Cerivastatin Activates Endothelial Calcium–Activated Potassium Channels and Thereby Modulates Endothelial Nitric Oxide Production and Cell Proliferation

CHRISTOPH RÜDIGER WOLFRAM KUHLMANN,* CHRISTINE GAST,* FANG LI,* MATTHIAS SCHÄFER,† HARALD TILLMANNS,* BERND WALDECKER,* and JOHANNES WIECHA‡
*Department of Cardiology and Angiology, Justus-Liebig-University of Giessen, Giessen, Germany; †Department of Physiology, Justus-Liebig-University of Giessen, Giessen, Germany; and ‡Department of Internal Medicine, Hospital Bad Orb, Bad Orb, Germany

Abstract. Statins are known to counteract the process of arteriosclerosis by exerting direct pleiotropic effects on vascular endothelium. The aim of this study was to investigate a possible effect of cerivastatin on endothelial Ca2+-activated K+ channels (BKCa) and to assess their contribution to cerivastatin-mediated changes of endothelial nitric oxide (NO) production and proliferation. Membrane potential was measured using bis-1,3-dibutylbarbituric acid-trimethine omonol–fluorescence imaging. Patch-clamp recordings of BKCa were performed on cultured human umbilical vein endothelial cells. NO production was measured using 4,5-diaminofluorescein–fluorescence imaging and a [3H]cGMP RIA. Proliferation was analyzed by means of cell counts and [3H]thymidine incorporation (TI). Cerivastatin (0.001 to 0.05 μmol/L) caused a significant membrane hyperpolarization (n = 30; P < 0.05). This effect was abolished using the BKCa inhibitor iberiotoxin (IBX; 100 nmol/L). The addition of mevalonate (500 μmol/L) blocked the BKCa activation induced by cerivastatin (n = 19; P < 0.05). Endothelial cGMP level was increased by acetylcholine (ACh; 1 μmol/L). The combination of ACh and cerivastatin additionally increased cGMP levels, with a maximum at 0.03 μmol/L cerivastatin (84%; n = 10, P < 0.01). ACh-induced increase of cGMP-level was significantly reduced by IBX (n = 10, P < 0.01) as it was with all combined administrations of ACh and cerivastatin. 4,5-Diaminofluorescein–fluorescence measurements revealed a significant increase of NO levels by cerivastatin, which was abolished by IBX (n = 30; P < 0.05). Cell counts and TI demonstrated significant inhibition of human umbilical vein endothelial cell proliferation with a maximum at 0.03 μmol/L (cell count, −32.2%; TI, −70%; n = 12; P < 0.01). These data show that cerivastatin activates endothelial BKCa, which plays an important role in the signaling of cerivastatin-mediated endothelial NO production and proliferation.

Cardiovascular risk factors such as hyperlipidemia, diabetes, hypertension, and smoking have been shown to cause endothelial dysfunction, which is characterized by an inadequate vascular response to vasorelaxant stimuli such as acetylcholine (ACh) (1,2). This is of great importance because endothelial dysfunction is the initiating step in the pathogenesis of arteriosclerosis (3,4). Because of the ischemia and resulting organ damage that it produces, arteriosclerosis is the number-one cause of death in Western countries.

Statins are known to reduce both atherosclerotic lesions and cardiovascular morbidity and mortality (5). However, their beneficial effects are not fully explained by their ability to reduce LDL (5,6). Thus, they have been described to exert a number of pleiotropic effects on the endothelium, thereby directly counteracting the process of arteriosclerosis (7,8). This has been explained by the fact that statins not only inhibit cholesterol formation but also reduce intermediate isoprenoid products of the mevalonate pathway known to regulate cellular signaling cascades such as cell differentiation and proliferation (8). Statins have been reported to reduce endothelial proliferation directly and thus to inhibit the intimal neovascularization responsible for plaque growth (9,10).

Accordingly, inhibition of rho isoprenylation by statins has been shown to play a decisive role in statin-induced increase of endothelial NO synthase (eNOS) expression (11). An increased NO bioavailability is observed within the first few weeks of statin treatment (12) and is essential for improving vascular function.

Conversely, many essential endothelial pathways are known to be modulated by membrane potential (MP) (13), which, in turn, is largely controlled by endothelial potassium channels.
Thus, endothelial potassium channels influence intracellular calcium levels (14) involved in the regulation of endothelial functions such as proliferation, migration, and permeability (15–17). Furthermore, the presence of intracellular calcium has been shown to be essential for agonist-induced NO formation (18). Recently, Ca\(^{2+}\)-activated K\(^+\) channels of large conductance (BK \(_{Ca}\)) have been directly associated with the regulation of endothelial proliferation (19) and the regulation of NO synthesis (20,21). Therefore, the aim of our study was to investigate the effect of cerivastatin on endothelial BK\(_{Ca}\) and to find a possible contribution of BK\(_{Ca}\) to cerivastatin-mediated endothelial NO synthesis and proliferation.

## Materials and Methods

### Isolation and Culture of Human Umbilical Vein Endothelial Cells

Endothelial cells were isolated from human umbilical cord veins by a collagenase digestion procedure and grown in culture as described previously (22). The endothelial cell basal medium (EBM; PromoCell, Heidelberg, Germany) was enriched with 10% FCS (PAA, Linz, Austria) and the following substances: 0.4% ECGS/H, 0.1 ng/ml epidermal growth factor, 1 \(\mu\)g/ml hydrocortisone, 1 ng/ml basic fibroblast factor, and 50 \(\mu\)g/ml gentamicin (PromoCell). The culture medium was changed every 48 h. Cell proliferation studies were carried out using endothelial cells from subcultures 4 to 8.

### Bis-1,3-Dibutylbarbituric Acid-Trimethine Oxonol—Fluorescence Imaging

HUVEC were grown on glass coverslips. MP was assessed using the fluorescent dye bis-1,3-dibutylbarbituric acid-trimethine oxonol (DiBAC\(_{4}\); Molecular Probes, Leiden, Netherlands). Cells were incubated in the same bath solution as for electrophysiologic recordings containing 0.5 \(\mu\)mol/L DiBAC\(_{4}\) for 15 to 30 min. Coverslips were mounted into an incubation chamber adapted to a fluorescence microscope (IX 70; Olympus, Hamburg, Germany). After 3 min, cerivastatin at concentrations of 0.001 to 0.05 \(\mu\)mol/L was added. Fluorescence intensities were acquired during intervals of 6 s and averaged over 1 min. Fluorescence was excited at 490 nm, and emitted light was detected at 535 nm. Changes in endothelial cell MP were analyzed with the TILL Photonics imaging system ( Martinsried, Germany). Data were expressed as DiBAC\(_{4}\)-fluorescence intensity.

### Electrophysiologic Recordings

Single-channel membrane currents were measured by means of the patch-clamp technique (23) in the cell-attached configuration. Patch pipettes of borosilicate glass ( Hilgenberg, Malsfeld, Germany) with a final resistance of 5 to 8 M\(\Omega\) when filled with pipette solution were used. For the recording, an L/M-PC patch-clamp amplifier (List, Darmstadt, Germany) was used. The data obtained were low-pass filtered at 1 kHz (6-pole Bessel filter), digitized (sample rate, 10 kHz) using a Digidata 1200A (Axon Instruments, Foster City, CA) A/D converter, and captured on the hard disk of an IBM-compatible personal computer. Analysis of the unitary currents was performed with pClamp 6.0.3. software (Axon Instruments). Open-state probability (NPO) was calculated from the ratio between channel open time and total recording time. The mean amplitude of the unitary currents was obtained for individual patches by fitting simple Gaussian distributions to the all-points histogram.

Intermittent recordings of BK\(_{Ca}\) were made up to 30 min after the application of cerivastatin in concentrations of 0.03 and 0.01 \(\mu\)mol/L and of cerivastatin (0.03 \(\mu\)mol/L) plus mevastatine (500 \(\mu\)mol/L). After the application of 100 nmol/L iberiotoxin (IBX) to the pipette solution, records were obtained after 1 min of exposure to IBX.

### \(^{3}\)H/cGMP RIA

A \(^{3}\)H/cGMP-RIA (Amersham, Freiburg, Germany) was used to analyze NO production. HUVEC were seeded at a density of 5000 cells/cm\(^2\). After 48 h, cells were stimulated for 30 min by replacing the culture medium with bath solution supplemented with the following substances: 1 mmol/L arginin (Sigma, Deisenhofen, Germany), 1 \(\mu\)mol/L ACh (Sigma), 0.03 and 0.01 \(\mu\)mol/L cerivastatin, and 100 nmol/L IBX. Stimulation was stopped by adding 98% ethanol ( Riedel-de Haen, Seelze, Germany). The cell lysate was centrifuged, and measurements of cGMP levels of the supernatant were performed using a cGMP-RIA.

### 4,5-Diaminofluorescein—Fluorescence Imaging

In addition, production of NO was examined by measurements of 4,5-diaminofluorescein (DAF)-fluorescence. HUVEC were loaded with 0.5 \(\mu\)mol/L DAF (Molecular Probes). After an incubation period of 15 to 30 min, extracellular DAF was removed and the medium was exchanged with bath solution. Cells were stimulated with cerivastatin (0.01 or 0.03 \(\mu\)mol/L) and/or IBX (100 nmol/L). An excitation wavelength of 488 nm was used. Emitted light was detected at 512 nm, and background was subtracted. Values represent DAF-fluorescence intensity.

### Cell Proliferation

Proliferation of HUVEC was measured by means of cell counts with the help of a Neubauer chamber and by using a \(^{3}\)H/thymidine incorporation assay (Amersham). For the cell counts, HUVEC of confluent primary cultures were seeded at a density of 5000 cells/well. On the first day (day 0), the cells were incubated in the above-mentioned basal medium. After 24 h (day 1), the medium was changed to serum-free EBM with supplemental hydrocortisone (1 \(\mu\)g/ml) and gentamicin (50 \(\mu\)g/ml). The next day (day 2), the medium was changed to normal EBM containing only 2% FCS and cerivastatin (0.03 and 0.01 \(\mu\)mol/L), and/or IBX (100 nmol/L). A serum-starved group was included as negative control. Cell counts were carried out on day 3 using a Neubauer chamber. For each sample, the mean value of two counts was used for statistical analysis.

\(^{3}\)H/thymidine incorporation assay was performed using 5000 cells/well. Cells were serum-starved for 24 h. Thereafter, cells were stimulated with the same supplements used in the cell count procedure before adding 0.2 \(\mu\)g/ml \(^{3}\)H/thymidine (0.25 MBq) to the medium. After 20 h, \(^{3}\)H/thymidine was added to each well. Four hours later, cells were washed with ice-cold PBS three times, fixed with 100% cold methanol for 15 min at 4°C, precipitated with 10% cold TCA for 15 min at 4°C, washed with water three times, and lysed with 200 \(\mu\)L of 1 N NaOH for 30 min at room temperature. The lysate was then transferred to scinti-vials, and the content of \(^{3}\)H/thymidine was measured using a \(\beta\) counter (Canberra-Packard, Dreieich, Germany). For each sample, the mean value of three counts was used for statistical analysis, and results were expressed as counts per minute.

### Solutions and Chemicals for Electrophysiologic Studies

For our electrophysiologic experiments, HUVEC were maintained in an extracellular (bath) solution containing (in mmol/L) 140 NaCl, 5 KCl, 0.5 MgCl\(_2\), 5.5 D-glucose, 10 HEPES, and 1.5 CaCl\(_2\) (pH 7.3; titrated with NaOH). For cell perfusion, the following substances were added to the bath solution: 0.03 and 0.01 \(\mu\)mol/L cerivastatin (Bayer,
The standard pipette solution contained (in mmol/L) 110 K+-aspartate, 30 KCl, 5 HEPES, and 1 MgCl₂ (pH 7.3; titrated with KOH). IBX (100 nmol/L; Sigma) was supplemented to the pipette solution to test its ability to block BKCa under cerivastatin perfusion. All experiments were conducted at room temperature (20 to 22°C).

**Statistical Analyses**

Statistical significance of the repeated measurements of NPo after cerivastatin application was determined using a Friedman test (P < 0.05; SPSS for Windows, release 10.0) followed by multiple comparisons (Nemenyi test). Data obtained from proliferation studies, cGMP-RIA, DiBAC₄, and DAF measurements were analyzed by means of ANOVA (P < 0.05, SPSS for Windows, release 10.0) followed by post hoc Tukey test. Results are expressed as mean values ± SEM.

**Results**

**Effects of Cerivastatin on Membrane Potential**

Endothelial cell MP was measured using DiBAC₄-fluorescence imaging. Cerivastatin was added at the following concentrations: 0.001, 0.003, 0.005, 0.01, 0.03, and 0.05 μmol/L. The application of cerivastatin at a concentration of 0.03 μmol/L resulted in the strongest hyperpolarization of the cell membrane (n = 30; P < 0.05). Because the membrane potential of endothelial cells is strongly influenced by BKCa, the BKCa inhibitor IBX (100 nmol/L) was able to block cerivastatin-induced hyperpolarization (Figure 1).

**Identification of BKCa**

BKCa has been identified extensively in our laboratory previously (19). It has been shown to be voltage dependent at test potentials from 20 mV to 120 mV with a single-channel slope conductance of 170.3 ± 2.1 pS (n = 7) well within the range of BKCa's characteristic conductance of 150 to 250 pS (24). The ion channel's typical Ca²⁺ dependence was demonstrated with half-maximal channel activation at pCa 5.7 (n = 4). Furthermore, the highly specific BKCa blocker IBX (100 nmol/L) was seen to cause a complete block of unitary outward currents observed in cultured human endothelial cells, establishing that these unitary outward currents are indeed carried through Ca²⁺-activated K⁺ channels. This electrophysiologic study to identify Ca²⁺-activated K⁺ channels in HUVEC was consistent with the description of BKCa in other endothelial cells (24).

**Effect of Cerivastatin on BKCa**

To test whether cerivastatin can modulate BKCa, recordings in cell-attached patches were performed. After a control measurement in cerivastatin-free bath solution, repetitive recordings of BKCa were elicited during a 30-min continuous perfusion with cerivastatin in concentrations of 0.01 and 0.03 μmol/L. This was followed by a 10-min washout period. Because BKCa activity was very low at low depolarizing test potentials, we studied only channel behavior at pipette potentials of 80 mV and 100 mV. A representative recording of BKCa activity during cerivastatin perfusion is shown in Figure 2A. Perfusion with both cerivastatin concentrations resulted in a similar pattern of BKCa activation with a significant increase of single-channel NPo after 15 min at a test potential of 100 mV and after 20 min at 80 mV (shown for the concentration of 0.03 μmol/L in Figure 2B). The significant increase in BKCa open probability respectively persisted throughout the 30-min recording time and was not reduced during washout (Figure 2A). Consistent with the DiBAC₄ measurements, the increase of BKCa open probability was greatest at the cerivastatin concentration of 0.03 μmol/L (NPo 0.225 ± 0.095 at 100 mV after 15 min; n = 19), followed by the concentration of 0.01 μmol/L (NPo 0.2376 ± 0.011; n = 18).

To demonstrate that BKCa activation is not caused by a direct or nonspecific effect of cerivastatin, we used mevalonate in a concentration of 500 μmol/L. The product of HMG-CoA reductase was added to the cerivastatin perfusion of 0.03 μmol/L, which on its own had caused maximal BKCa activation. Subsequently, mevalonate was seen to inhibit completely...

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**Figure 1.** Cerivastatin-induced hyperpolarization in HUVEC. (A) Dose-response relationship of cerivastatin (C; 0.001 to 0.05 μmol/L) on membrane potential. Results are expressed as means of DiBAC₄-fluorescence intensity. (B) Iberiotoxin (IBX; 100 nmol/L) significantly inhibits cerivastatin (0.03 μmol/L)-induced membrane hyperpolarization (n = 30; *P < 0.05 versus control; #P < 0.05 versus C 0.03 μmol/L).
the increase of single-channel open probability caused by cerivastatin (Figure 3).

To test whether cerivastatin (0.03 μmol/L) directly activates BKCa, we performed measurements using the inside-out configuration. In inside-out patches, BKCa NPo was significantly increased from 0.002 ± 0.002 (control) to 0.152 ± 0.092 (cerivastatin 0.003 μmol/L) at a pipette potential of +100 mV (n = 19; *p < 0.05 versus control).

To verify that the highly selective BKCa blocker IBX is sufficient to block cerivastatin-induced BKCa activation, we added 100 nmol/L IBX to the pipette solution and carried out cell-attached measurements of BKCa. Within 2 min after gigaseal formation, we observed a total block of initial BKCa openings, demonstrating the existence of BKCa in the patch. The following application of cerivastatin did not alter the blockade of BKCa by IBX (n = 10, data not shown).

The effect of cerivastatin on NO synthesis was measured by means of [3H]cGMP RIA (25). In the control group, HUVEC were incubated with arginin (1 mmol/L). As expected, the addition of ACh (1 μmol/L) significantly increased endothelial cGMP level as compared with the control group. It is interesting that the combination of ACh and cerivastatin resulted in an additional dose-dependent significant increase of the cGMP level. Remarkably, this increase was highest at the cerivastatin concentration of 0.03 μmol/L (84%; n = 10, P < 0.01), which also caused maximal increase of BKCa open state probability. When BKCa was blocked using IBX (100 nmol/L), ACh-induced increase of cGMP level was significantly reduced (n = 10, P < 0.01). Likewise, the increase of cGMP level caused by the combined administration of ACh and cerivastatin was significantly diminished by addition of IBX (n = 10, P < 0.01), demonstrating a significant involvement of BKCa. The results are summarized in Figure 4A.

To investigate whether cerivastatin has a direct effect on endothelial NO formation, we carried out DAF-fluorescence measurements without the addition of ACh. The significant increase of NO production induced by cerivastatin was completely blocked by IBX (100 nmol/L). These data are shown in Figure 4B.

Effect of Cerivastatin on Cell Proliferation

Addition of cerivastatin to the basal culture medium resulted in a significant dose-dependent decrease of the endothelial cell
number, with a maximal reduction at a cerivastatin concentration of 0.03 μmol/L (−32.2%, n = 12; P < 0.05). For assessing a possible contribution of BKCa to cerivastatin-mediated endothelial proliferation, the highly selective BKCa blocker IBX (100 nmol/L) was supplemented to the culture medium. It is interesting that the addition of IBX significantly but not completely reduced cerivastatin-mediated inhibition of cell proliferation. For excluding a direct cytotoxic or proliferation-enhancing effect of IBX on HUVEC, 100 nmol/L IBX was added to the basal culture medium. Compared with the control group (basal medium, without IBX), no changes in cell number were observed.

For further confirmation of our results obtained in the cell counts, endothelial proliferation on DNA level was analyzed by measurement of [3H]thymidine incorporation. In correspondence with the cell counts, incubation of HUVEC with cerivastatin resulted in a significant dose-dependent reduction of endothelial proliferation with a maximal inhibition at 0.03 μmol/L cerivastatin (−70%, n = 12; P < 0.01). Addition of 100 nmol/L IBX again partially but significantly reversed this effect on proliferation. Accordingly, in comparison with the control group, no significant changes in [3H]thymidine incorporation were observed when HUVEC were incubated with IBX alone.

Our data demonstrate that cerivastatin-induced reduction of endothelial proliferation is in part mediated by the activation of BKCa. The results of the cell counts and [3H]thymidine incorporation are shown in Figure 5.

Figure 4. Effect of cerivastatin on endothelial NO formation. (A) Acetylcholine (Ach, 1 μmol/L)-induced cGMP levels are significantly increased compared with the control group (⁎P < 0.01). Addition of cerivastatin (0.01 and 0.03 μmol/L) further increases Ach-induced cGMP levels significantly (⁎⁎P < 0.01). Application of iberiotoxin (IBX; 100 nmol/L) significantly reduces Ach- and cerivastatin-induced cGMP levels (⁎P < 0.01). cGMP levels shown in pmol/well as means ± SEM (n = 10). (B) Direct effect of cerivastatin on endothelial NO synthesis. Addition of cerivastatin (0.01 and 0.03 μmol/L) increases NO levels measured by DAF-fluorescence imaging. The effect of cerivastatin is completely abolished in cells treated with iberiotoxin (IBX; 100 nmol/L). Data are expressed as means of DAF-fluorescence intensity (n = 30; ⁎P < 0.05 versus Control; ⁎⁎P < 0.05 versus cerivastatin).

Figure 5. Effects of cerivastatin on HUVEC proliferation. Cell growth was analyzed by means of cell counts (A) and [3H]thymidine-incorporation assay (B). Number of cells are expressed per well ± SEM (n = 12; ⁎P < 0.05 versus control; ⁎⁎P < 0.05 versus cerivastatin) (A) and as percentages of the proliferation for [3H]thymidine-incorporation assay (n = 12; ⁎P < 0.01 versus control; ⁎⁎P < 0.01 versus cerivastatin) (B).
Discussion

Apart from their lipid-lowering properties, statins are known to exert direct pleiotropic effects on vascular endothelium, thereby counteracting the process of arteriosclerosis. These effects include an increase in NO bioavailability as well as a reduction of cell proliferation.

In the field of electrophysiology, it is a well-established fact that activation of Ca\(^{2+}\)-activated K\(^+\) channels can influence essential endothelial functions by the modulation of intracellular calcium homeostasis. Therefore, the aim of our study was to investigate whether the statin cerivastatin modulates endothelial Ca\(^{2+}\)-activated K\(^+\) channels and thereby influences endothelial NO formation and cell proliferation.

Measurements of the MP revealed a dose-dependent effect of cerivastatin with the strongest hyperpolarization at a concentration of 0.03 \(\mu\)mol/L. This effect was abolished by pre-incubation with the BK\(_{Ca}\) inhibitor IBX.

In our patch-clamp experiments, we could demonstrate for the first time that cerivastatin significantly increases BK\(_{Ca}\) NPo. This effect was dose-dependent with a maximum of activation at 0.03 \(\mu\)mol/L cerivastatin. Therapeutic cerivastatin serum levels were measured to range between 0.002 and 0.05 \(\mu\)mol/L (26). Thus, the concentrations of 0.03 \(\mu\)mol/L and 0.01 \(\mu\)mol/L lie within this therapeutic margin. The results of the inside-out recordings have shown that cerivastatin directly activates the endothelial BK\(_{Ca}\). The addition of mevalonate to the cerivastatin concentration of 0.03 \(\mu\)mol/L, which on its own activated BK\(_{Ca}\) maximally, completely inhibited the increase of BK\(_{Ca}\) NPo. This serves to show that BK\(_{Ca}\) activation must be a class effect of statins, because their common mechanism lies in the inhibition of HMG-CoA reductase and thus the formation of mevalonate. The use of isolated endothelial cells excludes an influence of altered plasma lipid levels on our findings. Principal mechanisms shown to activate BK\(_{Ca}\) in endothelial cells include changes in voltage and intracellular calcium (27,28). In smooth muscle cells, the modulation of a channel-linked receptor by G proteins leading to subsequent BK\(_{Ca}\) activation has been described (29,30). Therefore, we can speculate that a modification of G proteins resulting from an inhibition of the mevalonate pathway (31) could be essential for BK\(_{Ca}\) activation in HUVEC, assuming that these channel-linked receptors also exist in endothelial cells. However, the exact mechanism of channel activation remains as yet unclear.

The focus of our study was on finding a possible contribution of BK\(_{Ca}\) activation to statin-mediated changes of endothelial NO production and cell proliferation. An increase of NO bioavailability is one of the earliest effects observed with statin treatment (12,32). This effect is achieved both by an augmented expression of eNOS and by decreased NO inactivation.

However, impaired vasodilation to the known eNOS agonist ACh is an early indicator of endothelial dysfunction (1,2). In endothelial dysfunction, NO bioavailability is diminished partly as a result of increased eNOS translocation to the caveolae of the cell, where eNOS forms an inactivating complex with caveolin (33). Statins have been shown to inhibit caveolin and thus to increase NO synthesis (34) and to improve ACh-induced blood flow in vivo (35). Therefore, we examined cerivastatin’s ability to increase ACh-induced NO formation in HUVEC and investigated a possible role of BK\(_{Ca}\) in this process.

BK\(_{Ca}\) has been described to play a part in the regulation of NO synthesis by controlling membrane potential and increasing intracellular calcium (36–38). In endothelial cells, BK\(_{Ca}\) activation leads to hyperpolarization of the cell membrane as a result of an outward potassium current. This hyperpolarization in turn builds an electrochemical gradient for prolonged transmembranous calcium influx (14,36). Relevantly, it could be shown that the presence of intracellular calcium is essential for NO release after agonist stimulation (18).

We confirmed that BK\(_{Ca}\) contributes to ACh-induced NO formation in HUVEC, as the increase of cGMP under ACh stimulation was significantly reduced by addition of the selective BK\(_{Ca}\) blocker IBX. We previously showed ACh to activate BK\(_{Ca}\) in HUVEC directly, an effect that was again completely blocked by the addition of IBX.

Cerivastatin was seen to augment significantly ACh-induced NO synthesis as measured by cGMP RIA at all concentrations. This seems especially relevant considering the early impairment of ACh-mediated NO synthesis in arteriosclerosis. Remarkably, the highest increase in cGMP formation could be observed upon stimulation with 0.03 \(\mu\)mol/L cerivastatin, which also caused maximal BK\(_{Ca}\) activation. Channel blockade by IBX partially but significantly reduced cerivastatin-mediated cGMP synthesis at all concentrations, indicating a significant role of BK\(_{Ca}\) in the signaling of cerivastatin-mediated NO realize.

It can be assumed that simultaneous activation of BK\(_{Ca}\) by cerivastatin and ACh is greater than the activation observed for each single substance. This is likely to cause increased consecutive calcium influx, which could explain the part of increased NO synthesis that is due to BK\(_{Ca}\) activation, i.e., the part that was reduced by addition of IBX. Independent of channel activation, another mechanism involved in increasing NO formation could be a greater dissociation of eNOS from its inhibitory complex with caveolin in the caveolae of the cell membrane. ACh-induced vasodilation has been seen to be accompanied by a 60% reduction of membrane-bound eNOS but an unchanged concentration of caveolin (39), whereas statins have been described to prevent caveolin expression (34). It is interesting that cerivastatin increased NO formation also without substituting ACh. As demonstrated in the DAF measurements, the cerivastatin-specific effect on NO synthesis was completely blocked in IBX-treated cells. This finding points to the importance of BK\(_{Ca}\) in cerivastatin-induced increase of NO bioavailability.

The process of arteriosclerosis is also counteracted by a reduction of cell proliferation. Several studies have revealed statins to reduce endothelial as well as smooth muscle cell growth (40,41). Relevantly, a decreased endothelial proliferation has been shown to reduce neovascularization and thus inhibit the growth of atherosclerotic plaques as well as tumors (10,42,43). Furthermore, studies with transfilter co-cultures have revealed that statin-mediated reduced endothelial cell
proliferation also diminishes smooth muscle cell growth (41), which, in turn, is a decisive factor in plaque formation (3).

Recently, Weis et al. (43) described a biphasic effect of statins on the proliferation of human dermal microvascular endothelial cells with an increase of proliferation at cerivastatin concentrations between 0.005 and 0.01 μmol/L and a reduction only at concentrations between 0.05 and 1 μmol/L. Our results differ from these findings in that we already observed a reduction of cell proliferation at a cerivastatin concentration of 0.01 μmol/L. We found this decrease of proliferation to be more pronounced at 0.03 μmol/L. These discrepancies might be explained by the different cell types, different culture media, and/or different experimental techniques used. Because our results matched on a quantitative level and on a DNA level, it seems certain that in HUVEC, cerivastatin causes a reduction of cell proliferation at all the concentrations that we used.

It is interesting that addition of the highly specific BKCa blocker IBX partially but significantly inhibited the reduction of cell proliferation, as observed both in the cell counts and in the [3H]thymidine incorporation. Because IBX on its own was seen to have no effect on cell proliferation, a possible cytotoxic or proliferation-enhancing effect of the substance can be ruled out. Thus, it can be concluded that cerivastatin-induced reduction of endothelial cell proliferation must be partially mediated by BKCa activation.

In contrast to this, so far BKCa activation and raised intracellular calcium have been implicated in increased rather than in decreased endothelial cell proliferation (19,44). A possible explanation for this new finding is that cerivastatin apparently increases NO formation in part through an activation of BKCa. Relevantly, NO has been described to have a significant anti-proliferative effect on endothelial cells and on HUVEC in particular (45). We saw that the application of IBX led to a decrease of cerivastatin-induced NO synthesis at the same time as inhibiting cerivastatin-mediated reduction of cell proliferation. Thus, cerivastatin activating BKCa thereby increasing NO synthesis, could well be the relevant mechanism for the cerivastatin-induced reduction of cell proliferation that was also seen to be mediated by BKCa.

In summary, our study demonstrates for the first time that cerivastatin activates calcium-activated potassium channels of large conductance in human endothelial cells, which is highly likely to be a class effect of statins because the effect was fully reversed by the addition of mevalonate. The activation of BKCa significantly contributes to both the statin-induced increase of NO formation and the statin-mediated reduction of cell proliferation. Thus, this BKCa modulation by a statin can be regarded as a new pleiotropic effect on the endothelium associated with the vasculoprotective properties of statins.

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