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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 μ mol/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 (+45%) and agonist-stimulated (+107%) 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.¹ 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,² diminished cardiovascular mortality even in patients with average cholesterol levels,^{3,4} and restored normal endothelial function independently of their effects on serum cholesterol levels.⁵ 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 apoptosis⁶ and inhibition of vascular smooth muscle cell migration⁷ and proliferation,^{8,9} downregulation of

monocyte chemotaxis and neutrophil-endothelial interaction,¹⁰ increase in fibrinolytic activity,¹¹ plaque stabilization,¹² and upregulation of endothelial NO synthase (eNOS) expression^{13,14} and/or activity.¹⁵ 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.

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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.¹⁶ In addition, the balance between external and internal cholesterol is maintained through the efflux of free cholesterol to HDLs, a

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process involving discrete plasmalemmal microdomains called caveolae.^{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.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.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 $\mu\text{mol/L}$) 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 subfractions and lipoprotein-deprived serum were prepared as previously described.¹⁸ Freshly prepared LDL subfractions were supplemented with 50 $\mu\text{mol/L}$ 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 nmol/L to 10 $\mu\text{mol/L}$) in DMEM containing or without LDL subfraction. Incubations were carried out in the presence of 100 $\mu\text{g/mL}$ Cu/Zn superoxide dismutase (SOD), and medium was replaced every 12 hours. In some experiments, incubations were carried out in the presence of 1 mmol/L mevalonate (Sigma) or 25 $\mu\text{mol/L}$ *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.^{18,19} For eNOS/Hsp90 coimmunoprecipitation experiments, cells were instead homogenized in the presence of 0.4% Triton X-100 and 20 mmol/L sodium molybdate as reported by Bender et al.²⁰

NO_x Measurements and NO Detection

Quantitative analysis of nitrate and nitrite (NO_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

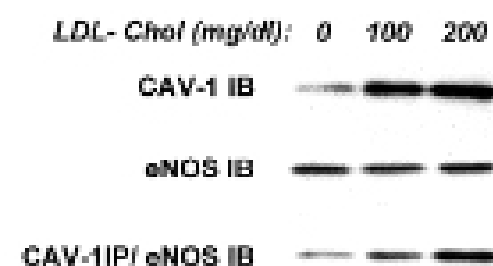


Figure 1. Effect of LDL-Chol on caveolin-1 (CAV-1), eNOS protein expression, and their interaction in ECs. Changes in caveolin-1 (top) and eNOS (middle) abundance analyzed by immunoblotting (IB) and in amount of eNOS coimmunoprecipitated (IP) with caveolin (bottom) are shown. Blots are representative of 3 to 5 separate experiments.

collected at different time intervals and processed through a cadmium-based microreductor 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.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 are presented for convenience as mean \pm SEM. By convention, we have used the term "NO_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 *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,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 ($-75 \pm 13\%$ with 0.01 $\mu\text{mol/L}$ atorvastatin; $P < 0.01$, $n = 3$).

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