturbed barrier function in vitro (authors’ unpublished data, 2000). One explanation for this finding may be that because statins are known to inhibit cell proliferation, nonconfluent monolayers were not able to reach confluence in the presence of simvastatin. Furthermore, in vascular smooth muscle cells, statins are known to induce apoptosis at high concentration. Taken together, these findings suggest that care has to be taken to use statins when endothelial cells are in a proliferative state, eg, in wound healing.

Treatment of patients suffering from prolonged edema has been less successful with current therapies until now. Although β-adrenergic agents have been shown to be effective in acutely induced vascular leakage, when they are administered in the presence of capillary leakage syndrome, desensitization to β-adrenergic agents occurs after 1 day. Future studies are necessary to investigate whether statin treatment could reduce vascular leakage under these conditions. The recent finding that simvastatin reduces leukocyte-endothelium interactions indicates that statins also have an effect on the microvascular endothelium. Our finding may also bear importance for stented vascular areas, inasmuch as previous work has demonstrated prolonged leakage in these areas.

In conclusion, we found that simvastatin, in a relatively high concentration, reduces endothelial permeability and that this decrease in permeability was accompanied by a decrease in cell F-actin content. These findings may have implications for the treatment of patients with a high risk of developing atherosclerosis and of those with implanted stents and possibly of patients with capillary leakage syndrome.

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