EXPERIMENTAL STUDIES

HMG-CoA Reductase Inhibition Improves Endothelial Cell Function and Inhibits Smooth Muscle Cell Proliferation in Human Saphenous Veins

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Coronary bypass grafting using autologous internal mammary arteries and saphenous veins (SVs) is routinely used for treatment of patients with multivessel coronary artery disease. The patency rates of the arterial and venous grafts are, however, quite different. Occlusions are rare in the internal mammary artery, whereas they occur more frequently in the vein (1,2). Venous bypass graft failure is primarily related to biological properties of endothelial cells (ECs) and smooth muscle cells (SMCs) (3,4). The endothelium of the SV releases much less nitric oxide (NO) compared with that of the internal mammary artery (3,4). Nitric oxide is produced from L-arginine via endothelial nitric oxide synthase (eNOS) and is a potent vasodilator, platelet inhibitor and anti-proliferative agent (5). In the arterial circulation, the venous endothelium is unable to prevent platelet-vessel wall interaction, which favors thrombus formation and vascular occlusion (6). Furthermore, SMCs from SVs grow excessively in response to several growth factors such as platelet-derived growth factor (PDGF) and thrombin (4,7,8). This may contribute to the lower long-term patency rate of venous grafts.

Although mechanisms of cell growth regulation have not yet been fully understood, recent evidence suggests that signal transduction molecules such as MAPK, PI-3K and decades, the increasing incidence of venous bypass graft disease made the development of approaches to treat such patients an important therapeutic challenge.

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Although mechanisms of cell growth regulation have not yet been fully understood, recent evidence suggests that signal transduction molecules such as MAPK, PI-3K and
p70S6K, which are activated by tyrosine kinase receptors such as PDGF receptors, are important for cell cycle progression (9–11). Cell cycle progression is positively regulated by the orderly activation of cyclin-dependent kinases (Cdks) and negatively regulated by several Cdk inhibitors known as CKIs (12–14). p27Kip1 is most likely involved in cell cycle control in human SV SMC (8). Cyclin D-Cdk4/Cdk6 regulates G1 progression, and cyclin E-Cdk2 is essential for G1/S transition by phosphorylating and inactivating the tumor suppressor gene product, pRB (12–14), which causes release and activation of E2F transcription factor and in turn regulates several proteins required for cell proliferation (14).

Lipid-lowering therapy with 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors strikingly decreases cardiovascular morbidity and mortality (15–18). The effects of those agents have been attributed to inhibition of cholesterol synthesis and lowering of low-density lipoprotein plasma levels. However, not all the clinical benefits of the agents can be explained by their lipid-lowering effects (19). Moreover, whether all the effects of statins could be explained by inhibition of HMG-CoA reductase is not clear. A recent study indicates that HMG-CoA reductase inhibitors could possess biological effects independent of HMG-CoA reductase (20). In the clinical setting no optimal therapy for venous bypass graft disease is available (21). This may be related, at least in part, to the fact that antiproliferative drugs may not be able to improve endothelial function and at the same time inhibit SMC proliferation. In this study, we hypothesized that the new HMG-CoA reductase inhibitor cerivastatin could meet these criteria in human SVs and therefore may have the important clinical impact of improving venous bypass graft function. Moreover, we also investigated whether cerivastatin affects EC and SMC function in human SVs solely by the classic mechanism (i.e., inhibition of HMG-CoA reductase).

**METHODS**

Chemicals and materials. Bovine serum albumin (BSA, 7.5%), monoclonal antibody against smooth muscle α-actin, and all chemicals for immunoblotting were from Sigma (Buchs, Switzerland); recombinant human PDGF-BB was from (R&D Systems GmbH, Wiesbaden, Germany); all tissue culture materials were from Gibco (Basel, Switzerland); [3H]-methyl-thymidine was from Amersham (Zürich, Switzerland); trichloroacetic acid was from Fluka (Buchs, Switzerland); rabbit polyclonal anti-human p42mapk (C14), p27Kip1 (C19), and Cdk2 (M2) were from Santa Cruz Biotechnol (Basel, Switzerland); mouse monoclonal antibody against human p21Cip (Clone EA10) was from Calbiochem (JURO Supply AG, Lucerne, Switzerland); mouse monoclonal antibody against human eNOS (N30020) was from Transduction Laboratories (Basel, Switzerland); rabbit polyclonal anti-human phospho-pRB (Ser807/811) was from New England BioLabs (Schwalbach/Taunus, Germany); mevalonate was from Sigma (Buchs, Switzerland); and cerivastatin was kindly supplied by Bayer AG (Leverkusen, Germany).

**Cultivation of ECs and SMCs.** Endothelial cells were isolated from human SVs as described (4). Briefly, fresh blood vessels were harvested in a cold sterile RPMI-1640 medium with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The vessels cleaned of connective tissue and adventitia were incubated with collagenase type II 75 U/ml for 15 min in phosphate-buffered saline (PBS). Cell pellets were then collected by centrifugation at 1,000 rpm for 10 min and seeded in culture dishes coated with 25 μg/ml human fibronectin and cultured in RPMI-1640 supplemented with 20 mmol/liter L-glutamine, HEPES buffer solution, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml endothelial cell growth supplement, 25 μg/ml heparin and 20% fetal calf serum (FCS). The day after, cells were washed with the medium to eliminate blood cell contamination. Endothelial cells were characterized by typical cobblestone and nonoverlapping appearance and indirect immunofluorescence staining using specific antibodies against von Willebrand factor. Cells of third and fourth passage were used.

Vascular SMC were cultivated from SVs obtained from patients undergoing coronary bypass surgery using explant technique as previously described (4,8). Briefly, the cells were cultured in Dulbecco’s modified eagle medium containing 20% FCS supplemented with 20 mmol/liter L-glutamine and HEPES buffer solution, 100 U/ml penicillin and 100 μg/ml streptomycin, 50 μg/ml endothelial cell growth supplement, 25 μg/ml heparin and 20% fetal calf serum (FCS). The day after, cells were washed by trypsinization (0.01% EDTA). Experiments were performed between passages five to eight. The SMC were characterized by typical morphological pattern (multilayer sheets and “hills and valleys”) and indirect immunofluorescence staining using specific mouse monoclonal antibodies against human SMC α-actin (Sigma).

**The eNOS expression.** Confluent ECs were rendered quiescent for 24 h by changing the medium to RPMI-1640 with the same ingredients as described above except that EC growth supplement and heparin were avoided and only 0.5% FCS was added. The cells were then treated with cerivast-
Cerivastatin and Human Venous Bypass Grafts

Effects of cerivastatin on eNOS. In cultured human SV-EC, treatment of the cells with cerivastatin (10^{-9} to 10^{-6} mol/liter; 24 h) up-regulated the eNOS protein level in a concentration-dependent manner (Fig. 1, top panel). Densitometry showed that the increase in eNOS protein level reached about two-fold (Fig. 1, lower panel, n = 3). This up-regulation of eNOS expression by cerivastatin was fully reversed by mevalonate (2 \times 10^4 mol/liter) (Fig. 1A, B). Accordingly, NO production stimulation by PDGF-BB was also increased by mevalonate (2 \times 10^4 mol/liter) in the cells treated with cerivastatin (10^{-6} mol/liter) for 24 h. In all experiments, n equals the number of patients from which vessels were obtained. The Student t test for paired observations and analysis of variance followed by the Scheffe test for repeated measurements were used. A two-tailed p value smaller than 0.05 was considered significant.

RESULTS

Effects of cerivastatin on eNOS. In cultured human SV-EC, treatment of the cells with cerivastatin (10^{-9} to 10^{-6} mol/liter; 24 h) up-regulated the eNOS protein level in a concentration-dependent manner (Fig. 1, top panel). Densitometry showed that the increase in eNOS protein level reached about two-fold (Fig. 1, lower panel, n = 3). This up-regulation of eNOS expression by cerivastatin (10^{-6} mol/liter) was fully reversed by mevalonate (2 \times 10^4 mol/liter) (Fig. 1A, B). Accordingly, NO production stimulated by Ca^{2+} ionophore (10^{-6} mol/liter) in the cells treated with cerivastatin (10^{-6} mol/liter) for 24 h was also markedly increased (from 393 \pm 55 nmol/liter to 840 \pm

Figure 1. Effects of cerivastatin on eNOS expression in human saphenous vein endothelial cells: Immunoblotting demonstrated up-regulation of eNOS by the HMG-CoA reductase inhibitor cerivastatin (Ceri) in a concentration-dependent manner. Densitometry showed about two-fold increase in eNOS protein level (n = 3).
42% nmol/liter, n = 3, p < 0.01) as measured by the porphyrinic microsensor (Fig. 2C). Similar to eNOS protein expression, the enhanced NO release by cerivastatin was also fully reversed by treatment of the cells with mevalonate (2 × 10^{-4} mol/liter; Fig. 2C, 372 ± 678 nmol/liter, p < 0.01 vs. cerivastatin-treated cells), whereas mevalonate alone had no effects.

**Effects of cerivastatin on SMC proliferation.** In cultured human SV SMC, the growth factor PDGF-BB (5 ng/ml) markedly enhanced 3H-thymidine incorporation (298 ± 23% above control, n = 4), which was concentration-dependently inhibited by cerivastatin (data not shown). The inhibitory effects of cerivastatin (10^{-6} mol/liter; 164 ± 11%) was only partially reversed by co-incubation of the cells with mevalonate (2 × 10^{-4} mol/liter; Fig. 3; n = 4, p < 0.05 vs. cerivastatin plus PDGF). Mevalonate alone had no effects on 3H-thymidine incorporation.

**Effects of cerivastatin on MAPK and p70S6K activation.** After stimulation of SMC with PDGF-BB (10 ng/ml, 10 min) both p42 MAPK and p70S6K were activated, as demonstrated by a slower mobility of the activated (phosphorylated) kinase on Western blots (Fig. 4). Cerivastatin (10^{-6} mol/liter) did not exhibit inhibitory effects on either p42 MAPK or p70S6K activation in response to PDGF-BB (Fig. 4). By contrast, activation of p42 MAPK was specifically inhibited by PD98059 (5 × 10^{-5} mol/liter), a specific MAPK kinase (MEK) inhibitor. The p70S6K activation stimulated by PDGF-BB (10 ng/ml) was prevented by rapamycin (10^{-5} mol/liter), a specific inhibitor of p70S6K, and also by wortmannin (10^{-5} mol/liter), a specific inhibitor of PI3-K (Fig. 4).

**Effects of cerivastatin on cell cycle regulators.** Western blotting demonstrated that stimulation of the SV SMC with PDGF-BB (10 ng/ml) for 24 h induced activation of Cdk2 accompanied by an increase in its electrophoretic mobility.
The molecular mechanism of increase in eNOS expression is not clear. It might be due to the different cell types used. It is possible that other cell-cycle regulatory proteins are still inhibited by cerivastatin independently on HMG-CoA/mevalonate pathway. This issue needs additional investigation.

Furthermore, results of the present study showed that p27Kip1, but not p21Cip1, was down-regulated by PDGF.
This confirmed our previous study (8) showing that p27Kip1 is most likely involved in cell growth regulation of these cells. Several studies showed that inhibition of HMG-CoA reductase prevents p27Kip1 or p21Cip1 down-regulation in established cell lines (32,33). To our surprise, the present study demonstrated that cerivastatin was unable to prevent the down-regulation of p27Kip1, indicating that HMG-CoA/mevalonate pathway is not involved in the regulation of the cell-cycle inhibitor in these particular human cells.

In IIC9 cells, a subculture of Chinese hamster embryo fibroblasts and also in FRTL-5 cells, a strain of rat thyroid cells, p27Kip1 down-regulation or degradation is dependent on Cdk2 activation through RhoA (29,34). However, this mechanism seems not to contribute to the down-regulation of the Cdk inhibitor in our cells, because inhibition of Cdk2 activation by cerivastatin did not correlate with up-regulation of p27Kip1. Our data contrast with the observation by Laufs et al. (35), who did show prevention of p27Kip1 down-regulation by another statin simvastatin in cultured SMCs from human aorta and SV. Unfortunately, this study did not specify the cell types from which the results were raised. It is also possible that simvastatin may have different effects on p27Kip1 than cerivastatin. This issue will be further investigated.

Conclusions. A recent clinical post-CABG trial showed that the progression of atherosclerosis in SV grafts was delayed by aggressive lovastatin treatment but not by moderate treatment (36). Because NO release in the human SV is very low, and excessive SMC proliferation importantly contributes to the poor long-term patency rate of venous grafts, the effects of cerivastatin on human SV EC and SMCs as shown in this study may be beneficial for patients with venous bypass grafts.

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REFERENCES


