Hydroxy-Methylglutaryl–Coenzyme A Reductase Inhibition Promotes Endothelial Nitric Oxide Synthase Activation Through a Decrease in Caveolin Abundance

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Hydroxy-Methylglutaryl–Coenzyme A Reductase Inhibition Promotes Endothelial Nitric Oxide Synthase Activation Through a Decrease in Caveolin Abundance

Olivier Feron, PhD; Chantal Dessy, PhD; Jean-Pierre Desager, PhD; J.-L. Balligand, MD, PhD

Background—Hypercholesterolemia is causally associated with defects of endothelial nitric oxide (NO)–dependent vasodilation. Increased uptake of cholesterol by endothelial cells (ECs) upregulates the abundance of the structural protein caveolin-1 and impairs NO release through the stabilization of the inhibitory heterocomplex between caveolin-1 and endothelial NO synthase (eNOS). Therefore, we examined whether the hydroxy-methylglutaryl–coenzyme A reductase inhibitor atorvastatin modulates caveolin abundance, eNOS activity, and NO release through a reduction in endogenous cholesterol levels.

Methods and Results—ECs were incubated with increasing doses of atorvastatin in the absence or in the presence of human LDL cholesterol (LDL-Chol) fractions in the presence of antioxidants. Our results show that atorvastatin (10 nmol/L to 1 μmol/L) reduced caveolin-1 abundance in the absence (−75%) and in the presence (−20% to 70%) of LDL-Chol. This was paralleled by a decreased inhibitory interaction between caveolin-1 and eNOS and a restoration and/or potentiation of the basal (145%) and agonist-stimulated (1107%) eNOS activity. These effects were observed in the absence of changes in eNOS abundance and were reversed with mevalonate. In the presence of LDL-Chol, atorvastatin also promoted the agonist-induced association of eNOS and the chaperone Hsp90, resulting in the potentiation of eNOS activation.

Conclusions—We provide biochemical and functional evidence that atorvastatin promotes NO production by decreasing caveolin-1 expression in ECs, regardless of the level of extracellular LDL-Chol. These findings highlight the therapeutic potential of inhibiting cholesterol synthesis in peripheral cells to correct NO-dependent endothelial dysfunction associated with hypercholesterolemia and possibly other diseases. (Circulation. 2001;103:113-118.)

Key Words: cholesterol ■ nitric oxide ■ endothelium ■ atorvastatin

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (or statins) were shown to substantially reduce cardiovascular morbidity and mortality in clinical primary and secondary prevention trials. Although it was reasonable to attribute most (if not all) of these therapeutic benefits to the reduction in atherogenesis secondary to their effect on serum lipid profile, recent studies suggested otherwise. Indeed, statins reduced clinical end points before any measurable regression in atherosclerotic plaques, diminished cardiovascular mortality even in patients with average cholesterol levels, and restored normal endothelial function independently of their effects on serum cholesterol levels. These clinical benefits, apparently unrelated to the central (hepatic) effect of statins in reducing LDL cholesterol (LDL-Chol), have been explained by several mechanisms (the so-called pleiotropic effects of statins), including prevention of intimal thickening through induction of vascular smooth muscle cell apoptosis and inhibition of vascular smooth muscle cell migration and proliferation, downregulation of monocyte chemotaxis and neutrophil-endothelial interaction, increase in fibrinolytic activity, plaque stabilization, and upregulation of endothelial NO synthase (eNOS) expression and/or activity. Although in most of these studies, the effect of statins have been ascribed to the inhibition of the mevalonate-dependent geranylgeranylation of Rho GTPase proteins, the causal relationship between this phenomenon and the protective effect of statins on vessel function remains elusive. Nevertheless, these studies highlighted the importance of the cholesterol synthesis pathway in peripheral cells as a target for the therapeutic effect of statins.

In peripheral cells, cholesterol homeostasis is achieved primarily through feedback regulation of the expression of key proteins involved in sterol flux and metabolism, eg, LDL receptor and HMG-CoA synthase and reductase. In addition, the balance between external and internal cholesterol is maintained through the efflux of free cholesterol to HDLs, a
process involving discrete plasmalemmal microdomains called caveolae.\textsuperscript{16,17} Recently, we demonstrated in endothelial cells (ECs) that the level of expression of caveolin-1, the main structural component of caveolae, is directly related to the amount of extracellular LDL-Chol and subsequent cholesterol uptake by these cells.\textsuperscript{18} Importantly, we documented that the increase in caveolin abundance induced by high LDL-Chol promotes its inhibitory interaction with eNOS, resulting in a decrease in NO production.\textsuperscript{18} This mechanism of cholesterol-induced impairment of NO production may participate in the pathogenesis of endothelial dysfunction and in the proatherogenic effects of hypercholesterolemia. One could therefore hypothesize that by reducing circulating LDL-Chol or directly inhibiting cholesterol synthesis in ECs (see above), statins could reverse endothelial dysfunction by decreasing caveolin expression and promoting NO release through the destabilization of the inhibitory caveolin/eNOS complex.

To test this hypothesis, we incubated ECs with increasing doses of the HMG-CoA reductase inhibitor atorvastatin and studied the effects on caveolin protein expression levels, caveolin/eNOS interaction, and eNOS activity. These experiments were performed in the absence and in the presence of human LDL-Chol fractions to verify the modulation of NOS activity by the statin in conditions of significant cholesterol influx from an extracellular source. Our results show that very low doses of atorvastatin (0.01 to 0.1 \textmu mol/L) significantly reduced caveolin abundance and restored basal and agonist-stimulated NOS activity by altering the stoichiometry of eNOS complexation with caveolin and heat shock protein (Hsp) 90, thereby underlying a novel regulation of eNOS activity by atorvastatin at the posttranslational level.

**Methods**

**Cell Culture and Treatments**

Human LDL fractions and lipoprotein-deprived serum were prepared as previously described.\textsuperscript{18} Freshly prepared LDL fractions were supplemented with 50 \textmu mol/L DTPA and used to prepare stock media at final concentrations of 100 and 200 mg/dL cholesterol.

Bovine aortic ECs (BAECs) were cultured to confluence in 3.5-cm dishes in DMEM containing 10% serum and were serum-starved for 24 hours. Cell monolayers were then exposed for 48 hours to atorvastatin (10 \textmu mol/L to 100 \textmu mol/L) in DMEM containing or without LDL subtraction. Incubations were carried out in the presence of 100 \textmu g/mL Cu/Zn superoxide dismutase (SOD), and medium was replaced every 12 hours. In some experiments, incubations were carried out in the presence of 1 \textmu mol/L mevalonate (Sigma) or 25 \textmu mol/L N-acetyl-leu-leu-norleucinal (ALLN) (Boehringer Mannheim).

**Immunoprecipitation and Immunoblotting**

ECs were collected and homogenized in an octylglucoside-containing buffer and processed for immunoblotting or immunoprecipitation as described previously.\textsuperscript{18,19} For eNOS/Hsp90 coimmunoprecipitation experiments, cells were instead homogenized in the presence of 0.4% Trition X-100 and 20 mmol/L sodium molybdate as reported by Bender et al.\textsuperscript{20}

**NO Measurements and NO Detection**

Quantitative analysis of nitrate and nitrite (NO\textsubscript{x}) was used as an index of NO production in our different cell systems. Briefly, aliquots of the medium bathing intact ECs or cell lysates were collected at different time intervals and processed through a cadmium-based microreducer chamber (WPI) to quantitatively reduce nitrate to nitrite. Acidic iodide was then used to convert nitrite to NO, which was electrochemically measured with an NO-selective microsensor (WPI), as recommended by the manufacturer. In some experiments, agonist-stimulated NO release was directly monitored by the NO sensor positioned above intact cell monolayers, as previously described.\textsuperscript{18} All the experiments were carried out in the presence of 7.5 U/mL SOD, and adequate controls with either vehicle or NOS inhibitors were routinely performed in parallel. Data are normalized for the amount of protein in the dish or in the lysate and presented as mean±SEM. By convention, we have used the term “NO\textsubscript{x}” to refer to measurements derived from nitrate and nitrite estimation and “NO” when release of nitric oxide was directly determined from the extracellular medium of agonist-stimulated cells. Statistical analyses were done with Student’s \textit{t} test or 1-way ANOVA where appropriate.

**Results**

**LDL-Chol Upregulates Caveolin and Its Interaction With eNOS in Quiescent ECs**

By exposing confluent, serum-starved ECs for 48 hours to culture medium containing or without LDL subfractions isolated from human serum (100 or 200 mg/dL cholesterol content), we examined the extent of the modulatory effect of LDL-Chol on caveolin abundance and caveolin/eNOS interaction. As in our previous study using non–serum-starved ECs,\textsuperscript{18} we found that although eNOS expression was not altered by the different treatments, caveolin protein expression dose-dependently increased with the levels of LDL-Chol present in the culture medium (Figure 1). In parallel to the increase in caveolin abundance, the association between the 2 proteins, as reflected by the fraction of eNOS immunoprecipitated by caveolin antibodies, was augmented proportionally to the extracellular LDL-Chol levels (Figure 1, bottom lane).

**HMG-CoA Reductase Inhibition Leads to a Reduction in Caveolin Expression**

We next examined the effects of a reduction in intracellular cholesterol neosynthesis on the same parameters. Cells were incubated for 48 hours in the absence of extracellular LDL-Chol but with increasing doses of the HMG-CoA reductase inhibitor atorvastatin. As depicted in Figure 2A (top left), we observed a dramatic reduction in caveolin expression even with the lowest dose used in this study (−75±13% with 0.01 \textmu mol/L atorvastatin; \textit{P}<0.01, \textit{n}=3).

To examine whether the effect of atorvastatin on caveolin expression was maintained in the presence of an extracellular source of cholesterol, we repeated the above experiments...
In subsequent series of experiments, we examined whether the effect of atorvastatin was intermediate (see Figure 3, top). By contrast, when cells were exposed to 200 mg/dL LDL-Chol, atorvastatin reduced caveolin expression to a similar level (Figure 3, bottom). Importantly, atorvastatin (up to 1 μmol/L) did not induce any significant increase in eNOS abundance, which was observed only at the highest dose of the drug (10 μmol/L) (Figure 2A, right).

Figure 2. Concentration-dependent effect of atorvastatin on caveolin-1 (CAV-1) and eNOS expression at various levels of LDL-Chol (0, 100, and 200 mg/dL). A, Immunoblotting (IB) analyses of caveolin-1 (left) and eNOS (right) abundance are shown. B, Densitometric analyses of caveolin immunoblots (n=3 to 4) as illustrated in A. a.u. indicates arbitrary units.

with ECs exposed to either 100 or 200 mg/dL LDL-Chol. In cells exposed to 100 mg/dL LDL-Chol, we observed a dose-dependent inhibitory effect of atorvastatin on caveolin expression (Figure 2A, middle left). At 200 mg/dL LDL-Chol (Figure 2A, bottom left), despite the higher starting level of caveolin expression, a reduction in caveolin abundance was clearly detectable (see frame in Figure 2A, left). Furthermore, when densitometric analyses of immunoblots were performed on exposure-matched films, the absolute decrease in caveolin abundance was not significantly different in each LDL-Chol condition tested (Figure 2B). Importantly, atorvastatin (up to 1 μmol/L) did not induce any significant increase in eNOS abundance, which was observed only at the highest dose of the drug (10 μmol/L) (Figure 2A, right).

Caveolin Expression Is Regulated by Endogenous Cholesterol Synthesis and Is Sensitive to the Inhibition of SREBP Catabolism

To examine whether these effects of atorvastatin were directly mediated through inhibition of cholesterol neosynthesis, which could transcriptionally regulate caveolin expression through sterol regulatory elements (SRE) present in its promoter region, 17, 2 complementary sets of experiments were designed. First, ECs incubated in the absence or in the presence of LDL-Chol were exposed to 1 μmol/L atorvastatin or 25 μmol/L ALLN, a nonspecific inhibitor of SRE binding protein (BP) catabolism, and the extent of inhibition of caveolin expression was compared. In cells incubated in the absence of extracellular cholesterol, atorvastatin and ALLN inhibited caveolin expression to a similar level (Figure 3, top). By contrast, when cells were exposed to 200 mg/dL LDL-Chol, atorvastatin did reduce caveolin expression, but to a lesser extent than ALLN, whereas at 100 mg/dL LDL-Chol, the effect of atorvastatin was intermediate (see Figure 3, top).

In subsequent series of experiments, we examined whether mevalonate, the downstream product of HMG-CoA reductase, reversed these effects. As shown in Figure 3, bottom, although mevalonate had no effect on the repression of caveolin expression by ALLN, it completely reversed the inhibitory effect of atorvastatin on the expression level of caveolin in every condition tested and even led to an increase over basal amounts of caveolin in some experiments (compare, for instance, lanes 1 and 2 or 7 and 8 in Figure 3, bottom).

HMG-CoA Reductase Inhibition Leads to a Reduction in the Inhibitory Caveolin/eNOS Interaction in ECs and Promotes NO Production

We previously demonstrated, in the same model, 18 that the extent of caveolin/eNOS interaction is proportional to the abundance of caveolin (see also Figure 1). Therefore, we next verified whether the effect of atorvastatin on caveolin expression was associated with a reduction in its association with eNOS, in the absence of any detectable change in eNOS abundance. Figure 4A, top, shows that on coincubation with 0, 0.1, or 1 μmol/L atorvastatin and 100 mg/dL LDL-Chol, atorvastatin reduced the amounts of eNOS bound to caveolin in ECs, as reflected by the extent of eNOS coimmunoprecipitated by caveolin antibodies. Accordingly, more free, unbound eNOS was found in the supernatant of the coimmunoprecipitation (Figure 4A, bottom).

We also examined whether these changes in the extent of caveolin/eNOS interaction directly accounted for changes in eNOS activity in the same conditions. Therefore, we measured eNOS activity from total lysates and caveolin IP supernatants, ie, in caveolin-depleted lysates. As shown in Figure 4B, atorvastatin treatment led to a significant increase in NO production in the same proportion in supernatants and total lysates, consistent with the hypothesis that in this cell model, all of the enzymatic activity is supported by the fraction of caveolin-free eNOS.

HMG-CoA Reductase Inhibition Increases Both Basal and Stimulated eNOS Activity in Intact Cells

We next determined whether the decrease in caveolin abundance after statin treatment was paralleled by increases in basal and stimulated eNOS activity in intact ECs. Basal NO,
production measured from cells exposed to LDL-free medium was 0.98 ± 0.12 nmol·h⁻¹·10⁻⁶ cells (n=6). In this condition, coincubation with increasing doses of atorvastatin produced a 45% to 60% increase at concentrations between 0.01 and 1 μmol/L and a further 35% increase at the highest drug concentration (10 μmol/L) (Figure 5A, solid bars). Of note, when cells were coincubated with mevalonate, statin exposure failed to induce any increase in basal NOx production (not shown). In the absence of drug treatment, the 48-hour incubation in the presence of 100 or 200 mg/dL LDL-Chol led to an average 25% and 53% decrease in basal NOx production, respectively (Figure 5A). When cells were coincubated with atorvastatin, we observed a restoration of basal NOx production in cells exposed to the lower dose of LDL-Chol (100 mg/dL) but no significant increase in basal eNOS activity in cells exposed to 200 mg/dL LDL-Chol (Figure 5A).

We next examined the effect of atorvastatin on agonist-evoked eNOS activation. ECs were or were not preincubated with LDL-Chol and/or atorvastatin and then exposed for 5 minutes to the calcium ionophore A23187 (5 μmol/L), a receptor-independent agonist known to promote the binding of Ca²⁺-activated calmodulin to eNOS. In the absence of atorvastatin (Figure 5B, open bars), cell exposure to extracellular LDL-Chol led to a dramatic decrease in A23187-stimulated NO release consistent with the higher levels of caveolin and its inhibitory interaction with eNOS. Atorvastatin treatment (0.1 μmol/L) increased the level of agonist-induced NO release, but the relative extent of this augmenta-
reasonably rule out indirect effects on NO oxidation, although stimulated NO production. Our use of SOD allowed us to expression in cultured cells.
inhibitor of SREBP catabolism, fully abrogates caveolin-1 and Feron et al18) and others17 that ALLN, a nonspecific of the caveolin-1 gene17 and the observation by us (this study
inhibition by atorvastatin of cholesterol synthesis in ECs as neosynthesis. By contrast, our results directly implicate the mediates, regardless of the effect of statins on cholesterol
studies (see introduction) had highlighted the therapeutic effects on endothelial receptor coupling to eNOS can be excluded in our experiments using the receptor-independent calcium ionophore A23187. Of note, the statin was effective both in the absence and in the presence of exogenously added LDL-Chol, but with different efficiency in resting and stimulated cells (see Figure 5A and 5B). Accordingly, in unstimulated cells, ie, at low activated calcium-calmodulin levels, eNOS activity appeared to be determined primarily by the abundance of caveolin available for its inhibitory binding to eNOS. Indeed, we documented a stronger potentiation of basal NO release at zero extracellular LDL-Chol, ie, when the (low) caveolin pool is maximally sensitive to inhibition of endogenous cholesterol synthesis by atorvastatin (see Figure 5A). Conversely, in agonist-stimulated cells, in which large amounts of activated calcium-calmodulin may compete with caveolin for eNOS binding, the beneficial effect of atorvas-tatin on eNOS activity was more prominent in cells expressing high levels of caveolin (ie, in the presence of high levels of LDL-Chol) (see Figure 5B). Moreover, these small changes appeared to be sufficient to alter the ability of the enzyme to interact with other modulators as well, as demonstrated with the chaperone Hsp90 in the present study, resulting in substantial increases in eNOS sensitivity. More generally, our demonstration of the effect of atorvastatin to decrease caveolin-1 expression leaves additional possibilities to impact on disease processes involving the interaction of caveolin with a variety of signaling molecules, eg, tyrosine kinases, adenylly cyclase, or G protein–coupled receptors.22

Discussion
We identified a cholesterol-dependent mechanism of endothelial dysfunction that involves the posttranslational regulation of eNOS in the absence of changes in absolute eNOS abundance. Instead, cholesterol modulates the abundance in ECs of caveolin-1, which acts as an inhibitor of eNOS activation. Importantly, this study shows that the HMG-CoA reductase inhibitor atorvastatin restores eNOS activity through downregulation of caveolin-1 expression. This occurs at concentrations as low as 10 to 100 nmol/L and is fully reversed by addition of excess mevalonate, confirming the specific effect of the drug on the mevalonate pathway. Recent studies (see introduction) had highlighted the therapeutic potential of inhibiting the mevalonate pathway in peripheral cells through the reduction of downstream isoprenoid intermediates, regardless of the effect of statins on cholesterol neosynthesis. By contrast, our results directly implicate the inhibition by atorvastatin of cholesterol synthesis in ECs as the mechanism promoting NO release. This is consistent with the previous identification of SRE in the promoter sequence of the caveolin-1 gene17 and the observation by us (this study and Feron et al18) and others17 that ALLN, a nonspecific inhibitor of SREBP catabolism, fully abrogates caveolin-1 expression in cultured cells.

Atorvastatin treatment potentiated both basal and agonist-stimulated NO production. Our use of SOD allowed us to reasonably rule out indirect effects on NO oxidation, although the application of SOD in the extracellular medium cannot prevent a potential decrease (by oxidative stress) in the bioavailability of intracellularly released NO. Likewise, indirect effects on endothelial receptor coupling to eNOS can be excluded in our experiments using the receptor-independent calcium ionophore A23187. Of note, the statin was effective both in the absence and in the presence of exogenously added LDL-Chol, but with different efficiency in resting and stimulated cells (see Figure 5A and 5B). Accordingly, in unstimulated cells, ie, at low activated calcium-calmodulin levels, eNOS activity appeared to be determined primarily by the abundance of caveolin available for its inhibitory binding to eNOS. Indeed, we documented a stronger potentiation of basal NO release at zero extracellular LDL-Chol, ie, when the (low) caveolin pool is maximally sensitive to inhibition of endogenous cholesterol synthesis by atorvastatin (see Figure 5A). Conversely, in agonist-stimulated cells, in which large amounts of activated calcium-calmodulin may compete with caveolin for eNOS binding, the beneficial effect of atorvastatin on eNOS activity was more prominent in cells expressing high levels of caveolin (ie, in the presence of high levels of LDL-Chol) (see Figure 5B). Moreover, these small changes appeared to be sufficient to alter the ability of the enzyme to interact with other modulators as well, as demonstrated with the chaperone Hsp90 in the present study, resulting in substantial increases in eNOS sensitivity. More generally, our demonstration of the effect of atorvastatin to decrease caveolin-1 expression leaves additional possibilities to impact on disease processes involving the interaction of caveolin with a variety of signaling molecules, eg, tyrosine kinases, adenylly cyclase, or G protein–coupled receptors.22

Clinical Implications
A deficient NO-dependent vasorelaxation is central to coronary and peripheral ischemic diseases secondary to hypercholesterolemia and may result from either a decreased production of NO or an increase in NO catabolism.23 Interestingly, both processes can be restored (albeit not uniformly) by supplementation with L-arginine or tetrahydrobiopterin in vivo (for references, see Wever et al23), suggesting that eNOS is still expressed in the dysfunctional endothelium but is somehow inactivated. In this regard, the eNOS-“sensitizing” effect of atorvastatin may already operate at very early stages of endothelial dysfunction, at a time when the activation (but not the abundance) of the enzyme is downregulated. Our results suggest that this peripheral effect may occur at very low concentrations of the drug, ie, close to those achieved therapeutically in vivo. In addition, our observation of a marked potentiation of basal NO production at zero extracellular LDL-Chol may extend the clinical usefulness of atorvastatin (and perhaps other statins as well) to NO-dependent endothelial dysfunctions secondary to diseases other than hypercholesterolemia, such as hypertension or heart failure.

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