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Review

Cerivastatin: a cellular and molecular drug for the future?

D. I. Siegel-Axel

Department of Medicine III (Cardiology), University of Tübingen, Otfried-Müller St. 10, D-72076 Tübingen (Germany), Fax +49 7071 29 5749, e-mail: daaxel@med.uni-tuebingen.de

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Abstract. The 'statin story' began in 1987 when the first-generation, fungal HMG-CoA reductase inhibitor lovastatin received FDA approval in the USA. Ten years later, the sixth compound of this class came onto the world market – the fully synthetic statin cerivastatin. A number of clinical studies had confirmed its high pharmacological efficacy, its excellent pharmacokinetic properties with fast and nearly complete absorption after oral uptake, a linear kinetic over a broad concentration range, and its favorable safety profile. The greatest advantages, of cerivastatin, however, are its lipophilicity, its high bioavailability of about 60% after oral application and its potency at 100-fold lower doses compared to other lipophilic statins. Nevertheless, the most exciting findings are certainly its non-lipid-related, pleiotropic effects at the cellular and molecular level. Statin therapy was also found to reduce mortality in cases where cholesterol levels or atherosclerotic plaque formation remained unaltered. However, cerivastatin improves endothelial dysfunction, possesses anti-inflammatory, antioxidant, anticoagulant, antithrombotic, antiproliferative, plaque-stabilizing, immunmodulatory, and angiogenic effects, and may even prevent tumor growth, Alzheimer's disease, and osteoporosis. Most of these effects seem to be based on the inhibition of isoprenoid synthesis. Although cerivastatin is no longer on the market because of some problematic side effects, it could be one of the most potent cellular and molecular drugs for the future.

Key words. HMG-CoA reductase inhibitors; cerivastatin; isoprenoids; pleiotropic effects; atherosclerosis.

Introduction

Despite much progress in cardiovascular research in the last decade with the help of cellular and molecular biology, the leading cause of death in industrial societies is still coronary heart disease (CHD) resulting in angina and myocardial infarction. The alterations occurring inside the arterial vessel wall after exposure to risk factors over many years, predominantly the enrichment of lipids, are defined as atherosclerosis.

A great variety of epidemiological studies have established that elevated serum cholesterol levels, predominantly low-density lipoprotein cholesterol (LDL-cholesterol) and to a lesser extent also very low density lipoprotein cholesterol (VLDL-cholesterol) which carries the major part of the triglyceride fraction, are the most important risk factors for the development of atherosclerosis leading to both cardiovascular and cerebral diseases [1].

For that reason, many approaches have been taken to develop drugs reducing blood lipid levels and the accumulation of abundant cholesterol inside the arterial vessel wall [2]. Among all strategies, the inhibition of a key step in endogenous cholesterol biosynthesis, the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by the HMG-CoA reductase, brought the breakthrough for the treatment of hypercholesterolemia [3].

The first description of an inhibitor of HMG-CoA reductase, the rate-determining enzyme in cholesterol biosynthesis, came as early as 1976 [4]. The isolation of the

* Corresponding author.

common precursor of the class of HMG-CoA reductase inhibitors ('statins'), mevastatin (ML-236B), was described, a natural product of the fungus *Penicillium citrinum*, which is synonymous to compactin, isolated from *P. brevicompactum*. Furthermore, the pioneering observation was made that this newly isolated natural compound is an extraordinarily potent competitive inhibitor of HMG-CoA reductase, possessing hypocholesterolemic activity [5]. The Nobel prize winners of the year 1985, Brown and Goldstein, the most famous pioneers in cholesterol research, concluded that HMG-CoA reductase inhibitors might serve as very potent drugs for lowering blood lipid levels in patients with hypercholesterolemia [6].

The first statin that entered the clinic was lovastatin [3] which was originally an enantiomerically pure fermentation product of the fungus *Aspergillus terreus* [7]. To optimize the affinity for the enzyme, the chemical structure of lovastatin was altered in semisynthetic processes leading to the two semisynthetic compounds simvastatin and pravastatin [8]. Whereas the negatively charged compound pravastatin is an active, very hydrophilic, hepatoselective drug [9], lovastatin and simvastatin are hydrophobic pro-drugs with a lactone ring (water solubility: 1.3–1.5 mg/l) which must first be activated by liver enzymes. In contrast, the statins of the second and third generation, such as the racemic compound fluvastatin, atorvastatin, rosuvastatin, and also cerivastatin, are fully synthetic salts which are more or less hydrophilic [10]. The physicochemical properties of statins are of great relevance for their pharmacokinetic behavior [11], their potential to exert adverse drug interactions [12], and their ability to penetrate cell membranes of various tissues, the major prerequisite for their direct pleiotropic effects on vascular cells [13]. Pravastatin is the only statin which is too hydrophilic to cross cell membranes of nearly all tissues including the vascular vessel wall. Only liver cells possess active transport mechanisms for the uptake of this drug, whereas the disposition characteristics of other statins result from high hepatic extraction because of their lipophilicity [9].

The high efficacy and good tolerability of the currently available statins are the reasons for their being considered first line in the pharmacological treatment of hyperlipidemia, predominantly when a lipid-poor diet is insufficient to reduce LDL-cholesterol levels [14]. Between 1994 and 1998, the following landmark trials were published: the Scandinavian Simvastatin Survival Study (4S), the West of Scotland Coronary Prevention Study (WOSCOPS), the Cholesterol and Recurrent Events trial (CARE), the Air Force Coronary/Texas Atherosclerosis Prevention Study (AFCAPS/TexCAPS), and The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) [15]. These primary and secondary prevention study trials provided compelling evidence that statins of the first generation can reduce the risk of cardiovascular events and even decrease total mortality. Surprisingly, CARE and LIPID even demonstrated that cardiac patients with normal cholesterol levels also profit from the statin-induced benefits on survival rate.

Cerivastatin and cholesterol reduction

In 1997, the synthetic HMG-CoA reductase inhibitor cerivastatin (BAYw6228) was developed by the Bayer Pharmaceutical Division and claimed as a much more potent statin which expands HMG-CoA reductase inhibitor choices because it is highly effective at 100-fold lower doses than all other known statins. What properties made cerivastatin a new-generation HMG-CoA reductase inhibitor?

The main cause of elevated cholesterol levels is the familiar heterozygotic hypercholesteremia which is characterized by a reduced expression of LDL receptors. As a consequence, the elimination of LDL-cholesterol is not complete. In a regulatory manner, the activity of the HMG-CoA reductase increases to produce more cholesterol to elevate blood cholesterol levels which leads again to down-regulation of LDL receptors. This results in a viscious circle with fatal consequences for the patient. HMG-CoA reductase inhibitors bind with higher affinity to the enzyme than does the substrate mevalonic acid and inhibit it competitively, thus breaking the cycle. A feedback mechanism leads to an elevated expression of LDL receptors with an increased elimination of LDL-cholesterol. The aim in synthesizing the new compound BAYw6228 (cerivastatin) was to create a statin with a very high affinity for the HMG-CoA reductase. The membrane-bound HMG-CoA reductase from a native microsomal fraction of rat liver was inhibited with an inhibition constant (K_i) of 1.3×10^{-9} mol/l whereas the K_i for lovastatin was determined as 150×10^{-9} mol/l [16]. As a result, the third-generation compound cerivastatin reduces cholesterol synthesis in vivo at 100-fold lower doses than the statins of the first generation. Furthermore, cerivastatin possesses an advantageous pharmacokinetic profile. It is not a racemate as are the natural statins, resorption from the intestine is nearly complete, and liver selectivity is high [15]. The detailed pharmacokinetic characteristics of cerivastatin can be described in brief as follows: a sodium salt ($C_{26}H_{33}FNO_5Na$, MW: 481.5) with high water solubility (>195 g/l at 25 °C) [17], high binding to plasma proteins (99%), low volume of distribution of 0.3 l/kg, rapid and complete absorption (>98%) with maximum plasma concentrations (C_{max}) reached at 2–3 h post oral application, absolute oral bioavailability of 60%, dose-proportional increase in AUC and C_{max}, linear pharmacokinetics with low intra- and interindividual variability [17]. The elimination half-life is about 2–3 h and the total body clearance is about 13 l/h [18]. Cerivastatin is subject to two oxidative bio-

transformation reactions catalyzed by cytochrome P450 (CYP) 2C8 and CYP3A4 leading to the metabolites M-1 and M-23 [17]. Since the clinical dosing conditions, such as food time and administration, do not influence the pharmacokinetics of cerivastatin, it is in general an uncomplicated drug with good compliance. Like atorvastatin, fluvastatin, and pravastatin, it is administrated as active drug, whereas lovastatin and simvastatin are prodrugs [19]. The majority of statins have low absolute bioavailabilities [19]: atorvastatin 12% [20], pravastatin 17% [21], fluvastatin 20–30% [22], simvastatin 5% [23], and lovastatin 5% [24]. In contrast, cerivastatin has the highest absolute bioavailability of 60% [25]. Finally, calculation of relative lipophilicity showed the following rank order: cerivastatin > simvastatin > fluvastatin > atorvastatin > rosuvastatin > pravastatin [26].

In the last 4 years, a number of large randomized multicenter studies in humans were performed with oral doses of cerivastatin between 0.1–0.4 mg/day [26, 27], as well as the pivotal North American and Canadian trial with more than 1000 hypercholesteremic patients receiving 0.8 mg cerivastatin for 8 weeks [28]. Cerivastatin was shown to cause dose-dependent reductions in LDL-cholesterol of 14.2–36.1% after a once daily dose of 0.025–0.4 mg/day [29]. Each doubling of the dose yielded an additional reduction in LDL-cholesterol of about 6% [30]. Along with the recommendation by the FDA [25], recent studies have focused on higher dosages of 0.4 and 0.8 mg/day which yielded LDL-cholesterol reductions of 33.4–44.0% [31]. With this dosing regime, 81–84% of patients could achieve the target LDL-cholesterol levels of the US National Cholesterol Education Program (NCEP) [25, 28, 32]. To date, there is no evidence that the absolute maximum LDL-cholesterol reduction is reached at 0.8 mg because higher doses have not yet been studied. However, with the doses of 0.4 and 0.8 mg/day, cerivastatin is either equivalent or better than the other available statins [27, 33, 34] and can reduce costs for the healthcare system because it is the least expensive statin [34, 35]. Over the last 3 years, cerivastatin (Lipobay, Baycol) has received marketing approval in many countries, including the USA, Canada, Europe, Japan, and Australia.

Non-lipid-related effects of cerivastatin

As the large clinical studies demonstrated clearly, elevated serum lipoprotein levels are strongly associated with coronary artery disease. Consequently, the inhibition of cholesterol synthesis with statins seems to be the fundamental mechanism underlying their beneficial effects. In the last few years, however, subgroup analysis of the pivotal studies indicated that at least some of the cardiovascular benefits could be explained by other effects beyond lipid lowering. Subgroup analysis of the WOSCOP and CARE studies indicated that serum cholesterol levels between statin-treated and placebo groups did not differ significantly but statin-treated patients showed a significantly lower risk of CHD [36]. Furthermore, reductions in cholesterol levels did not produce as impressive changes in the progression of atherosclerotic lesion formation as expected and the improvements were thus not proportional to the clinical benefits of statins [37]. Recently, some in vitro studies have even indicated protective effects on other non-vascular tissues and diseases which may lead to new indications for statin treatment in the future, e.g., for tumor therapy [38, 39] or for the increase of bone formation in osteoporosis [40, 41].

Cerivastatin mode of action

To understand the cellular and molecular mechanisms underlying the non-lipid-related direct effects of statins on various cell types, a consideration of the single steps in the cholesterol biosynthesis pathway is needed (see fig. 1). The main step leading to the reduction in cholesterol synthesis is the decrease in the precursor mevalonate by the inhibition of the HMG-CoA reductase. However, as the cascade in figure 1 shows, other isoprenoid intermediates are synthesized distal from mevalonate which have important functions as lipid attachment factors. Farnesylpyrophosphate (FPP) constitutes the major branch point in polyisoprene biosynthesis [42] and participates in several different upstream pathways: beside the biosynthesis of cholesterol, it is involved in the synthesis of ubiquinone and dolichol via the intermediate isoprenoid product geranylgeranylpyrophosphate (GGPP) [6, 43]. Beside the direct farnesylation of dolichol, FPP and GGPP modify a variety of small proteins, including nuclear lamins and small GTP-binding proteins belonging to the Ras, Rho/Rac/ Cdc42, and Rab family that have sequences at their COOH termini undergoing posttranslational modifications. Protein prenylation is a prerequisite for the firm membrane attachment of signal transduction proteins, for their subcellular localization, the transport of membrane-associated proteins, and the activation of downstream effectors. Small G proteins serve as molecular switches that transduce an upstream signal to a downstream effector. They are translocated between the plasma membrane and the cytosol and activate downstream effector kinases (e.g., Raf, Rho, MAP kinase, Rabphilins) which determine the temporal and also spatial distribution of specific cell functions, such as induction of gene expression involved in proliferation, migration, differentiation, morphology, and apoptosis of many cells [44]. The specific functions differ between the distinct families of small G proteins: proteins of the Ras family are predominantly involved in the regulation of gene expression, the Rho/Rac/ Cdc42 proteins of the Rho family regulate both gene expression and cytoskeletal reorganization, the Ran

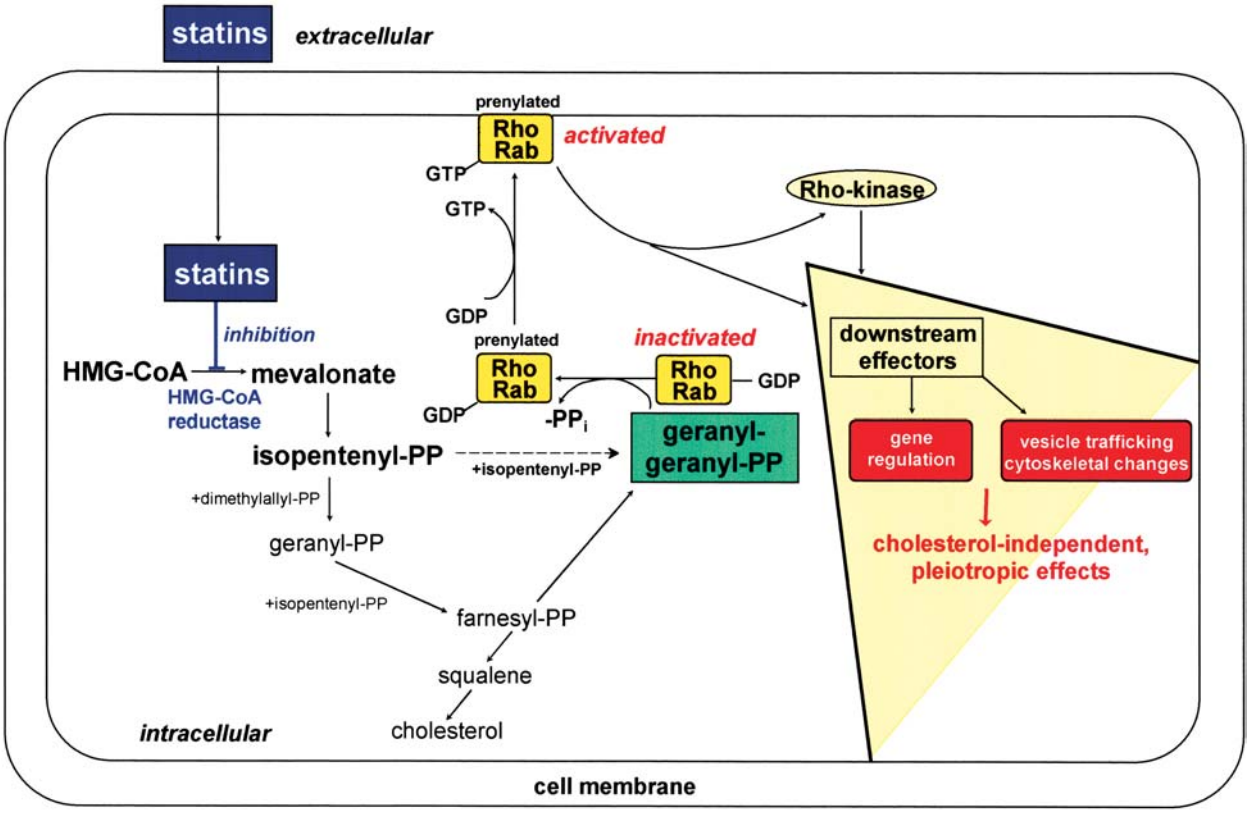

Figure 1. Regulation of the mevalonate pathway, and statin mode of action. By inhibiting of HMG-CoA reductase, the biosynthesis of two major downstream products of mevalonate is influenced: (i) cholesterol production and (ii) synthesis of isoprenoids, such as FFP and GGPP. GGPP activates Rho and Rab GTPases enabling membrane attachment by prenylation. Rho and Rab are delocalized from the membrane into the perinuclear region activating further kinases, such as Rho kinase, as well as downstream effectors. As a result, vesicule trafficking and cell migration, as well as gene expression of important cellular functions is regulated.

family members regulate nucleocytoplasmic transport processes during the G_1, S, and G_2 phases of the cell cycle and microtubule organization during the M phase, and finally the Rab and Sar1/Arf proteins regulate intracellular vesicle targeting/docking/fusion processes [44]. However, Rho/Rab proteins have also been shown to coordinately regulate cell adhesion and migration. Rab proteins form the largest branch of the small G protein superfamily and their lipid modification is also important for their binding to membranes and regulators, as well as for the activation of downstream effectors. One downstream effector, termed Rabkinesin-6 was recently found to be linked to the microtubule cytoskeleton, another effector, termed Rabphilin-11, is involved in cell migration, whereas Rabphilin-3 interacts with the actin cytoskeleton. Like Ras and Rho/Rac/Cdc42 proteins, Rab proteins stay in the cytosol in the GDP-bound form and translocate to their functioning sites upon activation. All three subfamilies possess sequences at their COOH termini that undergo posttranslational modifications by the attachment of farnesyl, geranylgeranyl, palmitoyl, and methyl moieties. In contrast, Arf proteins have an NH_2-terminal Gly residue that is modified with myristic acid, and Sar1/Ran do not have any sequence undergoing direct posttranslational modifications. Thus, the latter two subfamilies are not relevant for explaining statin-induced effects because they do not require isoprenylation for their activation.

Rab GTPases are posttranslationally modified by addition of geranylgeranyl moieties to carboxyl-terminal cysteine residues. Geranylgeranyltransferase type II (RabGGTase or GGTaseII), a 100-kDa heterodimer, catalyzes the transfer of two 20-carbon geranylgeranyl groups from GGPP onto C-terminal cysteine residues of Rab GTPases. However, it recognizes the Rab substrate only when it is bound to Rab escort protein (REP) which remains associated with the modified Rab until it is delivered to the appropriate subcellular membrane [45]. However, several Rab proteins (e. g., Rab8) contain carboxyl-terminal CaaL prenylation motifs typical of members of the Rho family, which are modified in a REP-independent manner by geranylgeranyltransferase type I (GGTaseI) [46]. Nevertheless, the question rises if Rab prenylation is influenced by statin treatment. Indeed, lovastatin-mediated depletion of intracellular mevalonate resulted in an fourfold increase in Rab5 and Rab7 protein levels [47].

However, concerning cardiovascular diseases, at present nearly all experimental studies with statins confirm the most important role of the small Rho proteins. Rho GTPases belong to the Ras superfamily of small GTP-binding proteins which consist of more than 14 members of the Rho, Rac, and Cdc42 subgroups. Among the different GTPases, Rho-GTPases are major substrates for the above-described isoprenylation by GGPP enabling the trafficking, activation, and binding of Rho to cell membranes [48]. Since activators of Rho, such as growth factors, cytokines, integrins, and hormones, trigger many important processes in atherosclerosis and restenosis, the beneficial effects of statins in general may be explained at least in part by the inhibition of Rho activation. However, future studies will probably provide further evidence for the role of the Ras and Rab superfamilies in explaining statin-induced pleiotropic effects.

Etiology of atherothrombosis

To understand where the pleiotropic effects of cerivastatin may play a role, I will first describe the basic mechanisms leading to the formation of atherosclerotic lesions. Facts about the direct effects and mechanisms of cerivastatin on each process are then explained in detail.

Atherogenesis is defined as a complex 'response-to-injury' process initiated by the functional or mechanical injury of the endothelium [49]. Some of the most important primary injurious noxes are, according to the classical risk factors, accumulated LDL-cholesterol at predilection sites (e. g., at branch points of small arteries), free radicals (cigarette smoking), infectious pathogens, elevated levels of glycosylation end products (diabetes), fluid shear stress (hemodynamic injury), and high blood pressure (hypertension) [50]. Atherogenesis then leads to the formation of a neointimal lesion that progressively occludes the arterial lumen. The oxidation and accumulation of plasma LDL in the subendothelial space is followed by the recruitment of circulating monocytes and T lymphocytes as a result of endothelial cell activation. Trapped monocytes then differentiate into macrophages which take up oxLDL and form foam cells. This further results in the activation of macrophages, T lymphocytes, endothelial and smooth muscle cells which proliferate and migrate from the media to the intima leading to neointima formation. Activation of these cells triggers the release of pro-inflammatory cytokines, which, combined with the secretion of metalloproteinases (MMPs) and expression of pro-coagulant factors, results in chronic inflammation and plaque instability. This can further evolve to plaque rupture and acute occlusion by thrombosis, resulting in myocardial infarction and stroke [50, 51]. The physical integrity of the plaque governs the most important clinical manifestations of atherosclerosis. The composition and stability of the plaque, rather than its volume, are recognized to determine the occurrence of serious coronary events. Weakening of fibrous plaques results in higher instability, predominantly at thin and macrophage-rich shoulder regions [52]. Beside lipid accumulation, enzymatic degradation of the extracellular matrix is the major cause of plaque weakening. Both smooth muscle cells and activated macrophages secrete MMPs [53] and urokinase-type plasminogen activator (uPA) [54]. There is a causal involvement of uPA in human coronary plaque stability determining the severity of atherosclerotic lesions [55]. Furthermore, oxidized lipoproteins are found to induce the expression of uPA on monocytes which results in increased plasmin generation and monocyte adhesion [56]. This observation confirms again that a high lipid content and an increased number of macrophages are two of the main characteristics of vulnerable plaques.

Another important pathophysiological process influencing the vulnerability of plaques is so-called 'plaque-angiogenesis' [57]. Neovascularization is an important process that is required for the progression of atherosclerosis and it is a major feature during plaque development [58]. Within complicated atherosclerotic plaques, the process of angiogenesis leads to destabilization of the extracellular matrix. Newly formed capillaries are more likely to rupture and may therefore trigger plaque rupture and acute coronary events [57]. Furthermore, histopathological studies of human stenosis samples revealed a higher number of neovessels in plaques from symptomatic than asymptomatic patients. Symptomatic plaques showed larger and irregularly shaped neovessels. This was accompanied by an increase in plaque necrosis and rupture in symptomatic plaques. Thus, beside lipid accumulation and matrix degradation, increased neovascularization within the atherosclerotic plaque also seems to contribute to plaque instability leading to plaque rupture and acute thromboembolic events [59].

Intimal thickening is due to the accumulation of cellular and extracellular substances in the space between the endothelial cell lining (intima) and the underlying medial smooth muscle cells. Therefore, the maintenance or recovery of the mechanical integrity of the endothelial cell lining, serving as a barrier between blood and vessel wall, is of great importance. Furthermore, endothelial dysfunction is characterized by an imbalance between relaxing and contracting factors, procoagulant and anticoagulant substances, and between pro-inflammatory and anti-inflammatory mediators [60].

The relaxing factors prostacyclin, endothelium-derived hyperpolarizing factor, nitric oxide (NO) and bradykinin, which induces NO release, play the predominant role in preventing vascular vessel diseases. NO interferes with key events in the development of atherosclerosis, such as vascular tone, monocyte and leukocyte adhesion to the endothelium, platelet-vessel wall interaction, and smooth

muscle cell proliferation [61]. For this reason, pharmaceutical or genetic approaches to restore endothelial function are the most promising strategies in the treatment of atherosclerosis.

Cerivastatin and endothelial function

Cholesterol lowering by cerivastatin, as well as its direct cellular effects are found to improve endothelial function. Which mechanisms are involved?

Effects on NO

Oxidized lipoproteins (oxLDL) after uptake by vascular cells and macrophages can inhibit the production of NO. Since cerivastatin-mediated reduction of LDL levels in blood also reduces oxLDL formation, the inhibitory effects of oxLDL on NO are consequently diminished. By this mechanism, cerivastatin improves impaired endothelium-mediated vasodilation. However, there is proof that cerivastatin also influences endothelial cell function directly at the molecular level. The expression of the endothelial NO synthase (eNOS) and NO release are found to be enhanced in cultured human endothelial cells by cerivastatin in response to Ca^{2+}-ionophore. This stimulation could be fully abrogated by the addition of mevalonate, indicating that the inhibition of the HMG-CoA reductase is responsible for this effect [62]. Furthermore, cerivastatin prevented tumor necrosis factor (TNF)-α-induced down-regulation of eNOS protein expression and the binding of cytosolic proteins to the 3′-untranslated region of eNOS mRNA which is associated with eNOS mRNA stabilization [63]. TNF-α is a pleiotropic cytokine that mediates inflammatory, proliferative, cytostatic, and cytotoxic effects in a variety of cell types, including endothelial cells [64]. Both of these cerivastatin-mediated effects could be reversed by coincubation with mevalonate. These findings are in line with other findings using simvastatin and lovastatin to upregulate eNOS expression [65, 66].

In addition, endothelial dysfunction can be studied in vivo using the hypercholesterolemic rabbit in which endothelium-dependent relaxation to acetylcholine and calcium is measured. Whereas the endothelium-dependent relaxation in controls was reduced by a hypercholesterolemic diet, vessel samples of cerivastatin-treated animals showed an upregulation of eNOS expression and reduction of the interaction of a 60-kDa cytosolic protein with the 3′-untranslated region of eNOS mRNA. The same effects were found in mononuclear cells. As a result, the endothelium-dependent relaxation was restored after treatment with cerivastatin for 3 weeks [67].

In recent years, an elegant method was applied for direct quantification of NO and O_2^- in a single endothelial cell with highly sensitive electrochemical microsensors [68]. Oxidative stress occurring as a result of hypercholesterolemia, the major risk factor for atherosclerosis, impairs NO bioactivity mainly by the accumulation of O_2^- known to be involved in the rapid breakdown of endothelium-derived NO [69]. Thus, the bioavailability of diffusible NO depends on both increased eNOS expression by endothelial cells and O_2^- release under oxidative stress conditions. For this reason, the kinetics of NO and O_2^- release were recorded in vitro to prove the effects of cerivastatin on the NOS system. Indeed, cerivastatin was capable of stimulating NO release and simultaneously scavenging aggressive O_2^- radicals which helped to preserve active NO concentrations. As a result, not only was a fast initial effect on NO release found after cerivastatin treatment but also long-term improvement of the L-arginine pathway resulting in a sustained increase of NO release. Interestingly, the long-lasting effect required only nanomolar doses of cerivastatin whereas initial effects were observed at 100-fold higher concentrations. Since the addition of L-mevalonate but not LDL-cholesterol reversed the sustained effect of cerivastatin, inhibition of endothelial HMG-CoA reductase and a deficit in nonsteroid isoprenoids may explain the observed changes in NO release [70].

Furthermore, cerivastatin could inhibit vascular inflammation and arteriosclerosis induced by chronic inhibition of NO synthesis [71]. Monocyte chemoattractant protein-1 (MCP-1) and transforming-growth factor-β1 (TGF-β1) gene expression serve as indicators because they are known to mediate early inflammatory and fibrotic processes. Treatment with cerivastatin augmented the reduced eNOS activity in rats after chronic NO synthesis inhibition. An explanation for the mechanism is the finding that cerivastatin decreased membrane translocation of RhoA with a concomitant accumulation of RhoA in the cytosol. Cerivastatin-induced augmentation of eNOS activity was concluded to be at least in part based on the reduced Rho activation which is in line with other studies [72].

Effects on superoxide anions

Among the effects of reduced NO synthesis, the production of superoxide anions contributes to endothelial dysfunction. A shift in the NO/O_2^- balance toward elevated superoxide anions reduces endothelium-dependent vasodilation [73] and even promotes the expression of proatherosclerotic genes [74]. Since the transfer of in vitro studies to the in vivo situation and the interpretation of functional consequences are difficult, the effects of several statins on O_2^- formation in endothelium-intact, healthy segments of rat aortas were studied. Cerivastatin inhibited O_2^- formation which was stimulated by phorbol ester treatment. A maximum inhibition of 70% was found 18 h after cerivastatin treatment at micromolar doses. To

elucidate the underlying mechanism, mevalonic acid was supplemented, which reversed the inhibitory effect. The inhibition could be mimicked by inactivation of p21 Rac but not by inactivation of p21 Rho. The authors concluded that statins, including cerivastatin, target the assembly of the NADPH oxidase by preventing the isoprenylation of p21 Rac. The NADPH oxidase was found to be the source of O_2^- generation in the rat aorta under the described experimental conditions [73].

Improvement of endothelial dysfunction in patients

A clinical study was recently published which provided some proof that the experimental findings about improvements in endothelial function are indeed of relevance for the clinical situation. Elderly diabetic patients were studied with or without mild hypercholesterolemia. Cerivastatin was given orally (0.15 mg/day) for 3 days. Several markers of endothelial function were assessed: endothelium-dependent flow-mediated dilatation, endothelium-independent dilatation by nitroglycerine in the brachial artery, NO-related products, such as nitrite/nitrate and cGMP, endothelium-related products, and 8-isoprostane as a marker of oxidant stress. Flow-mediated dilatation, nitrite/nitrate and cGMP levels were found to be significantly increased after cerivastatin treatment [75]. The change in flow-mediated vasodilation was surprisingly drastic after cerivastatin treatment for 3 days leading to vehement discussion [76, 77]. Two other statins, alone and in combination with antioxidant vitamins, did not bring such an improvement in brachial artery vasodilatation, not even after 1 year of therapy [76]. Beside some differences in patient profile (mild or severe hypercholesterolemia, diabetes, age) which could explain these differences in part, the data emphasize again that the potency of non-lipid-mediated effects between statins can differ enormously. This phenomenon must be due to the fact that cerivastatin possesses the highest bioavailability (60%) and lipophilicity among all HMG-CoA reductase inhibitors, which is the prerequisite for extrahepatic drug accumulation.

In addition, a prospective, double-blind study with overweight males aged between 40–60 years having combined hyperlipidemia but no other risk factor were treated either with fenofibrate, another lipid-lowering drug, or 0.2 mg cerivastatin for 6 weeks. With the help of high-resolution ultrasound, flow-mediated and nitroglycerin-induced dilation of brachial arteries was measured. An improvement of both endothelium-dependent and -independent dilation of the brachial artery was found but the effects on flow-mediated (endothelium-dependent) dilation were greater after cerivastatin treatment. This study underlines the beneficial effects of cerivastatin on arterial vasoreactivity also in patients with the strongest risk factor, hyperlipidemia [78].

Effects on eNOS-mediated collateral growth

As already mentioned, the increase of eNOS expression leads to elevated levels of NO which contributes to the beneficial vasodilating effects of statins. However, evidence has accumulated for the role of NO in mediating angiogenic effects of several important growth factors [79]. In fact, cerivastatin promoted collateral growth in ischemic tissues of $eNOS^{+/+}$ mice and eNOS activity was found to be essential for the enhanced recovery of blood flow after acute hind limb ischemia [80].

Effects on angiotensin II and its AT_1 receptor

One of the most potent vasoconstrictors serving as an antagonist of the vasodilator NO is angiotensin II. Angiotensin II induces hypertrophy, an increased production of extracellular matrix, and the expression of growth factors by arterial smooth muscle cells [81]. The effects of angiotensin II on the cardiovascular system are predominantly mediated by the angiotensin-1 receptor (AT_1-R) [82]. Increased expression of AT_1-R and the angiotensin-converting enzyme (ACE) is characteristic for atherosclerotic and restenotic lesions [83]. There is compelling evidence that statins influence the ACE system [84] and the expression of AT_1-R [85]. A recent in vitro study showed that the treatment of vascular smooth muscle cells with cerivastatin decreased AT_1-R mRNA and protein levels, acting at the transcriptional level [86]. Furthermore, in an in vivo study with hypertensive rats, cerivastatin was able to reduce not only blood pressure but also inflammatory responses and transcription factor activation [87]. In humans, the LDL-cholesterol-induced overexpression of AT_1 receptors elevating blood pressure could indeed be reduced by the decrease of AT_1-R density after treatment with statins for 6 weeks [85].

Cerivastatin and inflammation

The activation of inflammatory genes after endothelial dysfunction or injury is not only due to elevated angiotensin II levels but also to the expression of numerous inflammatory cytokines. Inflammatory processes are known to be prominent features of atherosclerosis involving a variety of different cell types: blood cells, such as monocytes, neutrophils, lymphocytes, and platelets, as well as vascular cells, such as endothelial cells and smooth muscle cells. Evidence has accumulated supporting the hypothesis that the complete atherosclerotic process may be an inflammatory event beginning with the earliest identifiable lesion, the so-called fatty streak, to advanced vulnerable plaques. Elevations of some clinical markers of inflammation, such as C-reactive protein (CRP), modified LDLs, homocysteine, and cytokines, have been identified as emerging risk factors

that may add prognostic information in patient management [88].

Effects on monocyte adhesion

The adhesion and invasion of monocytes upon vascular injury is a key step in atherosclerosis. Inflammatory cytokines and lipopolysaccharides (LPS) predominantly induce the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In vitro experiments with bovine aortic endothelial cells provided proof that cerivastatin suppresses LPS-induced ICAM-1 expression and this effect is reversible by supplementation of GGPP. Since the overexpression of a dominant-negative mutant of RhoA mimics this effect, blockade of geranylgeranylation of Rho by cerivastatin may again explain its effect [89]. Furthermore, monocyte adhesion to endothelial cells was impaired after cerivastatin treatment. Finally, cerivastatin decreased the induction of the transcription factor NF-κB which is involved in inflammatory processes [90].

Effects on pro-inflammatory factors

Another pro-inflammatory mediator is the terminal complement complex C5b-9. When human smooth muscle cells were stimulated with sublytic concentrations of single complement factors, C5b-9 was found to induce a nearly threefold increase in cell proliferation and a nearly fivefold activation of the MAP-kinase ERK (extracellular signal-regulated kinase). Cerivastatin inhibited the C5b-9-induced stimulation of smooth muscle cell proliferation and ERK activation, and the C5b-9-dependent release of interleukin (IL)-6 [91]. The effects of cerivastatin could be explained by previous experiments showing that the inhibition of C5b-9 induced NF-κB und AP-1 activation [92]. Again, coaddition of mevalonate reversed the effects. Thus, cerivastatin seems also to influence pro-inflammatory mediators which are involved in the pathogenesis of atherosclerosis.

Effects on inflammation after organ transplantation

Another clinical field in which inflammatory processes play a very important role is the allograft rejection response after organ transplantation. Current data indicate that cerivastatin may be able to suppress ICAM-1 expression in such acute inflammatory responses. An experimental study in rat cardiac allografts demonstrated the suppression of immune responses after cerivastatin treatment which was also indicated by lower IL-2 concentrations and a decrease in CD4-positive cells [93].

Effects on inflammation after infections

Evidence has emerged in the last 3 years indicating that infectious organisms, such as bacteria or viruses, may initiate or promote, at least in part, the atherosclerotic process. One mechanism may be the release of inflammatory and immune modulators which contribute to dissemination of the infection. One of the best-studied bacteria is *Chlamydia pneumoniae* which accumulates in alveolar macrophages during pulmonary infections and which can be cultured from coronary plaques [94]. Interestingly, in macrophage cultures, the infection rates with *C. pneumoniae* were lower after preincubation with cerivastatin and the secretion of the pro-inflammatory chemokines monocyte chemoattractant protein-1 (MCP-1) and IL-8 were reduced in both macrophages and endothelial cells after cerivastatin treatment [95]. Again, the latter effect was reversible by the addition of mevalonate.

Effects on CD40/CD40 ligand

Another important role seems to be played by CD40/CD40 ligand (CD154) interactions, which can also induce the synthesis of pro-inflammatory cytokines, chemokines, and adhesion molecules. The CD40 receptor is found on endothelial cells, monocytes, macrophages, and B cells. Patients with mild hypercholesterolemia showed marked elevations of CD154 and P-selectin on platelets and of CD40 on monocytes which paralleled elevated blood levels of MCP-1. However, CD40 on monocytes and MCP-1 levels were significantly down-regulated after cerivastatin treatment for a short period of 3 weeks. These results were supported by the in vitro observation that CD40 was down-regulated after pretreatment of endothelial cells or monocytes with cerivastatin [96]. These experiments support a potential for cerivastatin to reduce monocyte activation and adhesiveness of blood cells, explaining again some of its non-lipid-related beneficial effects on atherosclerosis.

Effects on adhesion and invasion of blood cells

Flow cytometric analysis of adhesion molecules is a frequently used method to study monocyte adhesiveness to endothelial cells and the expression of surface integrins. In such experiments, cerivastatin was found to reduce the firm adhesion of monocytes on endothelial cells and the monocytic expression of the integrins CD11a, CD18, and VLA4. Again, the effects could be restored by the addition of mevalonate and a decreased membrane translocation of RhoA could be observed [97].

Upon activation and adhesion to the endothelium, transmigration of leukocytes is the most important step contributing to chronic inflammation of the vascular wall. There is also proof that neutrophil activation is related to

acute coronary events, such as unstable angina and myocardial infarction [98]. The synthesis and release of reactive oxygen species and chemoattractants by migrated neutrophils inside the intima mediates further cytotoxic effects on the endothelium and enhances vessel injury. Apoptosis, programmed cell death, normally restricts the accumulation of neutrophils, but several immune mediators, such as granulocyte-macrophage colony-stimulating factor, delay apoptosis of neutrophils. Furthermore, smooth muscle cells migrate from the media into the intima where their subsequent proliferation and matrix production also contribute to intimal thickening [98]. Thus, pharmacological strategies inhibiting neutrophil transmigration and inducing apoptosis of blood cells and smooth muscle cells may protect against new lesion formation in atherosclerosis. In fact, cerivastatin was found to inhibit in vitro leukocyte chemotaxis via the inhibition of their cell function and induce neutrophil, monocyte, and smooth muscle cell apoptosis [98]. By this effect, the number of activated, pro-atherosclerotic cells at inflammatory sites may be decreased by cerivastatin. In line with several other studies described in this article, the effects of cerivastatin, e. g., on the migration of neutrophils, could be restored completely by the addition of mevalonate.

Cerivastatin and thrombosis

Beside monocytes and macrophages, platelets contribute mainly to the progression of atherosclerosis by the release of growth factors and by the formation of occlusive thrombi after plaque rupture. Thrombosis is one of the major complications of atherosclerosis and monocytes contribute to the progression of atherosclerotic plaque formation by their pro-coagulant activity via the expression of tissue factor.

Effects of platelet tissue factor

On one side, platelets initiate thrombus formation directly, on the other, they can stimulate monocytic expression of tissue factor by the release of mediators because tissue factor is normally not detectable in circulating cells of healthy subjects [99]. When whole blood and cellular preparations of hypercholesterolemic patients were investigated for tissue factor levels before and after cerivastatin treatment, tissue factor expression in monocytes and whole blood was significantly reduced. Since tissue factor expression of monocytes is enhanced in hypercholesterolemic patients due to the enhancement of monocyte production of tissue factor by activated platelets, the beneficial antithrombotic effects of cerivastatin can be explained by the counteraction of tissue-factor-induced mechanisms [100].

Effects on vascular tissue factor

Elucidating the actions of tissue factor has also underlined the relevance of tissue factor induction in vascular cells for coronary syndromes [101, 102]. A recent in vitro study revealed that statins exert preventive effects on tissue factor induction. In human aortic endothelial cells, simvastatin could prevent tissue factor induction by thrombin and increased tissue factor activity on the cell surface. The up-regulation of tissue factor expression and activity could also be inhibited in human aortic smooth muscle cells [103].

Effects of cerivastatin on prostacyclin signaling

One of the most potent vasodilators and inhibitors of platelet aggregation is prostacyclin which is released by endothelial cells. Prostacyclin also serves as a cytoprotective factor against vessel injury. The effects of prostacyclin are mediated by coupling at the specific G-protein-coupled receptor, the prostanoid IP receptor. For IP receptor activation of adenylyl cyclase and efficient binding to phospolipase C, posttranscriptional isoprenylation is required. Thus, the effects of cerivastatin on IP receptor function are also relevant. In human cell cultures expressing the IP receptor stably, nanomolar doses of cerivastatin were found to inhibit IP receptor signaling more potently than micromolar lovastatin concentrations. However, both statins significantly reduced IP-receptor-mediated cyclic AMP generation [104]. Mention must be made that IP isoprenylation does not influence binding of the ligand prostacyclin [104] and that only human embryonic kidney and erythroleukemia cell lines have been studied to date, and not relevant primary vascular smooth muscle cells or freshly isolated platelets. However, one must take into consideration that adverse effects after high-dose and long-term use of statins could occur, which might be due to their interference with IP receptor isoprenylation and receptor signaling [104]. However, to date no clinically relevant negative effects have been reported and the prominent thrombotic risk reduction which is mediated by the other described pleiotropic effects seems to ‘overcompensate’ the reduction in prostacyclin-mediated antiaggregation.

Effects on peroxisome-proliferator-activated receptors

Recent work has provided some evidence that anti-inflammatory and antithrombotic actions of the arterial wall might be mediated by peroxisome proliferator-activated receptors (PPARs) [105] and that cerivastatin might influence PPARs at the subcellular level [106]. PPARs are transcription factors which regulate gene expression in inflammatory responses and lipid homeostasis. They are expressed in different cell types of human atherosclerotic

tissues where they influence the recruitment and adhesion of leukocytes and monocytes to the atherosclerotic lesion. Furthermore, PPARs modulate genes which control the thrombogenicity associated with plaque rupture. Thus, PPARs seem to influence atherosclerosis development by acting at both metabolic and vascular levels [51]. Clinical trials have demonstrated that PPAR agonists lower the progression of atherosclerosis and that specific PPAR ligands decrease intimal thickenings in human carotid arteries [107]. The development of PPAR agonists with anti-inflammatory properties was based on the finding that PPAR activation inhibits TNF-α action. The cytokines TNF-α and IL-6 are released by monocytes and macrophages and PPAR ligands can block phorbol-ester-induced synthesis of IL-6 and TNF-α in monocytes [108]. On the other hand, PPARs can also repress gene expression by interfering with other signaling pathways, such as the AP-1 and NF-κB pathways. The binding of the AP-1 and NF-κB proteins to their DNA target sequences can be prevented by PPARs. Finally, the interaction of PPARs with c-jun and p65 has been described [51].

Beside the classical hypolipidemic fibrates which are well-known ligands for the three PPAR isotypes α, γ and δ [109], statins can also influence PPARs at the molecular level. Cerivastatin was found to induce PPARα and PPARγ mRNA expression and protein levels in endothelial cells and hepatocytes, which might be associated with the induction of several genes [106]. Moreover, cerivastatin induced the transcriptional activation of PPARα/RXRα, PPARδ/RXRα, and PPARγ/RXRα. When cerivastatin was applied in addition to bezafibrate, the transcriptional activation of PPARα/RXRα induced by bezafibrate was increased synergistically and dose dependently by cerivastatin. Supplementation with mevalonate, farnesol, geranylgeraniol, or cholesterol was found to decrease the cerivastatin-induced transcriptional activation of PPARα/RXRα, again indicating that the effects were based on inhibition of the cholesterol biosynthesis pathway. Finally, the combination of cerivastatin and fibrates also decreased the transactivation of NF-κB [109]. Thus, the regulation of the cellular cholesterol metabolic pathway and cellular PPAR activity, which is related to inflammatory processes, contributes to the beneficial effects of cerivastatin in the prevention of vascular diseases induced by both hyperlipidemia and inflammation. The cross-talk between the statin and PPAR pathway which is activated by fibrates was also confirmed in hepatoma cells and the involvement of GGPP was again demonstrated [110].

The antiatherogenic effects of statins are not only due to the lowering of LDL-cholesterol but also to the increase in HDL and its major component apolipoprotein (apo)A1. Indeed, cerivastatin was also able to increase apoA1 mRNA levels in human hepatoma cells and this effect was shown to be mediated at the transcriptional level through the activation of the apoA1 promotor. There is a statin response element which coincides with a PPARα response element activated after treatment with fibrates. Thus, cerivastatin and fibrates were found to act in a synergistic manner. A transient cotransfection method with a dominant-negative form of RhoA was able to provide proof that the activation of PPARα by cerivastatin is based on the inhibition of the RhoA signal transduction pathway.

Cerivastatin and the thrombolytic system

Data on the effects of cerivastatin on hemostatic factors are limited despite its importance for atherosclerosis through fibrin formation and thrombus development. Relevant measurable markers are tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) levels. In normal vessels, there is a well-controlled balance between the synthesis and release of these two opposing factors. PAI-1 concentrations increase significantly during the progression from normal vessels to fatty streaks to the developed atherosclerotic plaque, whereas tPA levels show the opposite trend, with lowest amounts in advanced plaques [111]. Interestingly, measurement of hemostatic factors in initially healthy humans was found to help to predict the future onset of atherothrombotic events [112]. Treatment of hypercholesterolemic patients with lovastatin, e.g., for 6 months, has been shown to reduce PAI-1 activity significantly [113]. In 2002, a very interesting in vitro study provided proof that cerivastatin significantly decreases PAI-1 mRNA expression and protein synthesis in human vascular cells. Cerivastatin was found to be the most potent inhibitor among all statins if they were compared on a molar basis. Surprisingly, cerivastatin showed no effect on a human hepatoma cell line, which indicates the specificity of this effect for vascular cells [114]. Unfortunately, no clinical study has yet been published providing data if these cerivastatin-mediated effects may be relevant for the clinical situation.

Cerivastatin and plaque stability

As already pointed out, pivotal clinical trials have provided evidence that the event rate in CHD is not related significantly to the magnitude of LDL-cholesterol level reduction [115]. However, the cholesterol content and the degree of cholesterol modification determines mainly the composition of the atherosclerotic plaque. Rupture of vulnerable plaques and erosions are the most prominent features leading to thrombosis, acute myocardial infarction, and sudden death [116]. Thus, rather than the extent of the lumen-narrowing plaque, the composition and

characteristics of the plaque material determines the acute appearance and severity of coronary events [117]. Stable and hard fibrous plaques lower the risk of acute rupture. They are characterized by a high amount of fibromuscular components, such as smooth muscle cells and extracellular matrix proteins, predominantly the structural collagens which strengthen the fibrous cap. The thickness and stability of the fibrous cap covering the edges of the plaque determine the risk for ulcerations along the endothelial surface upon shear stress. Smooth muscle cells are the major 'players' in the physiological wound-healing process of vascular tissues, because they are needed for the normal reparative process. Thus, injuries at the plaque surface induce smooth muscle cell proliferation and migration, which are accompanied by the synthesis of interstitial collagens [118]. These 'wound-healing' reactions are mediated by growth factors such as TGF-β1 or platelet-derived growth factor (PDGF) which are synthesized from adhering platelets or activated vascular cells themselves [119, 120]. Another important property of the stable, fibrotic plaque is the absence of highly active inflammatory cells which can alter plaque structure predominantly by the catabolism of extracellular matrix via the secretion of MMPs. This leads to the weakening of fibrous caps preferentially at the shoulder region, the deposition and aggregation of platelets, and acute thrombosis. In contrast to the stable plaque, the typical vulnerable and weak atheromatous plaque with a high risk of rupture is composed of an increased number of macrophages and T lymphocytes, a decreased number of smooth muscle cells and extracellular matrix, a large lipid core with a high accumulation of lipid droplets or aggregates containing modified LDL, and a very thin, unstable fibrous cap, predominantly in the shoulder region. The presence of macrophages leads to accelerated degradation of the stabilizing collagens by matrix-degrading proteinases and the release of interferon-gamma (IFN-γ) by T lymphocytes which inhibits smooth muscle cell growth and matrix synthesis [121]. As already pointed out in the Introduction, plaque angiogenesis is another important feature influencing plaque growth and fragilization [57]. The complicated atherosclerotic plaque of symptomatic coronary patients is characterized by a high density of neovessels leading to plaque necrosis, plaque destabilization, and finally also to serious plaque rupture [59].

Effects on plaque composition

Overall lowering of blood LDL-cholesterol levels helps to prevent the accumulation of LDL-cholesterol in the lipid core of plaques [122]. Interestingly, an increase in LDL oxidation resistance and LDL vitamin E levels, improved LDL antioxidant capacity, and reduced macrophage cholesterol ester accumulation was observed in humans after statin treatment for some months or years. However, the striking improvement in cardiovascular event reduction evaluated in the large clinical trials cannot be completely explained by cholesterol-dependent effects [123]. For this reason, experimental studies have been performed to elucidate more precisely the plaque-stabilizing effects of statins [124]. Cerivastatin, for example, reduced the development of atherosclerotic lesions and plaque size in hyperlipidemic rabbits (WHHL). Cerivastatin-treated animals also showed a significant decrease in macrophages and extracellular lipid deposits inside aortic lesions, and the percent area of macrophages in coronary lesions was diminished whereas the percent area of smooth muscle cells and collagens remained unaltered [124]. However, cerivastatin was capable of inhibiting not only macrophage infiltration into the neointima but also the extent of neointima thickening several weeks after mechanical injury of the endothelium [125].
Which mechanisms lead to the decrease of macrophage deposition? Cerivastatin diminishes the accumulation of macrophages in aortic lesions and the expression of the matrix metalloproteinases MMP-1, MMP-3, and MMP-9. Furthermore, the expression of the pro-coagulant and pro-thrombotic tissue factor can be reduced by cerivastatin which induces the actions of CD40 ligand. Cerivastatin reduces the proliferative activity of macrophages which results in a decrease in the proteolytic activity of metalloproteinases and diminishes tissue factor expression [126].

Effects on the expression of protease within the plaque

Beside MMPs, uPA is activated in macrophage-rich regions of the plaque. As already mentioned, both uPA and MMPs are related to the weakening of the plaque. Recent work showed that cerivastatin is able to reduce monocyte adhesion, plasmin generation, uPA and uPA receptor expression, and MMP-9 secretion, whereas the release of the inhibitor of metalloproteinase-1 (TIMP-1) remained unaltered. At low, nanomolar concentrations, only the overexpression of urokinase expressed on monocytes under oxidized LDL treatment and plasminogen activators returned to basal values. In contrast, higher doses decreased uPA even on unstimulated monocytes. A mechanistic explanation is provided by the finding that a delocalization of Ras from the monocyte membrane occurred after cerivastatin treatment which was accompanied by the inhibition of NF-κB translocation into the nucleus. NF-κB is a transcription factor for uPA and MMP-9 genes. The cerivastatin-induced effect could be reversed by FPP [127].
One step further to the clinical situation, a small patient study with hypercholesterolemic probands receiving cerivastatin for 1 year at a very low dose of 0.15 mg/day

confirmed some of the effects on plaque stabilization found in animals. Ultrasonography of the carotid artery showed a decrease in the amount of cholesterol esters within plaques, an increase in the mean percentage of fibrous plaque material, and a significant reduction in plaque height whereas no significant cholesterol lowering occurred. These results provide at least some proof for the alteration of plaque composition by cerivastatin in humans [128]. Finally, insights into the underlying molecular mechanisms were provided, since cerivastatin was found to inhibit the expression of the *ets-1* gene which is involved in MMP synthesis [129].

Cerivastatin and angiogenesis

As already pointed out above, the complicated and fragile atherosclerotic plaque of symptomatic coronary patients is characterized by a high density of neovessels. This is the result of 'angiogenesis', which is defined as the formation of new blood vessels by endothelial cells. Indeed, recent studies revealed that cerivastatin is able to inhibit capillary tube formation in vitro. This effect was based on a decreased locomotion of endothelial cells and could be reversed by GGPP. Again, the delocalization of RhoA from the cell membrane to the cytoplasm indicated that this effect of cerivastatin was related to the inhibition of downstream isoprenoids. However, FFP addition reversed cerivastatin-induced inhibitory effects on MMP-2 which is also involved in cell invasion. Thus, the effects of cerivastatin on endothelial cells seems to be related to both the RhoA and Ras pathways [130].

These observations were extended by data showing that cerivastatin repressed the proliferative activity of angiogenic factors on microvascular endothelial cell proliferation and actin stress fiber formation whereas the unstimulated, basal endothelial cell growth was not influenced. This growth inhibitory effect was again found to be related to a G_1/S arrest which was accompanied by an increase in the amount of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$. Since GGPP and mevalonate, but not FFP, were able to reverse these effects, a relationship between cerivastatin-induced inhibitory effects and RhoA inactivation was again shown. Indeed, the delocalization of RhoA from the cell periphery to the cytoplasm and the induction of actin depolymerization could be observed, which was reversible by GGPP coincubation. RhoA-dependent inhibition of cell proliferation was associated with the inhibition of focal adhesion kinase and Akt activations. These findings could be confirmed in two in vivo angiogenesis models [131].

Discrepancies exist in the literature regarding the pro- or antiangiogenic effects of statins and their downstream effects on Akt. The two studies with cerivastatin by Vincent et al. [130, 131] are in contrast to the study reported by Kureishi et al. [132], showing that simvastatin promotes angiogenesis at unstimulated endothelial cells which was accompanied by Akt activation. The authors explained that several differences existed between these two studies in the experimental models used (effects with or without stimulation by angiogenic factors) and the origin of endothelial cells (microcapillary endothelial cells which are representative of atherosclerotic plaque vascularization versus umbilical vein cells). Furthermore, in the report Kureishi et al. [132], Akt phosphorylation in smooth muscle cells was not influenced by statins at all. Nevertheless, Vincent et al. [131] provided direct proof for the antiangiogenic properties of cerivastatin in two independent in vivo models: (i) in bFGF-enriched matrigel introduced subcutaneously in mice and (ii) in the chick chorioallantoic membrane model [131]. These in vivo findings were also confirmed by Park et al. [133] using simvastatin at concentrations similar to those found in the serum of treated patients. Capillary tube formation was again inhibited in both vascular endothelial growth factor (VEGF)-stimulated chick chorioallantoic membranes and bFGF-stimulated mouse corneas [133]. Thus, there is striking evidence for the antiangiogenic activity of statins and the described contrasting in vitro effects are unlikely to be explained by the use of different HMG-CoA reductase inhibitors.

Interestingly, evidence hast also been provided that statin doses may influence the effects on angiogenesis. A very elegant set of recent experiments published several weeks ago investigated the effects of cerivastatin on angiogenesis in vitro and in vivo in a much broader concentration range than all other studies published previously. Interestingly, a biphasic, dose-dependent effect on angiogenesis was found when endothelial cell proliferation, migration, and differentiation were studied at low (0.005–0.01 μM) or high (0.05–1.0 μM) cerivastatin doses. At high doses, endothelial cell apoptosis was increased and accompanied by reduced secretion of the angiogenic VEGF. This effect was confirmed by in vivo data in murine models showing a reduced inflammation-induced angiogenesis at high cerivastatin concentrations. In contrast, low cerivastatin doses showed pro-angiogenic effects both in vitro and in vivo. These test concentrations represented serum levels in humans after oral application of low (0.2 mg/day) and high (0.8 mg/day) cerivastatin doses [134].

Cerivastatin and smooth muscle cell proliferation/migration

The contribution of antiproliferative and antimigratory effects to the pleiotropic effects of cerivastatin has been demonstrated clearly by three independent groups. However, determination of cell mitosis and cell numbers ver-

sus untreated controls in several in vitro assays showed that the potency differs between cerivastatin and the other statins.

Previously, we described some mono- and coculture systems having the advantage that primary cultures of human arterial endothelial (haEC) and smooth muscle cells (haSMCs) are isolated from human arteries and used in very early passages to preserve most of the in vivo properties of normal arterial cells in men [135–137]. Furthermore, we developed a transfilter coculture model to imitate the morphology of the arterial vessel wall enabling cell-to-cell interactions by direct cell-cell contacts through lamellipodia and by the secretion of PDGF-AB, TGF-β1, and other mediators [136, 138]. When cerivastatin was applied continously to the endothelial side of transfilter cocultures for 2 weeks, a dose-dependent inhibition of haSMC proliferation on the opposite filter side could be observed [139]. In transfilter cocultures, higher IC_{50}s were determined compared to monocultures because test compounds had to first cross the endothelial cell lining and the filter pores to reach the target cell, namely the haSMC multilayer. In addition, haSMC migration from the upper to the lower filter side toward the endothelial cell lining can be analyzed in this model which resembles migration of haSMCs from the media to the intima in vivo. The adequacy of this test system could be confirmed by immunohistochemical examinations showing the formation of haSMC multilayers on the lower filter side after 2 weeks standard cultivation covered by a confluent endothelial cell lining [136]. After treatment with cerivastatin, the multilayer was reduced significantly to one to two layers whereas the endothelial cell lining remained still intact. With respect to a potential inhibition of restenosis in humans, the use of cerivastatin seems to be the most promising strategy because the effective dose range could be determined at about 10 to 100-fold lower concentrations compared to all other statins [139–141]. Significant effects were already measurable at concentrations ≥ 0.005 µM and the IC_{50} was determined at 0.037 µM (mitochondrial activity) and 0.064 µM (proliferation in cocultures), which correspond nearly exactly with serum levels in humans (0.002–0.05 µM; table 1). In contrast, the IC_{50} after simvastatin, lovastatin, or atorvastatin treatment was calculated between 0.36–17.5 µM [139] which differs much more from the in vivo levels in humans (maximum: 0.002–0.2 µM) after oral administration of the highest dosage and after liver passage [20, 142] (see table 1). Furthermore, nanomolar cerivastatin doses were able to inhibit haSMC migration (IC_{50}: 0.04 µM) even when the test compound was applied exclusively to the endothelial side of transfilter cocultures which first requires diffusion across the filter pores to the haSMC-layers (table 1). To elucidate the subcellular mechanisms, immunofluorescence microscopic staining with specific antibodies against RhoA was performed after cerivastatin treatment for 24 h. In line with several other studies, we found a translocation of RhoA from the cell periphery, namely the cell membrane, into the cytoplasm, predominantly to the perinuclear region (see fig. 2).

Concerning cholesterol-reducing effects of statins in vitro, cerivastatin was found to display an equal cholesterol synthesis inhibitory potency to lovastatin, simvastatin, atorvastatin, and fluvastatin with an IC_{50} at about 0.002 µM. When compared to the IC_{50} for haSMC growth inhibition (about 0.04–0.06 µM), just a 20-fold difference to the IC_{50} of cholesterol synthesis was found whereas a 250 to 1500-fold difference was determined for the other lipophilic statins (IC_{50}: 0.5–3.0 µM) [140].

Cerivastatin and the extracellular matrix

The extracellular matrix (ECM) determines mainly the dimension, structure, and vulnerability of the intimal plaque [53, 143]. The fibrous proteins biglycan and collagen-1 are characteristic components of advanced atherosclerotic and late restenotic lesions [118, 144]. In contrast, the ‘matricellular’ glycoprotein TSP-1 is expressed immediately after vessel injury and functions as a kind of

Table 1. IC_{50} (µm) values of statins: in vitro cell growth and matrix inhibition versus C_{max} in blood.

	Pravastatin	Lovastatin	Simvastatin	Atorvastatin	Cerivastatin
MI: haSMC	83.8 ± 21.3	0.36 ± 0.06	0.38 ± 0.04	0.57 ± 0.10	0.037 ± 0.003
MI: haEC	95.0 ± 13.3	0.28 ± 0.04	0.17 ± 0.03	0.33 ± 0.03	0.008 ± 0.001
cocu: prol.	no effect	4.01 ± 1.3	0.65 ± 0.09	17.50 ± 2.9	0.064 ± 0.02
cocu: migr.	no effect	3.40 ± 0.5	0.77 ± 0.07	5.75 ± 1.7	0.040 ± 0.005
TSP-1 mRNA	no effect	0.5	0.5	not studied	0.03
C_{max} in men	0.1–0.5	0.02–0.05	0.02–0.08	0.002–0.2	0.002–0.05

Mitochondrial activity (MI) of human arterial smooth muscle cells (haSMC) and endothelial cells (haEC) after 4 days, proliferation (prol.) and migration (migr.) of human arterial smooth muscle cells in coculture (cocu) with activated endothelial cells for 14 days, and thrombospondin-1 (TSP-1) mRNA expression after 3 days treatment with increasing statin doses. C_{max} demonstrates maximum blood levels achieved in humans after oral application of 40–80 mg (except cerivastatin: 0.2–0.8 mg) of each statin.

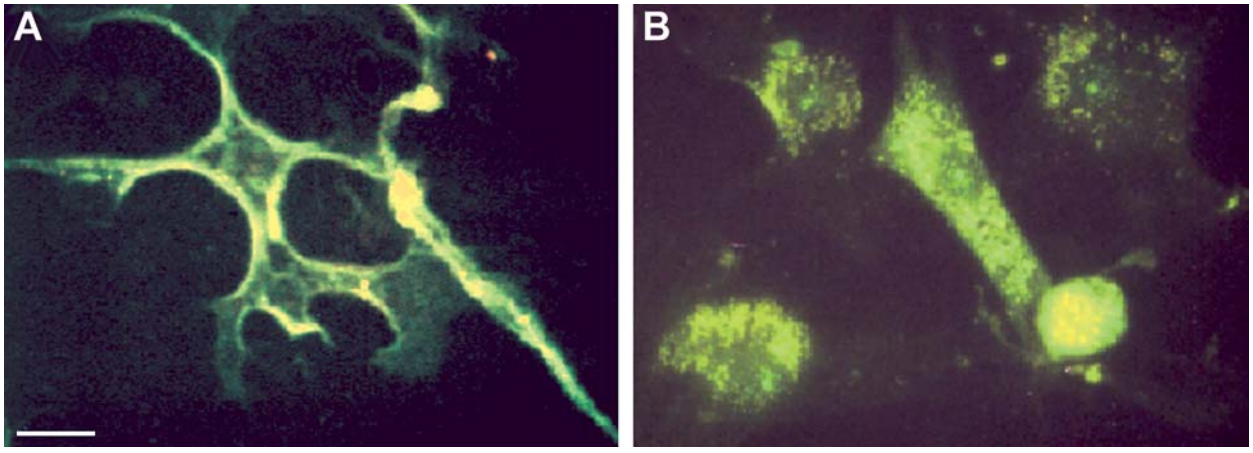

Figure 2. Key mechanism of cerivastatin's subcellular and molecular actions: delocalization of RhoA from the cell periphery to the perinuclear region of human arterial smooth muscle cells. (*A*) Control culture without cerivastatin showing distinct RhoA staining along the cell membrane and at the lamellipodia extensions of characteristic spindle-shaped cells (scale bar, 10 µm). (*B*) Treated culture 24 h after addition of 0.5 µM cerivastatin showing a punctate, diffuse staining of RhoA in the cytoplasm and an altered cell shape: most cells are rounded and lamellipodia have disappeared.

immediate-early protein [145]. TSP-1 modulates cell-matrix interactions, stimulates smooth muscle cell mitosis and contributes to the degradation of the ECM [146]. TSP-1 is secreted by thrombin-activated platelets, binds to the platelet surface, and interacts with integrins, as well as with integrin-bound fibrinogen and fibronectin [147]. Furthermore, it induces platelet aggregation through the FcR gamma chain signaling pathway and through agglutination [148]. In contrast to TSP-1, fibronectin is a structural glycoprotein which regulates cell adhesion, cell motility, and differentiation [144]. After statin treatment in the same dose range capable of inhibiting cell growth (see table 1), mRNA expression of the 'pro-atherothrombotic' ECM protein TSP-1 and the structural proteins collagen-1 and biglycan [146] was significantly reduced, whereas fibronectin and the matrix regulating components MMP-2 and TGFβ1 were not influenced. The mRNA expression of TIMP-2 was even stimulated by cerivastatin [149]. Thus, the stimulation of TIMP-2-mediated MMP inactivation may also contribute to the beneficial effects of statins preventing plaque rupture [53]. Finally, current experiments with the specific Rho kinase inhibitor Y-27632 reveal that the effects of cerivastatin on TSP-1 mRNA expression can be mimicked to some extent by blockade of Rho kinase [unpublished observations].

Cerivastatin and atherosclerosis-independent effects

Effect of cerivastatin on bone formation

Recent work has supported the hypothesis that statins may reduce the risk of bone fractures and even enhance new bone formation in vitro and in rodents by the inhibition of osteoclastic activity [150]. In a search for agents that enhance osteoblast differentiation and bone formation (30,000 natural compounds), lovastatin was found to be as the only product that specifically increased luciferase activity in cell cultures, indicating that the promoter of the bone morphogenetic protein-2 (BMP-2) gene was activated. Some epidemiological studies have already reported reductions in fracture risk [151], but clinical relevance is not clarified satisfactorily. Possible effects on bone fracture are an important clinical issue because of the increasing use of statins in the primary and secondary prevention of atherosclerosis in elderly, low- and high-risk patients. Some discrepancies in the present data may be due to the fact that the extent of statin uptake into the bone determines the results. Statins predominantly accumulate in the liver and most are biotransformed rapidly, undergo a high first-pass effect, and do not reach bones in sufficient amounts [152]. Second, oral doses used were probably not sufficiently high because only the use of higher doses decreased the fracture risk [153]. Interestingly, a protective effect for fractures of the hip, vertebral body, and foot in individuals aged 50 years and older after relatively short treatment periods was reported. This protective effect was much weaker or not present when other lipid-lowering drugs were used [154].

In a first case report about the treatment of a young woman with type I osteogenesis imperfecta with 0.8 mg cerivastatin per day for 6 months, bone-forming effects were reflected in an sharp increase in specific bone-formation markers after starting the treatment. The bone forming effect of cerivastatin is presumed to be induced by a higher deposit of calcium in bone [41]. Undoubtedly interesting would be to study long-term effects of cerivas-

tatin in larger, controlled, and randomized trials to confirm these very promising non-lipid effects of cerivastatin.

Effect of cerivastatin on central nervous system diseases

Epidemiological studies have provided a growing body of evidence that hypercholesterolemia is also a risk factor for some brain diseases, such as dementia [155]. Furthermore, some recent clinical reports found a strong association between statin therapy and a reduction in the occurrence of Alzheimer's disease and indicate that statins may also exert other beneficial effects on central nervous system (CNS) diseases beyond their vascular effects [156]. Isoprenoid-dependent, neuroprotective properties of statins have been studied [157]. The development of Alzheimer's disease was found to be associated with the accumulation and aggregation of amyloid-beta protein (Aβ) within the brain and statins were found to reduce intracellular and extracellular levels of Aβ peptides in neurons [158]. Thus, the use of statins may offer new therapeutic strategies for the treatment or prevention of Alzheimer's disease. Unfortunately, the possible impact of cerivastatin on this field cannot be examined because of its withdrawal from the market. Given the high bioavailability of cerivastatin compared to the other statins and its chemical properties allowing sufficient uptake into non-hepatic tissues and penetration of the blood/brain barrier, it would undoubtedly exert very potent effects on CNS functions.

Effects of cerivastatin on tumor growth

Retrospective studies of clinical statin trials have revealed that the incidence of cancer may be decreased with HMG-CoA reductase inhibitors. Patients under statin therapy were found to be 28% less likely to be diagnosed as having any cancer versus patients receiving bile acids [159]. Even the large 4S trial with simvastatin revealed a 27% reduction in cancer deaths for men and women versus placebo [160]. On the basis of some in vitro experiments, the inhibition of isoprenoid synthesis was again proposed to be the key step. Tumor HMG-CoA reductase possesses high sensitivity to the isoprenoid-mediated secondary regulation. The repression of mevalonate synthesis by specific isoprenoid endproducts was found to reduce ras and lamin B processing, arrest cells in G_1, and initiate cellular apoptosis [161]. Recently, the contribution of cerivastatin to Ras and RhoA inhibition was studied, when its inhibitory effects on a highly invasive and metastatic breast cancer cell line expressing Ras and RhoA were compared to a poorly invasive and non-metastatic breast cancer cell line. Cerivastatin was found to inhibit proliferation and invasiveness of the highly active, aggressive breast cancer cell line whereas the effects on the low-aggressive cell line were much weaker. The antiproliferative effect was reversible on the addition of GGPP but could not be reversed by FPP. Thus, the Rho-dependent rather than the Ras-dependent MAPK pathway seems to be involved in this process. Since the antiproliferative effect was found to be related to cell cycle arrest in the G_1/S phase of the cell cycle, this in vitro study with cerivastatin was in line with a previous lovastatin study showing an increase in $p21^{Waf/Cip1}$, a cyclin-dependent kinase inhibitor blocking G_1/S transition [39]. The geranylgeranylation of RhoA promotes cell growth, influencing the suppression of $p21^{Waf/Cip1}$ transcription. NFκB and AP-1 transcription are well known to be stimulated by the prenylation of RhoA [44, 87], both regulating the function of genes involved in cell growth and invasiveness.

In an attempt to screen for the growth inhibitory effects of cerivastatin, a panel of different human and murine tumor cell lines was studied. Cerivastatin-induced effects were compared to the growth inhibitory potency of lovastatin and simvastatin. Cerivastatin revealed the most potent antiproliferative activity, being 2.5–55 times more effective than the other statins. Similar to its effects on vascular smooth muscle cells, the IC_{50} values were determined at nanomolar up to low micromolar doses although slightly higher doses were needed to inhibit tumor than smooth muscle cell growth [38].

Furthermore, there is proof that statins can trigger apoptosis in some tumor cells, e.g., human acute myeloid leukemia (AML) cells. In the effective dose range, cerivastatin inhibited cell growth and apoptosis about ten times more potently than lovastatin. Cerivastatin-induced apoptotic effects could be abolished by the addition of mevalonate and GGPP, again suggesting the involvement of the isoprenoid biosynthesis pathway. The apoptotic effects of cerivastatin seem to be specific for transformed cells, since non-transformed human bone marrow progenitor cells were not influenced [162].

Effect of statins on circulating progenitor cells

The most exciting and innovative research at present involves examining effects of statins on human endothelial progenitor cells (EPCs). The development of therapeutic strategies with stem cells in general is currently the most promising but also the most discussed research field. One example is therapeutic neovascularization, an important goal to recover ischemic tissue, e.g., to restore blood flow in patients with limb ischemia. However, stimulation of angiogenesis is also an important therapeutic option to improve ischemic heart disease. As already mentioned, low doses of statins, including cerivastatin, can exert pro-angiogenic effects in vitro. Recent animal studies, however, have suggested that statins may

also promote angiogenesis in vivo in ischemic limbs and preserve ischemic-reperfused myocardium of the heart. To gain more insights into the underlying signal transduction mechanisms by which statins promote vasculogenesis, circulating EPCs were used [163]. EPCs are this leave the bone marrow in response to ischemic damage or by cytokine triggering and are recruited to the injured peripheral tissue to stimulate vasculogenesis. To date, the most important known supporter of this process is VEGF. However, its effects are imitated by statins. In vitro, statins augment the number of EPCs isolated from peripheral blood and induce EPC differentiation via the activation of the PI3-kinase/Akt pathway. Akt is a serine/threonine kinase already known to be involved in statin-induced angiogenesis [132]. Furthermore, statins enhance not only proliferation but also migratory activities and survival of EPCs. After 2 days in culture, 88% of EPCs become senescent. However, the addition of atorvastatin improved their viability significantly [164]. Since other HMG-CoA reductase inhibitor acted in the same manner, these actions seem to be class effects, and would probably also apply to cerivastatin. In contrast to most other pleiotropic effects of statins, statin-induced Akt activation in endothelial cells could be reversed by mevalonate but was nearly independent of the downstream isoprenoid GGPP or Rho kinase. Instead, the expression of cyclins, such as cyclin F, was found to be regulated by statins [164].

In vivo experiments in mice supported these data. In mice the recruitment of bone-marrow-derived endothelial progenitor cells could be demonstrated after simvastatin treatment [163].

Are there also data available about the effects of cerivastatin on EPCs? In an interesting animal study, hind limb ischemia was induced in a wild-type mouse, and mice were treated with cerivastatin (among others). EPCs that incorporated into new or enlarging vessels during ischemia-induced angiogenesis were identified with several detection methods. EPC incorporation in the control group was rare (1.6% of the vessels) whereas vessel density was significantly higher in animals treated with cerivastatin. In the cerivastatin group, EPCs could be detected in 15.5% of the vessels. Thus, EPCs did not significantly contribute to ischemia-induced angiogenesis in control animals but EPC incorporation could be enhanced enormously by cerivastatin treatment [165].

To provide some evidence that these effects may also be involved in patients and contribute to the benefits of statin therapy, 15 patients with stable coronary artery disease were treated with atorvastatin for 4 weeks and EPCs were isolated before and after initiation of statin treatment. After 1 week, a 1.5-fold increase in circulating EPC numbers was recorded, which increased to 3-fold levels after 4 weeks. In addition, isolated EPCs also exhibited a higher migratory activity. If one goes back to patients with early stages of coronary atherosclerosis, coronary flow reserve after cholesterol lowering could indeed be rapidly improved after 6 months treatment with statins.

Although these are preliminary data and the work in this field has been triggered enormously by the enthusiasm for stem cell research that the multipotent, pleiotropic actions of statins include EPC activation is not surprising. Unfortunately, the withdrawal of cerivastatin from the market makes it currently impossible to test it in patient studies. With its two unique properties – potent effects at 100-fold lower doses than other statins and highest bioavailability – there is no doubt that cerivastatin would show equal or even better effects on peripheral, extrahepatic processes, such as EPC recruitment from bone marrow to ischemic tissues.

Conclusions

In August 2001, cardiologists were shocked when they were deprived of one of their most potent tools for the prevention of cardiovascular mortality – the potent low-dose HMG-CoA reductase inhibitor cerivastatin was withdrawn from the world market. The reasons were some press releases of fatal rhabdomyolysis, a side effect which resulted in 31 deaths in the USA after overdosing and combination therapy with the lipid-lowering drug gemfibrozil [166]. Nevertheless, this overview of the various pleiotropic effects of cerivastatin demonstrates clearly that it is a very potent drug not only for cardiovascular medicine but probably also for the treatment of other diseases, such as bone diseases, tumors, and Alzheimer's disease. Most of these effects are based on a common mode of action, inhibition of the prenylation of small GTP-binding proteins belonging to the Ras superfamily (fig. 3). It is obvious that very efficient drugs also show side effects which, however, even confirm their potency. The great variety of effects at the cellular and molecular level emphasizes the important regulatory role of cerivastins in varying functions of different cell types and tissues. With the help of its high bioavailability, sufficient accumulation in extrahepatic tissues is also guaranteed. Moreover, it is the only statin showing a correspondence between effective in vitro concentrations in the nanomolar range and blood levels achieved in patients after oral application. Since side effects can be detected and avoided early by analyzing the blood picture of patients carefully and limiting drug combinations and overdosing, cerivastatin could still be a very valuable drug for the future.

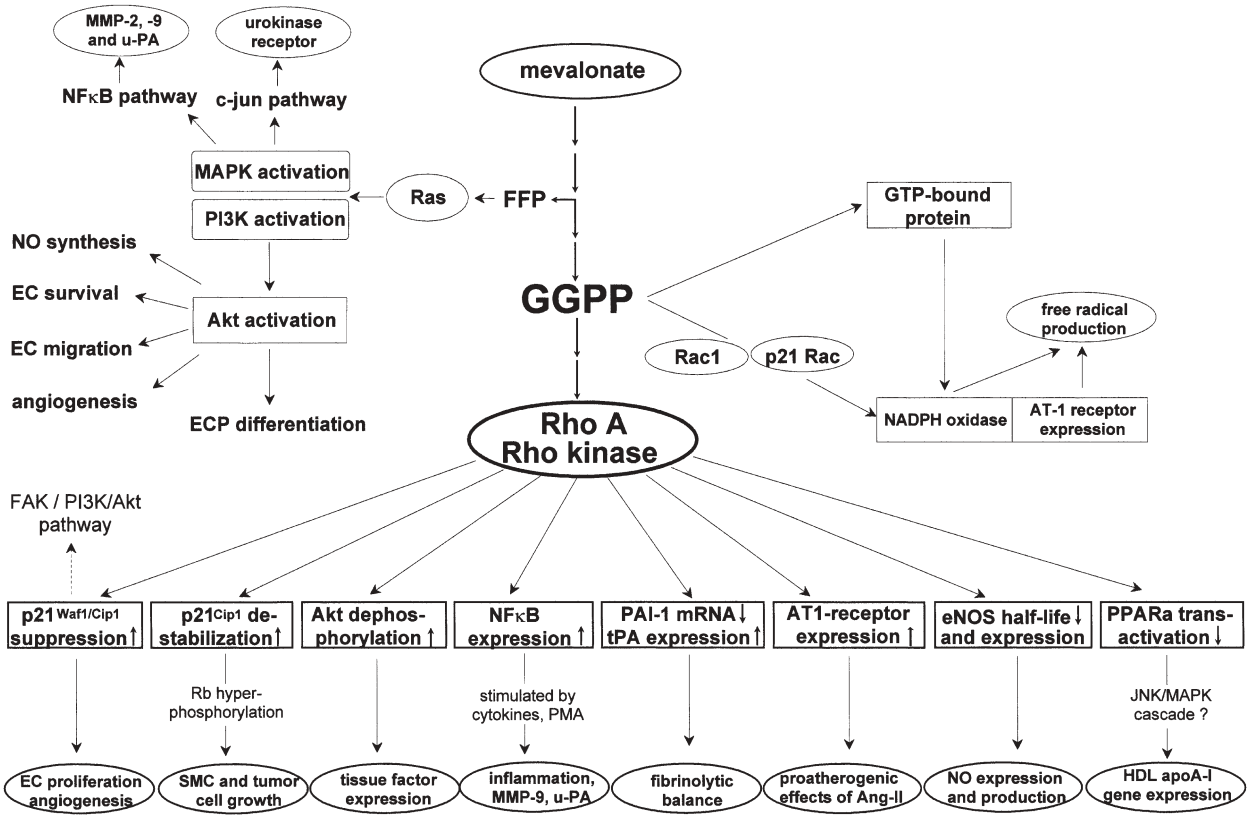

Figure 3. Summary of the pleiotropic effects as a result of isoprenoid biosynthesis from mevalonate with the focus on GGPP-induced Rho activation. After treatment with HMG-CoA reductase inhibitors, the effects are reversed: RhoA-stimulated processes (indicated as ↑) are blocked and actions which were suppressed by RhoA (indicated as ↓) are restimulated.

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