Increased Nitric Oxide Bioavailability in Endothelial Cells Contributes to the Pleiotropic Effect of Cerivastatin

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Background—Although statins preserve endothelial function by reducing serum cholesterol levels, it has been suggested they may also stimulate nitric oxide (NO) synthase in endothelium with concurrent increase in superoxide (O$_2^-$) generation, leading to impairment of NO activity. Therefore, measurements of biologically active NO and O$_2^-$ in endothelium after exposure to the HMG-CoA reductase inhibitor cerivastatin were undertaken to evaluate its potential effect on NO biological activity.

Methods and Results—Highly sensitive electrochemical NO and O$_2^-$ microsensors were placed near the surface of a single human umbilical vein endothelial cell, and the kinetics of NO and O$_2^-$ release were recorded in vitro. Cerivastatin demonstrated a time-dependent effect on NO release in endothelial cells. The initial release (approximately the first 3 minutes) was concentration-dependent (0.01 to 10 μmol/L) and was similar to that observed for typical NO synthase agonists calcium ionophore or acetylcholine. Cerivastatin stimulated NO release at a favorable rate and scavenged O$_2^-$, which led to the preservation of the active concentration of NO. The sustained effect (after ≈6 hours) of cerivastatin on endothelium was associated with an ≈35% increase in NO release as compared with the initial effect. In contrast to the initial effect, the sustained effect of cerivastatin was shown at concentrations ≈100-fold lower and was dependent on inhibition of endothelial HMG-CoA reductase.

Conclusions—These data provide direct evidence to prove that in the presence of cerivastatin, the NOS system in endothelium operates with high efficiency toward increasing NO activity by activation of NO release and by concurrent inactivation of O$_2^-$.

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Key Words: nitric oxide ● nitric oxide synthase ● endothelium ● cholesterol ● atherosclerosis

Recent completed primary and secondary intervention trials have shown that the significant reductions in LDL cholesterol (LDL-chol) achieved with HMG-CoA reductase inhibitors (statins) resulted in significant reductions in morbidity and mortality rates associated with coronary artery disease as well as reductions in the incidence of stroke and total mortality rates. This benefit occurs early in the course of statin therapy and has led to suggestions that these drugs may have antiatherogenic effects in addition to their capacity to lower atherogenic lipids and lipoproteins. In fact, there is increasing experimental evidence that statins have additional beneficial effects by acting directly on endothelial cells. It has been shown that statins upregulate endothelial nitric oxide synthase (eNOS) expression and reverse the downregulation of eNOS expression by hypoxia and oxidized LDL under cholesterol-clamped conditions. On the other hand, it has been reported that statins may potentiate but that l-arginine inhibits superoxide radical generation in the atherosclerotic vascular wall.

Risk factors for coronary atherosclerosis, such as hypercholesterolemia, impair NO bioactivity, mainly as the result of an oxidative stress by O$_2^-$, which rapidly inactivates endothelium-derived NO. NO and O$_2^-$ react rapidly to produce cytotoxic peroxynitrite. The presence of peroxynitrite in advanced plaques has been documented by the use of nitrotyrosine antibodies. Experiments in the hypercholesterolemic rabbit aorta suggested that the overall production of NO is not reduced but is rather markedly augmented. However, because of inactivation of NO by O$_2^-$, the concentration of bioavailable NO is significantly diminished in the endothelium. Recently, it has been shown that asymmetrical N$^\text{G}$-dimethyl-l-arginine (ADMA), an endogenous inhibitor of NOS, is accumulated in the plasma of hypercholesterolemic rabbits. ADMA may compete with l-arginine for the enzyme, especially when the intracellular availability of the amino acid in the vicinity of eNOS is not sufficient.

For the previously described reasons, it is not always the increase of eNOS expression in endothelial cells, which can be translated into the increase of NO bioavailability. Direct measurements of diffusible NO (which is capable of activating vascular smooth muscle relaxation), together with con-
current measurements of $O_2^-$, are required to assess bioavailability of NO stimulated with a pharmaceutical compound. Recently, the design and application of microsensors for direct in vitro electrochemical measurements of NO and $O_2^-$ in a single cell have been published.\textsuperscript{12,13} These microsensors, designed for cell culture,\textsuperscript{14} allow the direct quantification of NO and $O_2^-$ with high sensitivity. The purpose of this study was to determine, through the use of NO and $O_2^-$ microsensors, whether inhibition of endothelial HMG-CoA reductase is associated with an increase of NO biological activity in endothelial cells. We used cerivastatin, the newest member of the statin class (a third-generation compound), a totally synthetic, pure enantiomeric that is the most potent inhibitor of HMG-CoA reductase.\textsuperscript{1,2}

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (cat #CRL-1730) and cultured as described.\textsuperscript{9} The confluent cells (4 to 5 x 10\textsuperscript{6} cells/35-mm dish) were placed with minimum essential medium containing 5% human lipoprotein-depleted serum. In some experiments, cells were pretreated with different concentrations of cerivastatin for 1 to 24 hours with or without mevalonic acid or LDL. At the end of the incubation period, 10 $\mu$L of solutions of the test substances were injected to reach a final concentration in the cell medium as follows: 1 $\mu$mol/L calcium ionophore (Cal) A23187, 1 $\mu$mol/L acetylcholine (Ach), or 1 to 10 $\mu$mol/L cerivastatin. In the experiments with NOS inhibitors, the cells were pretreated for 20 minutes with 100 $\mu$mol/L L-NAME (N$^\text{G}$-nitro-l-arginine methyl ester), a selective eNOS inhibitor, or 100 $\mu$mol/L 1400W (N-[3-(aminomethyl)benzyl]acetamidine), a selective iNOS inhibitor.

**NO and Superoxide Measurement**

NO and superoxide microsensors were measured by previously described electrochemical methods.\textsuperscript{12,13} The microsensors of NO and $O_2^-$ (working electrodes) were positioned on the surface of the cell membrane by means of a computer-controlled micromanipulator, and the test substances were injected with a nanoinjector that was positioned at close proximity (5 $\pm$ 2 $\mu$m) from the cell membrane. Whether cerivastatin has scavenger properties in the xanthine–xanthine oxidase superoxide anion-generating system has been assessed according to the procedures published previously.\textsuperscript{14} In this experimental model, cerivastatin did not change uric acid production by xanthine oxidase, thereby indicating that the effect of the compound on the production of $O_2^-$ was not due to the inhibition of the enzyme activity. To assess the total NO production by endothelial cells, measurements of nitrates and nitrates (final products of NO decay) were performed with the Griess method.\textsuperscript{15}

**Data Analysis**

Values are expressed as mean$\pm$SEM, with a value of $P<0.05$ considered statistically significant. Statistical evaluation was performed by ANOVA, followed by the Student’s unpaired t test.

**Results**

Figure 1A shows a typical recording of NO concentration versus time (high-resolution amperogram) obtained from a single HUVEC after injection of cerivastatin. An initial effect of cerivastatin on endothelium (approximately the first 3 minutes) was manifested by the release of NO in the form of sinusoidal pulses; four well-defined peaks were observed in the time span of $\approx$40 seconds after injection of cerivastatin (1 $\mu$mol/L). The amplitude of the first peak was $\approx$70 nmol/L after stimulation of NO release with 1 $\mu$mol/L cerivastatin. The amplitude of subsequent peaks steadily increased, reaching maximum at the fourth peak of 120 nmol/L, $\approx$30 seconds after injection of the compound. The rate of the increase of NO concentration was in the range of 3 to 7 nmol/L per second for each of the four peaks. A steady NO concentration of $\approx$30 nmol/L was observed for at least 160 seconds after the appearance of the fourth peak. Because the activation of NO release by any eNOS agonists species in endothelial cells is always associated with an increase of $O_2^-$ release, the effect of cerivastatin on the concentration and the rate of $O_2^-$ release during production of NO was also evaluated. Amperograms of $O_2^-$ release were recorded simultaneously with amperograms of NO release after injection of the eNOS agonists. During the time course of cerivastatin-stimulated NO production, only a trace of $O_2^-$ was recorded. The first peak of $O_2^-$ concentration was observed $\approx$2 seconds later than the NO peak. The rate of $O_2^-$ release was $<1$ nmol/L per second.

The kinetics of release of NO followed by a release of $O_2^-$ after stimulation with cerivastatin is distinctively different from that observed for well-defined eNOS agonists Cal and Ach (Figure 1, B and C). Cal is a receptor-independent eNOS agonist that rapidly stimulated NO release within a few seconds. The single peak concentration of 445 $\pm$ 20 nmol/L was reached 1.5 seconds after injection of Cal (1 $\mu$mol/L). The peak concentration stimulated by Cal represents the maximum NO concentration that can be reached from a single HUVEC. The rate of concentration increase was 290 nmol/L per second. After reaching the sharp peak, a rapid decrease of NO concentration at a rate of 135 nmol/L per second was observed. After Cal injection, the $O_2^-$ peak concentration of 40 $\pm$ 4 nmol/L was elicited 1 second later than the peak of NO concentration. The rate of $O_2^-$ concentration increase was 18.2 nmol/L per second. Ach, a receptor-dependent NO agonist, produced an $\approx$40% ($262 \pm 14$ nmol/L)
lower peak of NO concentration than did Cal but in a relatively short period of time. An increase of NO concentration was recorded 5 seconds after Ach (1 μmol/L) injection. The rate of NO release amounted to 38 nmol/L per second, which was ≈7.5 times slower than the rate of Cal-stimulated NO release but several times faster than that observed for cerivastatin. A semiplateau developed after 15 seconds; after ≈25 seconds, a slow decay of NO concentration at a rate of 22 nmol/L per second was elicited. Ach-stimulated O$_2^-$ release reached a maximum of 18±2 nmol/L after ≈23 seconds. The rate of O$_2^-$ release was 2.4 nmol/L per second, which was ≈7.5 slower than that elicited for Cal.

The amount of O$_2^-$ released concurrently with NO after injection of cerivastatin was the lowest amount ever recorded in our laboratory after injection of an NOS agonist. The ratio of NO to O$_2^-$, when calculated with the total amount of release of both radicals, was ≈4 and 3 times higher for cerivastatin than for Cal and Ach, respectively (Figure 2A). To more fully investigate the scavenging properties of cerivastatin, the xanthine–xanthine oxidase system was used. This system limits the number of variables that appear in experiments with the cells. The concentration of O$_2^-$ measured electrochemically depends on the kinetics of its generation, the kinetics of the scavenging process, and the kinetics of the electrode reaction. All these processes are concentration dependent. As can be clearly seen in Figure 2, cerivastatin scavenges O$_2^-$ generated either by cells or by the xanthine–xanthine oxidase system.

Figure 3 shows the concentration-response curve of the peak NO release for cerivastatin. There is a linear response for the compound in the range of concentration between 0.1 and 1.0 μmol/L. The total concentration of NO released by HUVECs after injection of the lowest tested concentration of cerivastatin (0.1 μmol/L) was 25±2 nmol/L; this accounts for the 18-nmol/L increase of NO concentration as compared with basal spontaneous NO release of 7.2±0.2 nmol/L. At concentrations of cerivastatin >1.0 μmol/L, a significant deviation from the linear relation between NO concentration and cerivastatin concentration was demonstrated. A semiplateau was reached at concentrations of cerivastatin >4 μmol/L.

Apart from investigating the initial effect (approximately the first 3 minutes) of cerivastatin on NO release, a long-term effect (sustained effect) of cerivastatin on the l-arginine pathway was also examined. As shown in Figure 4, the incubation of HUVECs with cerivastatin resulted in the increase of basal NO concentration as well as eNOS agonist–stimulated NO release. Approximately 6 hours of incubation time was required to elicit a significant increase of NO release. After ≈12 hours of cell incubation with cerivastatin, the NO release (either basal or after stimulation with Cal) reached the maximal response (9.9±0.2 versus 7.2±0.2 nmol/L for spontaneous release and 600±25 versus 445±20 nmol/L for Cal, with or without cerivastatin, respectively, P<0.01 for both). The sustained effect of cerivastatin on NO release from the cells was dose dependent (Figure 5A). The 24-hour incubation concentration of cerivastatin that resulted in the highest Cal-stimulated NO release was 0.1 nmol/L. An enhanced cerivastatin-stimulated release of NO was observed after 24-hour incubation with cerivastatin (Figure 5B). The pattern of changes of the maximum NO concentrations with increased cerivastatin concentration was similar to that ob-
served for stimulation of NO release with CaI and Ach (not shown for Ach data).

To measure total NO concentration with incubation time within the period of 24 hours, the measurement of nitrates and nitrites (NO$_2^-$ and NO$_3^-$) was performed. Figure 6 depicts a relation between total concentrations of NO$_2^-$ and NO$_3^-$ produced by 1.2 $\times$ 10$^6$ endothelial cells (suspended in 2 mL of cell culture medium) incubated with different concentrations of cerivastatin (from 0.1 to 10 nmol/L) over a period of 24 hours. A substantial increase in the concentration of NO$_2^-$ and NO$_3^-$ after incubation with cerivastatin was observed. The incubation of the cells with 10 nmol/L of cerivastatin doubled the concentration of nitrogen species produced during the 24-hour period.

The mechanism of the stimulatory effect of cerivastatin on NO release in the endothelial cells was examined. The presence of either L-mevalonate (10 nmol/L) or LDL (1 mg/mL) in the cell incubation medium did not significantly change the initial effect of cerivastatin on NO release (data not shown). However, cotreatment with L-mevalonate (10 nmol/L) completely blocked the sustained effect of cerivastatin after a 24-hour incubation period; there was no increase in NO release after stimulation with either CaI (Figure 7A) or cerivastatin (Figure 7B) injection. Moreover, cotreatment with L-mevalonate (10 nmol/L) significantly blocked the increase in basal NO concentration after a 24-hour incubation of the cells with 0.1 nmol/L cerivastatin. By contrast, cotreatment with LDL (1 mg/mL) did not appreciably reverse the sustained effect of cerivastatin on NO release after stimulation with either CaI or cerivastatin injection. Treatment with L-mevalonate or LDL alone did not have any appreciable effect on either CaI- or cerivastatin-stimulated NO release. The release of NO after stimulation with either CaI or cerivastatin was inhibited by 100 $\mu$mol/L 1NAME (both by $\approx$70%), whereas it was not affected by 100 $\mu$mol/L of 1400W (a selective iNOS inhibitor).

**Discussion**

This study demonstrates for the first time direct simultaneous measurements of NO and O$_2^-$ obtained after cerivastatin.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Spontaneous (triangles) and CaI-stimulated (circles) NO (A) and superoxide (B) release after different incubation times with 0.1 nmol/L cerivastatin. Points represent peak NO and superoxide release after stimulation with Cal. n=6 at each incubation time point; *P<0.05 and **P<0.01 vs control (without cerivastatin, open symbols).

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Peak NO release stimulated by 1 $\mu$mol/L Cal (A) and 1 $\mu$mol/L cerivastatin (B) from HUVECs after 24-hour incubation with different concentrations of cerivastatin. n=6 at each bar; *P<0.01 vs control (without cerivastatin).

![Figure 6](http://circ.ahajournals.org/)

**Figure 6.** Sum of nitrite and nitrate concentrations in cell culture medium after 24-hour incubation of HUVECs with different concentrations of cerivastatin. n=6 at each bar; *P<0.05 and **P<0.01 vs control.
administration in endothelial cells. The use of isolated endothelial cells maintained in culture excluded the influence of cerivastatin actions in reduction of the hepatic production of LDL-chol. Furthermore, the presence of the endothelial cell response to cerivastatin in LDL-chol–depleted culture medium precluded that the response may be mediated through changes in direct actions of LDL onto endothelial cells. NO release was inhibited by l-NAME but not by 1400W, demonstrating specificity of the effect of eNOS. The limitation of all studies reported previously on statins ameliorating endothelial dysfunction is that the estimation of NO and/or \( \text{O}_2^- \) releases were suggested based on the comparison of vascular smooth muscle relaxation or on bioassays relying on secondary effects and not based on direct measurement of NO and \( \text{O}_2^- \). The measurement of NO and \( \text{O}_2^- \) concentrations in biological systems is a challenging analytical problem. The detection in real time of NO and \( \text{O}_2^- \) in the location with the highest concentration, the surface of the endothelial cell membrane, is the most efficient and accurate method of measuring endogenous levels of both radicals. This is because a major source of either NO or \( \text{O}_2^- \) in the vessel wall is the endothelium itself. Moreover, the near diffusion-limited reaction of \( \text{O}_2^- \) with NO to form peroxynitrite is one of the fastest chemical reactions known to occur in the liquid phase (\( k = 9.6 \times 10^9 \text{ L/mol per second} \)), with the rate of \( \approx 5 \) times faster than the dismutation of \( \text{O}_2^- \) with superoxide dismutase. 6,8,9

Cerivastatin elicited a double effect on NO release in endothelial cells: first, the initial effect within the first few minutes, and second, the sustained effect after several hours since the onset of the exposure to the compound. The initial effect of cerivastatin on NO release was similar to that seen with Ach, a known receptor-dependent eNOS agonist, and different from that observed for Cal, a receptor-independent eNOS agonist. The rapid time course of the endothelial cell response to cerivastatin made it unlikely that the initial effect could be a result of cerivastatin effects on cholesterol metabolism in the cultured cells. Endothelial cell eNOS activity is known to decrease with elevation of membrane cholesterol, but it is very unlikely that significant alterations in endothelial cell plasma membrane cholesterol could occur within several dozen seconds of cerivastatin addition to the culture. This concept can be confirmed by the experiments that both l-mevalonate (reaction product of HMG-CoA reductase) and LDL-chol failed to inhibit the initial effect of cerivastatin on NO release. It can be speculated that cerivastatin in the initial phase may act on a specific cell membrane receptor to allow entry of extracellular calcium activating eNOS, or it might act on membrane components rather nonspecifically to activate eNOS or induce release of another eNOS agonist. Whatever the process, the data presented here demonstrate that cerivastatin causes an immediate release of NO from the endothelial cells.

Considering the effect of potent eNOS agonists, it is worthwhile to note that activation of NO release by any eNOS agonist in endothelial cells is always associated with increased release of \( \text{O}_2^- \). 14 For instance, the rapid kinetics of NO release by Cal is followed by extensive release of \( \text{O}_2^- \). The process of \( \text{O}_2^- \) production can be triggered by insufficient concentration of L-arginine in the proximity of the enzyme. The significance of some other Ca\( ^{2+} \)-dependent sources of \( \text{O}_2^- \) is limited because the enhanced NO concentration may actually inhibit other enzymatic sources of \( \text{O}_2^- \), such as NADPH oxidase. 16

The present in vitro studies show some unusual and highly favorable kinetics of NO release by cerivastatin; this is due to the modest stimulation of NO release by the compound with concomitant scavenging of \( \text{O}_2^- \). In the experiments with \( \text{O}_2^- \) generated by the xanthine–xanthine oxidase system, the radicals were efficiently scavenged by cerivastatin at concentrations equal to the concentrations required for stimulation of NO release. Hence, the action of NO is prolonged in the presence of cerivastatin, and the eNOS system operates with high efficiency with probably negligible formation of toxic peroxynitrite. This dual, concurrent action of cerivastatin may contribute to the protection of the cardiovascular system against injuries induced by atherosclerosis. In this regard, peroxynitrite has been shown to be a potent, active oxidant that can attack many types of biological molecules. 6,8,9

The sustained effect of cerivastatin on endothelial cells was revealed through an increase of NO release either spontaneously or after stimulation with an exogenous eNOS agonist, including cerivastatin itself. In the present study, the sustained effect of cerivastatin on NO spontaneous release in the endothelial cell was confirmed by increasing the accumulation of NO decay products (\( \text{NO}_2^- \) and \( \text{NO}_3^- \)).
revealed the sustained effect on NO production at nanomolar concentrations that were approximately 2 orders of magnitude lower than the concentrations required for the initial effect of cerivastatin. The diminished NO release in response to Ca2+ was observed in the cells incubated with the high concentration (100 nmol/L) of cerivastatin for 24 hours. This effect was not elicited after 12-hour incubation of the cells with 100 nmol/L cerivastatin (data not shown), suggesting negative feedback regulation of eNOS by NO and accumulated nitroso compounds. In this context, excess NO production may be considered undesirable.

The range of concentrations achieved for the sustained effect correlate with complete inhibition of cholesterol biosynthesis by cerivastatin in human arterial cells (IC50=4 nmol/L in human arterial myocytes). Indeed, the sustained effect of cerivastatin occurs most probably through the inhibition of endothelial HMG-CoA reductase because the effects of cerivastatin on NO release from endothelial cells were reversed in the presence of l-mevalonate. Interestingly, l-mevalonate alone did not produce any change in NO release, indicating that basal intracellular l-mevalonate levels may be sufficient to limit NO release. The supply of cholesterol in the form of LDL, at a concentration sufficient to support cellular processes without decreasing uptake of l-arginine in endothelial cells, was unable to revert the cerivastatin-mediated sustained effect. It suggests that a deficit in nonsteroid isoprenoids of the mevalonate pathway may be involved in the changes observed in NO concentrations. Among others, mevalonate is the precursor of geranylgeranylated proteins, which is required for geranylgeranylation of Rho GTPase proteins in endothelial cells. In addition, it has been shown that Rho negatively regulates eNOS expression and that statins upregulate eNOS expression by blocking Rho geranylgeranylation, which is necessary for its membrane-associated activity.

The data presented in this study provide direct evidence of how treatment with cerivastatin, and on a broader scale to include other statins, may give clinically observed normalizing effect of endothelium-dependent relaxation in atherosclerotic arteries before significantly lowering serum cholesterol levels. The dual action of cerivastatin on the biological activity of NO (by enhancing its production due to initial and sustained phase of the compound action, and by reducing the rate of oxidative inactivation by O2•-) may explain the strong antithrombotic effects of cerivastatin. Cerivastatin may be seen as an antithromogenic agent that will affect overall coronary heart disease risk even when the LDL-cholesterol level is not the most prominent problem within the risk profile (eg, in asymptomatic patients with multiple risk factors). These two mechanisms of cerivastatin action may clearly extend the clinical applications of the drug beyond hypercholesterolemia and atherosclerosis but also to other pathological conditions (eg, hypertension, diabetes) associated with diminished NO production on the one hand and increased O2•- production in endothelium on the other. The importance of clinical impact of the dose-distinguishable initial and sustained effects of cerivastatin deserve further investigation, including safety analyses concerning the risk of side effects.

In conclusion, our findings demonstrate that cerivastatin is potent for increasing NO biological activity in endothelium cells through either activation of NO release or concurrent scavenging of O2•-. In contrast to the initial effect of cerivastatin, the sustained effect of cerivastatin on NO release is associated with inhibition of HMG-CoA reductase and is achieved at ≈100-fold lower concentrations (in the nanomolar range).

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**References**

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