HMG-CoA Reductase Inhibitor Increases GTP Cyclohydrolase I mRNA and Tetrahydrobiopterin in Vascular Endothelial Cells
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Objective—Endothelial nitric oxide synthase (eNOS) activity is supported by tetrahydrobiopterin (BH4), which appears to be important for generating protective NO but decreases uncoupling formation of superoxide. We investigated the effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, in terms of BH4 metabolism in human umbilical vein endothelial cells (HUVECs).

Methods and Results—We measured the mRNA levels of GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in the first step of de novo BH4 synthesis, by real-time polymerase chain reaction. The mRNA of GTPCH, as well as of eNOS, was upregulated in HUVECs treated with cerivastatin. This increase was time and dose dependent. Fluvastatin was also observed to enhance GTPCH and eNOS mRNA levels. In parallel with this observation, cerivastatin increased intracellular BH4. Incubating HUVECs with tumor necrosis factor (TNF-α) was observed to increase GTPCH mRNA while decreasing eNOS mRNA. In the presence of cerivastatin, the TNF-α–mediated increase in GTPCH mRNA was enhanced, and the TNF-α–mediated decrease in eNOS mRNA was attenuated. Cerivastatin increased the stability of eNOS mRNA. However, it did not alter the stability of GTPCH mRNA but increased GTPCH gene transcription, as shown by nuclear run-on assays. Preteatment of HUVECs with the selective GTPCH inhibitor, 2,4-diamino-6-hydroxypyrimidine, caused a decrease in intracellular BH4 and decreased citrulline formation after stimulation with ionomycin. Furthermore, the potentiating effect of cerivastatin was decreased by limiting the cellular availability of BH4.

Conclusions—Our data demonstrate that statins elevate GTPCH mRNA, thereby increasing BH4 levels in vascular endothelial cells. In addition to augmenting eNOS expression, statins potentiate GTPCH gene expression and BH4 synthesis, thereby increasing NO production and preventing relative shortages of BH4. (Arterioscler Thromb Vasc Biol. 2003;23:176-182.)

Key Words: statins • cytokines • nitric oxide • tetrahydrobiopterin • endothelial cells

The effectiveness and speed with which 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statin medications) can favorably influence vascular biology by mechanisms other than causing a reduction in plasma cholesterol have been demonstrated in a number of reports. During the past several years, numerous additional effects of statins on vascular cells have been identified, which appear to modulate atherogenesis, plaque rupture, or thrombosis. Some of these appear to operate independently of the cholesterol-lowering mechanism. For example, statins may upregulate nitric oxide (NO) expression by interfering with posttranscriptional regulation of endothelial NO synthase (eNOS). Evidence regarding the importance of this mechanism in vivo was provided by the observation that statins inhibit ischemic cerebral stroke induced by occlusion of the middle cerebral artery in normal but not e-NOS–deficient mice. Tetrahydrobiopterin (BH4) is 1 of the most potent, naturally occurring reducing agents and an essential cofactor for enzymatic NOS activity. A suboptimal concentration of BH4 reduces NO formation and favors uncoupling of NOS, leading to NOS-mediated reduction of oxygen and the formation of superoxide anions and hydrogen peroxide. Recent findings suggest that accelerated catabolism of BH4 in arteries exposed to oxidative stress may contribute to the pathogenesis of endothelial dysfunction in arteries of hypertensive individuals, as well as in those suffering from hypercholesterolemia, diabetes, smoking, or ischemia/reperfusion injury. Beneficial effects of short- and long-term BH4 supplementation on endothelial function have been reported in animals and humans.

The synthesis of BH4 occurs through 2 distinct pathways: a de novo synthetic pathway, in which GTP is a required...
precursor, and a salvage pathway, which uses available dihydropteroins. The GTP cyclohydrolase I (GTPCH), the rate-limiting enzyme in the first step of de novo BH4 synthesis, catalyzes the formation of dihydropteroin triphosphate from GTP. Subsequently, dihydropteroin triphosphate is converted to BH4 by the actions of 6-pyruvyltetrahydropterin synthase and sepiapterin reductase, with concomitant formation of tetrahydropterin intermediates. Immunostimulant-evoked BH4 synthesis occurs with a time course that is preceded by an increase in GTPCH mRNA. The intracellular concentration of BH4 appears to be rate limiting for NO synthesis. In the present study, we have examined the effect of HMG-CoA reductase inhibitors on GTPCH mRNA and intracellular biopterin in vascular endothelial cells.

Methods

Cell Culture and RNA Extraction
Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion from normal umbilical cords, as previously described. Cells were grown in human EC culture medium with EC growth supplement (Nissui Co), heparin, and 10% fetal bovine serum. Cells were used at passages 2 to 4.

Taqman/Real-Time PCR
For quantitative measurement of mRNA, 2 μg total RNA was treated with DNase I for 15 minutes and subsequently used for cDNA synthesis. Reverse transcription was performed with a SuperScript preamplification system (Gibco BRL) with random oligonucleotide primers. TaqMan probes and primers for GTPCH and eNOS were designed with the use of Primer Express (Applied Biosystems) and synthesized by Applied Biosystems. For GTPCH, the following primers were used: forward primer, 5′-GTGATGATTGAGGAGTCTAC-3′; reverse primer, 5′-CTCCAGGTCTTTGGATCCT-3′. The TaqMan probe was as follows: 5′-FAM-CACAAACAATGGACAGG-CACAATGTG-TAMRA-3′. For eNOS, the following primers were used: forward primer, 5′-TCTCCGCTCCTAGT-3′; reverse primer, 5′-AGCCATACAGGATTGTCGCC-3′. The TaqMan probe was as follows: 5′-FAM-CACGGTGATGCGGATG-TAMRA-3′. Taqman probes and primers for control (GAPDH) are commercially available and were purchased directly from Perkin-Elmer. A typical reaction (50 μL) contained 1/20 of reverse transcription cDNA, 200 to 400 nmol/L primers, and 100 nmol/L probes in 1× TaqMan master buffer. Polymerase chain reactions (PCRs) were carried out on an ABI Prism 7700 system (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. PCR results were analyzed with the Sequence detector 1.6 program (Perkin-Elmer).

Assay of Biopterin
Biopterin (BH4 and more-oxidized species) was measured essentially as described by Fukushima and Nixon. Measurements of intracellular and extracellular (cell supernatant) biopterin and pterin (2-amino-4-hydroxypteridine [AHP]) were performed by C18 reversed-phase high-performance liquid chromatography with fluorescence detection, with authentic biopterin or AHP as standards. The amount of 5,6,7,8-BH4 was calculated on the basis of the difference in biopterin concentration after oxidation in acid (total biopterins) and base (7,8-dihydrobiopterin [BH2] + biopterin). Biopterin levels were expressed as picomoles per milligram of cell protein. Protein concentration was measured by the method of Lowry et al with bovine serum albumin as the standard. Biopterin released by the cells into the medium of a 90-mm-diameter dish was expressed in terms of picomoles per well.

Nuclear Run-On Assay
Isolation of nuclei and in vitro transcription proceeded as described. Linearized plasmids containing target cDNAs dissolved in 0.4 mol/L NaOH were immobilized onto Hybond-N+ nylon membranes (Amersham Pharmacia) and hybridized with the labeled RNA as described. Blots were exposed to an imaging plate (Fuji Photo Film Co), and signal intensity was quantified with a Fujix bioimaging analyzer (BAS2000II).

Measurement of NO Synthesis
Citrulline synthesis was measured by modification of a previously described technique. Cell monolayers were incubated at 37°C for 30 minutes in Hanks’ balanced salt solution (pH 7.4) containing 0.5% fetal bovine serum. Subsequently, cells were stimulated with 2 μmol/L ionomycin in the presence of 10 μmol/L l-arginine and 3.3 μCi/mL L-[3H]arginine. After 15 minutes, the reaction was stopped with cold phosphate-buffered saline containing 5 mmol/L L-arginine and 4 mmol/L EDTA, after which the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 50 mmol/L HEPES and 5 mmol/L EDTA (pH 5.5) and applied to 2-mL columns of Dowex AG50WX-8 (Na+ form). Radioactivity corresponding to [3H]citrulline within the eluate was quantified by liquid scintillation counting. Agonist-induced [3H]citrulline production was calculated from the difference in radioactivity observed among ionomycin-stimulated cells and unstimulated cells. This was expressed as femtomoles per milligram of cell protein. Basal [3H]citrulline synthesis was determined from the nitro-l-arginine methyl ester (1 mmol/L, 30-minute preincubation)–inhibitable radioactivity in unstimulated cells, which was not always detectable.

Statistical Analysis
Data are presented as mean±SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher’s protected least-significant-difference test. A value of P<0.05 was considered statistically significant.

Results
We first examined the mRNA levels of GTPCH and eNOS quantitatively by using real-time PCR in HUVECs treated with cerivastatin. As shown in Figure 1A, cerivastatin increased GTPCH mRNA at 6 hours, after which it increased further and remained elevated for at least 24 hours. Treatment of the cells with different concentrations of cerivastatin significantly increased GTPCH mRNA levels to between 0.01 and 1 μmol/L (Figure 1A). As shown in Figure 1A, an increase in eNOS mRNA with cerivastatin was also observed at 6 hours, after which the level of eNOS mRNA continued to increase and remained elevated for at least 24 hours. Treatment of the cells with different concentrations of cerivastatin significantly increased eNOS mRNA levels to between 0.01 and 1 μmol/L (Figure 1A). In another experiment, we examined the effect of another statin, fluvastatin, on GTPCH and eNOS mRNA levels. Fluvastatin also increased the mRNA levels of both GTPCH and eNOS (Figure 1B).

Incubating HUVECs with TNF-α increased GTPCH mRNA levels while decreasing eNOS mRNA levels. In the presence of cerivastatin, the TNF-α–mediated increase in GTPCH mRNA was enhanced, and the TNF-α–mediated decrease in eNOS mRNA was attenuated (Figure 2).

We next determined whether cerivastatin might increase cellular biopterin levels. Treatment of the cells with cerivastatin increased cellular biopterin levels in a concentration-dependent manner (Figure 3A). The BH4 content of the cells
was significantly higher than that of controls, whereas cellular 7,8-BH2 levels were similar (Figure 3B). In contrast, a similar high level of AHP was observed in media from control and cerivastatin-treated (0.5 \(\mu\)mol/L) cells (Figure 3B).

We then examined whether or not an increased stability of mRNA could explain alterations in GTPCH induction by cerivastatin. Figure 4A shows that the rate of decay of GTPCH mRNA was not altered by incubating cells with cerivastatin. A half-life approximating 1.5 hours was observed. We also evaluated eNOS mRNA stability under conditions in which cells were incubated with cerivastatin. eNOS mRNA levels decreased with time in control cells and were not altered by treatment with cerivastatin (Figure 4A).

We further examined whether or not the increased transcription of GTPCH mRNA could serve as the basis for the altered GTPCH mRNA induction by the statin. The transcription rate of the GTPCH gene assessed by nuclear run-on assay was substantially increased 6 hours after exposure to cerivastatin (Figure 4B).

To identify which product of the HMG-CoA reductase reaction is necessary for the effect of statins, mevalonate,
Farnesylpyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP) were added to cells treated with cerivastatin. Mevalonate is a cholesterol precursor, and FPP and GGPP are involved in farnesylation and geranylgeranylation of proteins, respectively. Mevalonate (0.1 mmol/L) completely reversed the statin-induced increase in GTPCH mRNA (Figure 4A).

Similarly, GGPP (10 μmol/L) completely reversed the statin-induced increase in GTPCH mRNA, whereas FPP (10 μmol/L) did not block this effect of cerivastatin (Figure 5A).

Figure 3. A, Cellular biopterin (BP) concentrations in cerivastatin-treated HUVECs. Cells were incubated with cerivastatin (0.01 to 1 μmol/L) for 24 hours, after which cellular biopterin (BH4 and more-oxidized species) was measured. B, Cellular and extracellular biopterin (BH4 and BH2/BP) and pterin concentrations in cerivastatin-treated HUVECs. Cells were incubated for 24 hours in the absence (white bars) or presence (black bars) of cerivastatin (0.5 μmol/L). The amount of BH4 was calculated from the difference in biopterin measured after oxidation in acid (total biopterins) and that measured in base (BH2+BP). Data represent the mean±SEM (n=3). **P<0.01 compared with control.

Figure 4. A, Effect of cerivastatin on GTPCH and eNOS mRNA stability. Cells were exposed to actinomycin D (Act.D.; 5 μg/mL) in the absence (open circles) or presence (closed circles) of cerivastatin (0.5 μmol/L). After the indicated times, mRNA levels were measured by real-time PCR. Data represent the mean±SEM (n=3). **P<0.01 compared with control. B, GTPCH gene transcription in cerivastatin-stimulated HUVECs. Nuclear run-on analysis was conducted 6 hours after incubation in the absence or presence of cerivastatin (0.5 μmol/L). Transcription of the isolated nuclei was analyzed by hybridizing 32P-labeled RNA to GTPCH and GAPDH cDNAs immobilized on nylon membranes. Representative of 3 experiments performed with similar results (top). Signals of GTPCH were quantified and normalized to those of GAPDH by using a bioimaging analyzer (bottom). Data represent means of triplicate determinations (±SEM).
Mevalonate, FPP, and GGPP alone did not affect basal GTPCH mRNA levels (data not shown). Similarly, we determined the effect of mevalonate, FPP, and GGPP on eNOS mRNA levels in cells treated with cerivastatin. Mevalonate (0.1 mmol/L) and GGPP reversed the statin-induced increase in eNOS mRNA, whereas FPP (10 μmol/L) did not block this effect of cerivastatin (Figure 5A). Rho is an important geranylgeranylated protein. To determine whether inhibition of Rho is related to upregulation of GTPCH mRNA levels by cerivastatin, we incubated cells with C3 exoenzyme, which ADP-ribosylates and inactivates Rho. As shown in Figure 5B, C3 exoenzyme increased GTPCH mRNA levels in a dose-dependent manner. Similarly, C3 exoenzyme increased eNOS mRNA.

The effect of GTPCH inhibition on cerivastatin-mediated increases in BH4 levels and eNOS activity was assessed. The selective GTPCH inhibitor, 2,4-diamino-6-hydroxypyrimidine (DAHP), inhibits de novo synthesis of BH4 by acting as an analogue of the first pyrimidine intermediate formed in the GTPCH reaction. Accordingly, preincubation of the cells with DAHP (1 mmol/L, 24 hours) caused a decrease in intracellular BH4 in control cells. Cerivastatin enhanced the BH4 levels, which were also attenuated with treatment with DAHP to the same levels as in the control cells with DAHP. In the presence of cerivastatin, ionomycin-stimulated citrulline formation was enhanced. The potentiating effect of cerivastatin on citrulline formation was substantially attenuated by DAHP, inasmuch as the cellular availability of BH4 had fallen, under these conditions (Figure 6).

**Discussion**

Beneficial effects of short- and long-term BH4 supplementation on endothelial function have been reported in animals and humans. Furthermore, it appears that the beneficial effects of some antioxidants (e.g., vitamin C) on vascular...


function might be mediated by increases in intracellular BH4.  The present study demonstrates that statins increase cellular biopterin by inducing GTPCH gene expression. This appears to enhance NO formation and to reduce uncoupling of NOS, leading to NOS-mediated oxygen reduction and formation of superoxide anions, along with hydrogen peroxide. Statins may increase eNOS activity by inducing eNOS as well as by increasing the amount of available BH4. Upregulation of GTPCH mRNA by statins appears to result from transcriptional activation, whereas eNOS mRNA levels appear to increase owing to stabilization of eNOS mRNA. Statins elevate BH4 levels, with a resultant increase in the ratio of BH4 to cellular 7,8-BH2. This creates favorable conditions for the production of NO from eNOS, because it is thought that the ratio of oxidized to reduced BH4 metabolites might regulate superoxide formation from eNOS.  

Incubating HUVECs with TNF-α increased GTPCH mRNA levels while decreasing eNOS mRNA levels. In the presence of cerivastatin, the TNF-α-mediated increase in GTPCH mRNA was enhanced, and the TNF-α-mediated decrease in eNOS mRNA was attenuated. This is consistent with a previous report in which inflammatory cytokines were observed to increase eNOS activity in cultured human ECs by increasing BH4 levels during a decrease in the total expression of eNOS enzyme, thus suggesting that statins might act against inflammation.

In the presence of BH4, NOS dimers secrete small amounts of O2- and couple heme and O2 reduction to NO synthesis. Significant amounts of O2- may be produced because of heme-catalyzed reduction when BH4 concentrations fall below those required to saturate the enzyme. Indeed, subsaturating levels of BH4 can lead to endothelial dysfunction as a result of decreased NO production in ECs, and insufficient BH4 can also result in NOS-uncoupled production of reactive oxygen intermediates, such as O2- and H2O2. NO may generate both NO and O2- when concentrations of BH4 are absolutely low or low relative to NOS. When the steady-state flux of O2- is high, evidence indicates that ONOO- is formed, which may be the reactive species responsible for many of the toxic effects of NO. Thus, intracellular levels of BH4 dictate the rate of NO synthesis by eNOS and even determine the end product, be it NO or O2-. Therefore, the observation that statins maintain BH4 at a level sufficient for eNOS saturation may be important with regard to their ability to increase NO formation. BH4 may prevent eNOS uncoupling through stabilization of eNOS in its dimer form, that BH4 is directing electron flow to l-arginine rather than to oxygen, and that BH4 itself may act as an antioxidant.

The present study found that mevalonate prevents the stimulatory effect of statins and that GGPP reverses the effects of statins. Furthermore, C3 exoenzyme was observed to upregulate GTPCH mRNA levels as well as those of eNOS. These results suggest that decreases in GGPP levels secondary to mevalonate depletion and subsequent Rho inactivation are related to the stimulatory effect of statins on both GTPCH and eNOS expression. The present study also found that inhibition of BH4 formation by DAHP, an inhibitor of GTPCH, substantially decreased BH4 levels in both control and statin-treated ECs. Likewise, inhibition of Ca2+-dependent NO synthesis by DAHP was seen, and the potentiating effect of statins on ionomycin-stimulated citrulline formation was abolished by pretreatment with DAHP. These observations suggest that statins might increase eNOS activity in part by increasing intracellular BH4.

In addition to augmenting eNOS expression, cerivastatin and fluvastatin were observed to potentiate GTPCH gene expression and BH4 synthesis of GTPCH in vascular ECs. Thus, statins increase NO production and prevent relative shortages of BH4. This may shift the balance toward NOS-catalyzed generation of protective NO and away from the production of deleterious reactive oxygen species.

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