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Research Article

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Effects of the 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitors, Atorvastatin and Simvastatin, on the Expression of Endothelin-1 and Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells

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Abstract

Endothelial dysfunction associated with atherosclerosis has been attributed to alterations in the L-arginine-nitric oxide (NO)–cGMP pathway or to an excess of endothelin-1 (ET-1). The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been shown to ameliorate endothelial function. However, the physiological basis of this observation is largely unknown. We investigated the effects of Atorvastatin and Simvastatin on the pre–proET-1 mRNA expression and ET-1 synthesis and on the endothelial NO synthase (eNOS) transcript and protein levels in bovine aortic endothelial cells. These agents inhibited pre–proET-1 mRNA expression in a concentration- and time-dependent fashion (60–70% maximum inhibition) and reduced immunoreactive ET-1 levels (25–50%). This inhibitory effect was maintained in the presence of oxidized LDL (1–50 μg/ml). No significant modification of pre–proET-1 mRNA half-life was observed. In addition, mevalonate, but not cholesterol, reversed the statin-mediated decrease of pre–proET-1 mRNA levels. eNOS mRNA expression was reduced by oxidized LDL in a dose-dependent fashion (up to 57% inhibition), whereas native LDL had no effect. Statins were able to prevent the inhibitory action exerted by oxidized LDL on eNOS mRNA and protein levels. Hence, these drugs might influence vascular tone by modulating the expression of endothelial vasoactive factors. (*J. Clin. Invest.* 1998. 101:2711–2719.)

Key words: statins • endothelin • nitric oxide • HMG-CoA reductase • endothelium

Introduction

The discovery, several years ago, of a new class of drugs termed 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase inhibitors and their usage as therapeutic agents, has resulted in the beneficial treatment of hyperlipidemia and coronary artery disease. This group of drugs is familiarly known as "statins" and their common mechanism of action is their ability to inhibit cholesterol synthesis in the liver by blocking the conversion of HMG-CoA to mevalonate, the rate-limiting step in the mevalonate pathway (1, 2), and in the long term to lower LDL plasma levels (3, 4). Decrements in LDL cholesterol levels are associated with longer survival and less incidence of acute coronary disease (5).

Although much has been learned about the pathogenesis of atherosclerosis from the molecular standpoint, several critical steps in the formation of the atheroma plaque remain to be understood (6). One fact, which has been established, is that atheromatous vessels show endothelial dysfunction with an impaired response to endothelium-mediated vasodilatation (7). An alteration in either the expression or function of the endothelial vasoactive factors, endothelin-1 (ET-1), and nitric oxide (NO), has been proposed as a potential explanation for this phenomenon, given their crucial role in the regulation of vascular tone (8–10). ET-1 is a 21–amino acid component of a family of related peptides, which is the major isopeptide synthesized by endothelial cells, with powerful vasoconstrictive effects among others (11). ET-1 is generated from a 38–amino acid precursor, big ET-1, itself derived from a series of intracellular proteolytic steps of a propeptide, pre–proET-1, through the action of specific metalloproteinases called endothelin-converting enzymes. After it is released abluminally from endothelial cells, it acts upon vascular smooth muscle through the G-protein–coupled receptor subtype, ET_A, leading to vasoconstriction in human resistance vessels (12).

NO is a labile-free radical with multifaceted actions, wide tissular distribution, and ubiquitous presence in higher organisms (13). Most probably, basal release of NO along the vascular tree contributes to maintain a vasodilatory tone in a fundamental way (14) through its interaction with and subsequent activation of soluble guanylate cyclase, as the intracellular generation of cyclic guanosine monophosphate in smooth muscle cells leads to vascular relaxation. NO in endothelial cells is synthesized from L-arginine through the action of NO synthase type 3 or endothelial NO synthase (eNOS), an enzyme with a complex regulatory pattern both at the transcriptional and posttranslational levels (15, 16). Of interest, inactivation of endothelium-derived NO may be mediated by oxidized lipoproteins (oxLDL), a major chemical component of the initial proatherogenic milieu (17, 18).

Several clinical studies have recently confirmed that statins

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1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; ECL, enhanced chemiluminescence; eNOS, endothelial NO synthase; ET-1, endothelin-1; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NO, nitric oxide; oxLDL, oxidized low density lipoprotein.

may just not only represent cholesterol-lowering agents, but also contribute to ameliorate the endothelial dysfunction present in atherosclerosis (19). Using vascular endothelial cells in culture, we have investigated if statins could modulate the expression of the vasoactive factors ET-1 and NO, both in the absence and presence of oxLDL. Our results show that statins significantly reduce the synthesis of ET-1 and the expression of its precursor, the pre–proET-1 mRNA, in a process specifically related to the inhibition of HMG-CoA reductase. Furthermore, we have observed that statins prevent the downregulation of eNOS mRNA and protein levels associated to oxLDL.

Methods

Materials. Cell culture media, calf serum, glutamine, and penicillin/streptomycin were purchased from Bio-Whittaker (Walkersville, MD); cell culture plates from Becton Dickinson (France); Atorvastatin and Simvastatin were a gift of Parke-Davis Pharmaceuticals Research (Ann Arbor, MI). Deoxycytidine 5′-triphosphate α-^{32}P-(3,000 Ci/mmol) was from Amersham (Aylesbury, UK). L-[U-^{14}C] arginine (sp act 317 mCi/mmol) was from Amersham International (Buckinghamshire, UK). Dowex AG 50WX-8 cation exchange resin (100–200 mesh) was from Bio-Rad Laboratories (Hercules, CA). X-OMAT S X-ray film was from Eastman Kodak Co. (Rochester, NY). L-citrulline, calcium ionophore A23187, mevalonate, and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Bovine aortic endothelial cells (BAEC) were isolated from thoracic aortas using previously described methods (20). Characterization was based on their typical cobblestone appearance and uniform uptake of fluorescent acetylated LDL. Cells were maintained in RPMI 1640 supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 95% O_2 and 5% CO_2. Experiments were performed on confluent monolayers at passages 3–6, made quiescent by serum deprivation. Specifically, cells were kept in 0.5% serum for 24 h before starting the procedure and in serum-free medium during incubations.

Cell viability. Cellular toxicity of statins was tested by trypan blue exclusion. Furthermore, potential induction of apoptosis was studied by DNA fragmentation analysis in agarose gels and by analysis of the cell cycle distribution assessed by flow cytometry in the presence of propidium iodide (21). No significant changes in either of these parameters were observed with Atorvastatin or Simvastatin at the concentration range used in this work.

RNA isolation, Northern blotting, and hybridization. Total cellular RNA was isolated from BAEC with the guanidinium thiocyanate-phenol-chloroform method (22) subjected to electrophoresis in 1% agarose gels containing 0.66 M formaldehyde, transferred to nylon membranes (Hybond, Amersham International, Buckinghamshire, UK), UV cross-linked (UV stratalinker 1800 from Cultek, Stratagene, La Jolla, CA), and hybridized as previously described (23), except that prehybridization solution was used during hybridization. A full-length bovine ET-1 cDNA (24) or a bovine eNOS cDNA (25) (GenBank/EMBL/DDBJ Accession No. M89952) were used as probes. Pre–proET-1 and eNOS blots were washed at final stringency conditions of 42°C, 1 × SSC, 0.5% SDS and 65°C, 0.1 × SSC, 0.1% SDS, respectively, and exposed on X-OMAT S film using intensifying screens at −80°C. Equivalent amount of RNA loading was ensured by ethidium bromide staining of ribosomal RNA or, where indicated, by rehybridization with a rat β-tubulin cDNA (24). The density of autoradiographic signals or the band intensities of 28 and 18 S ribosomal RNA were quantitated with a UMAX-VISTA-T630 image scanner using the public domain software package National Institutes of Health (Bethesda, MD) Image 1.55. Levels of pre–proET-1 or eNOS mRNA were normalized to RNA loading and expressed in relative densitometric units with respect to control values.

Quantitation of ET-1 protein. ET-1 was measured by enzyme-linked immunosorbent assay (Amersham International), according to the kit instructions (Biotrak Endothelin-1 ELISA system, RPN 228). Previously, ET-1 was extracted on Sep-Pak C18 cartridges (Waters Associates, Milford, MA). 4 ml conditioned medium of BAEC grown in 100-mm culture plates were acidified with 2 ml acetic acid (4% final concentration) and applied to cartridges preactivated with methanol, distilled water, and 4% acetic acid. Cartridges were then washed with distilled water and 25% ethanol, and ET-1 was eluted twice with 1 ml 4% acetic acid in 86% ethanol. The eluted ET-1 was then concentrated to dryness and reconstituted for ELISA.

Oxidation of LDLs. LDL was isolated from the plasma of humans using sequential buoyant density centrifugations techniques (26, 27). LDL (200 μg of protein/ml) was oxidized as described (28) by incubation with 5 μM $CuSO_4$ in phosphate-buffered saline plus 1 mM Ca^{2+} and 1 mM Mg^{2+} for 24–48 h at 37°C. Oxidation was arrested by refrigeration and addition of 1 mM EDTA and 10 μM butylated hydroxytoluene. LDL was then extensively dialyzed against 0.15 M NaCl, 1 mM EDTA, and 1 μM BHT, and stored at 4°C. Protein concentrations of lipoprotein preparations were determined using the Lowry method. Oxidative modification of LDLs was assessed by three different methods: (*a*) determination of thiobarbituric acid reactive substances as described previously (29), which were at least 10-fold higher in oxLDL compared to native, (*b*) fluorescence of LDLs, measured with excitation and emission wavelenghs of 365 and 425 nm, respectively (30), was fivefold higher in the case of oxidized lipoproteins, and (*c*) electrophoretic mobility under nondenaturing conditions showed a single band with a twofold faster migration rate for oxLDL.

SDS-polyacrylamide gel electrophoresis and immunoblotting. For SDS-polyacrylamide gel electrophoresis, BAEC were homogenized by sonication in buffer 1 (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM β-mercaptoethanol, containing 2 μg/ml each of protease inhibitors: leupeptin, pepstatin, trypsin inhibitor, and aprotinin). Soluble (cytosolic) and particulate (membrane) subcellular fractions were resolved by crude homogenate ultracentrifugation at 100,000 *g* at 4°C for 1 h; the membrane fraction was washed and then resuspended in buffer 1 containing 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate. Aliquots containing 10 μg of protein were electrophoresed on 8% polyacrylamide gels and transferred to Immobilon-P membranes using a semidry electroblotting system (Trans-Blot SD; Bio-Rad Laboratories). Blots were probed with anti-eNOS mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) and the eNOS protein was visualized using an enhanced chemiluminescence (ECL) detection system from Amersham. The density of ECL signals were quantitated with an AGFA StudioStar image scanner using the public domain software package National Institutes of Health Image 1.55.

Determination of eNOS activity. Except where indicated, BAEC were incubated with L-[^{14}C]arginine (0.5×10^6 cpm, 0.8 μM) plus 10 μM L-citrulline in an L-arginine–free medium for 30 min and with calcium ionophore A23187 (10 μM) for 15 min at 37°C at the end of each experimental period. Samples were processed as described (31). L-[^{14}C]citrulline was detected by liquid scintillation counting following cation exchange chromatography over Dowex AG50W-X8 resin. Activity in crude homogenates was determined by citrulline assay in accordance with previously described methods (32), using L-[^{14}C]arginine (0.5×10^6 cpm, 4 μM) as substrate.

In some experiments, the accumulation of nitrite (NO_2^-) in the cell culture supernatant of BAEC was taken as an index of eNOS activity. After treatment with the various agents for 24 h, nitrite was determined by the Griess reaction as described previously, (33) using sodium nitrite as a standard.

Data analysis. Unless otherwise indicated, data are expressed as means±SEM obtained in at least three separate experiments. Comparisons were made with analysis of variance followed by Dunnett's modification of the *t* test, whenever comparisons were made with a common control and the unpaired two-tail Student's *t* test for other

comparisons. The level of statistically significant difference was defined as $P < 0.05$.

Results

Statins inhibit the expression of the pre–proET-1 gene in a concentration- and time-dependent fashion. Intracellular levels of ET-1 are considered to be essentially regulated at the transcriptional level, as no preformed intracellular deposits have been identified (34). Therefore, we first evaluated if statins were capable of modifying the expression of pre–proET-1 mRNA. As shown in Fig. 1, both HMG-CoA reductase inhibitors, Atorvastatin and Simvastatin, significantly reduced the steady state levels of pre–proET-1 mRNA, when compared to basal after 24-h treatment. This effect was maximal (52±15% for Atorvastatin- and 57±12% for Simvastatin-mediated inhibitions) with the concentration of 10 μM and already detectable at 2 μM Atorvastatin (44±16% inhibition) and 5 μM Simvastatin (37±13% inhibition). Fig. 2 *A* demonstrates that the inhibition of pre–proET-1 mRNA expression elicited by both statins at 10 μM concentration was already noticeable at the first timepoint studied (8 h) and maximal between 16 and 24 h of exposure to the drugs, with a partial recovery of the expression after 48 h that was significant in the case of Atorvastatin. To discard the possibility that a destabilization of Atorvastatin in the culture medium could account for the effect observed, the drug was re-added for the last 24 h of a 48-h incubation period. As shown in Fig. 2 *B*, the recovery of pre–proET-1 transcript was not reduced. In fact, a lesser degree of Atorvastatin-mediated inhibition of pre–proET-1 expression was observed when compared to no re-addition conditions. This result implies that the recovery effect observed is not merely due to pharmacological modifications of Atorvastatin half-life. We next performed experiments devoted to correlate the levels of pre–proET-1 mRNA with immunoreactive ET-1 synthesis. As it is shown in Fig. 3, treatment with either Atorvastatin or Simvastatin resulted in a significant reduction in ET-1 levels (~25% for Atorvastatin and 50% for Simvastatin) in the BAEC culture medium conditioned for a 4-h period after 24-h pretreatment with either of the two drugs.

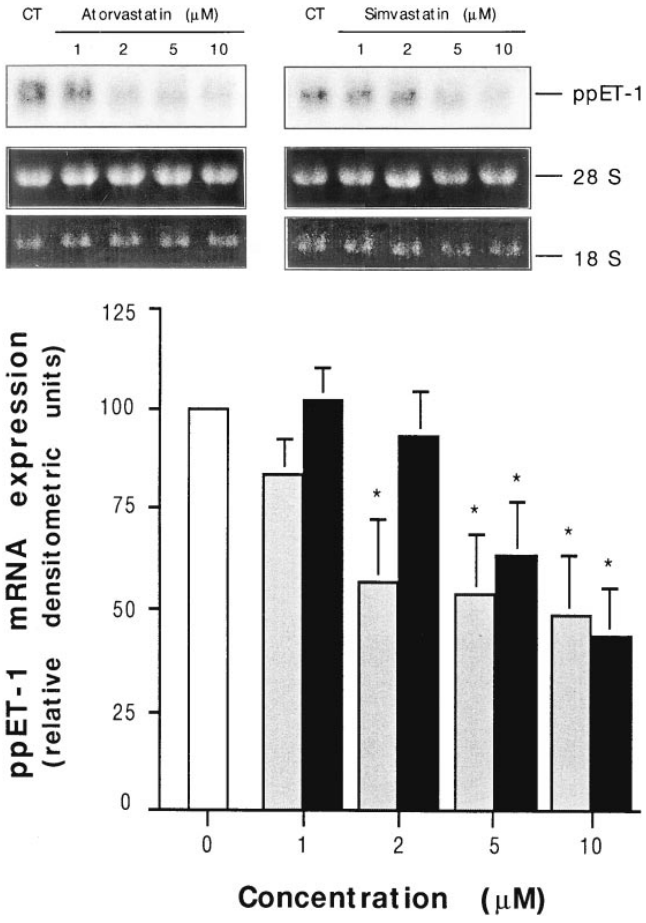


Figure 1. Concentration-response of the effect of Atorvastatin and Simvastatin on pre–proET-1 mRNA expression BAEC. *Top*: Northern blot analysis (10 μg total RNA/lane) of pre–proET-1 transcript levels after 24-h treatment. *Bottom*: densitometric band intensities were normalized to 28 S ribosomal RNA ethidium bromide staining (relative densitometric units) as described in the methods section and plotted as a function of Atorvastatin (*gray bars*) and Simvastatin (*black bars*) concentration. Data are means±SEM of three independent experiments. *$P < 0.05$ versus control (*white bar*).

Simvastatin does not destabilize pre–proET-1 mRNA. To evaluate the potential effect of Simvastatin on pre–proET-1 mRNA stability, we determined its half-life after inhibition of total RNA synthesis with Actinomycin D in BAEC. As shown in Fig. 4, treatment with Simvastatin per se resulted in an early inhibition of pre–proET-1 mRNA expression (27 and 39% inhibition after 2- and 4-h treatment, respectively). However, no significant changes in pre–proET-1 mRNA half-life were observed in the absence or presence of Simvastatin (control: 24 min; Simvastatin: 27 min, as estimated from the plot shown in Fig. 4). These data strongly suggest that the decrement observed in pre–proET-1 steady state mRNA levels after Simvastatin treatment, is due to transcriptional rate-related events.

Mevalonate but not native LDL prevents the inhibitory effect of statins on pre–proET-1 transcript levels. To assess whether statin-mediated reduction of pre–proET-1 mRNA levels was due to specific inhibition of HMG-CoA reductase, BAEC were exposed to mevalonate, the product of enzymatic conversion of HMG-CoA. As shown in Fig. 5, mevalonate (100 μM, 24 h) completely reversed the inhibitory action of Simvastatin (10 μM, 24 h), whereas no effect on pre–proET-1 expression was observed with mevalonate alone. Mevalonate also prevented the inhibitory action elicited by 24-h treatment with 10 μM Atorvastatin (data not shown). We then investigated whether the putative inhibition of cholesterol synthesis by statins was responsible for the downregulation of pre–proET-1 transcript. However, cholesterol supplement by addition of 2 μg protein/ml native LDL was unable to prevent the effect of statins. This suggests that a product(s) of mevalonate metabolism other than cholesterol could play a role in the control of steady state levels of pre–proET-1 mRNA. The different agents and major metabolic steps intervening in the cholesterol biosynthetic pathway are depicted in Fig. 5, lower panel.

The inhibitory effect of statins on pre–proET-1 mRNA levels is maintained in the presence of proatherogenic factors. We then explored whether the effect of statins on pre–proET-1 mRNA levels could be modified by the presence of a well known pathogenetic agent in the development of atherosclerosis, namely oxLDL. As shown in Fig. 6, statins were effective at inhibiting pre–proET-1 expression in the presence of a range of concentrations of oxLDL (1–50 μg/ml, 24 h) that are comparable to levels to which endothelial cells are exposed in

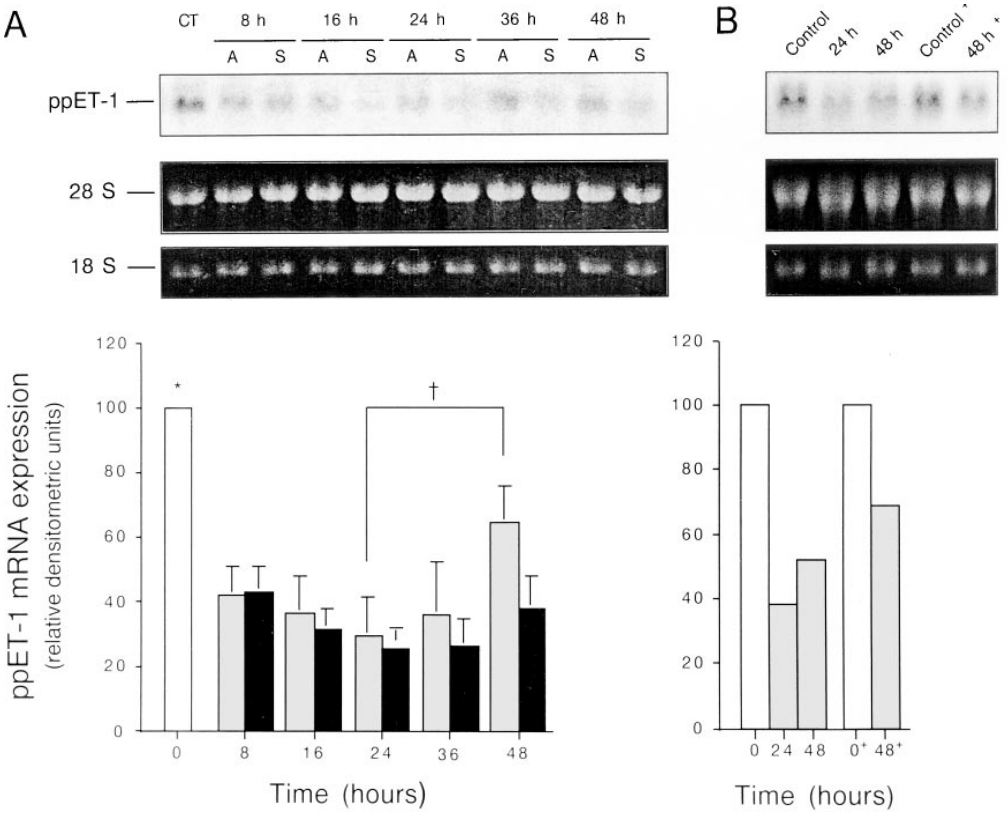
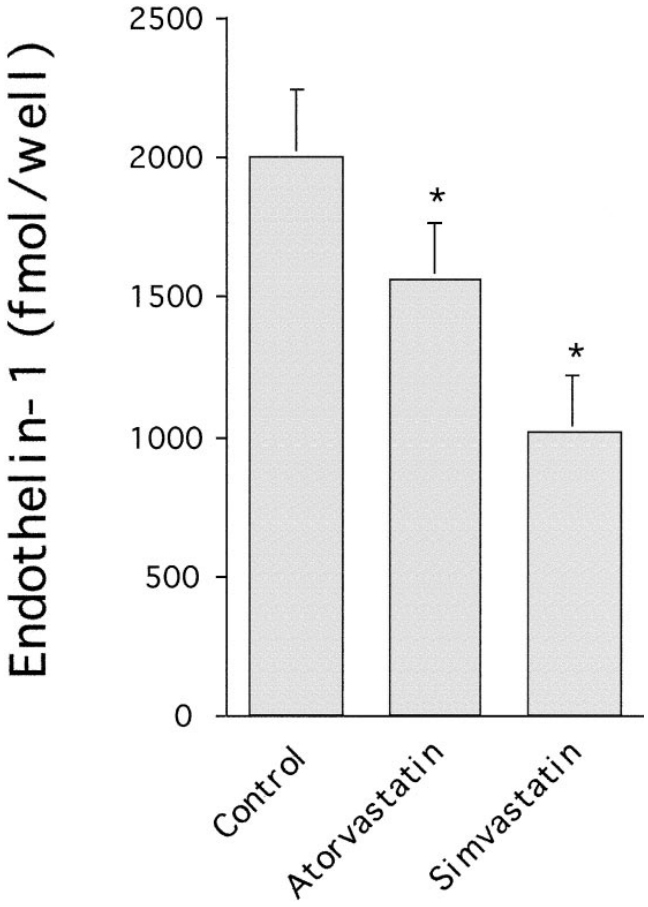


Figure 2. Time course of pre–proET-1 mRNA expression in Atorvastatin- and Simvastatin-treated BAEC. *A*: *Top* shows a representative Northern blot (10 μg total RNA/lane) at the indicated times of treatment with 10 μM Atorvastatin (*A*) or 10 μM Simvastatin (*S*). *Bottom*: bar graph showing the densitometric analysis as a function of the time of exposure to Atorvastatin (*gray bars*) and Simvastatin (*black bars*). Columns represent mean±SEM of three independent experiments. $*P < 0.05$, control (*white bar*) versus each statin treatment. $^{\dagger}P < 0.05$ compared with the indicated value. *B*: Northern analysis of pre–proET-1 transcript levels at the indicated times of treatment with 10 μM Atorvastatin. Where + is indicated, 24 h before the end of each experimental period, the medium was discarded and fresh medium plus 10 μM Atorvastatin were added.



vivo. At the higher concentrations of oxLDL, Atorvastatin was slightly more effective than Simvastatin in maintaining this inhibitory effect.

Statins prevent the downregulation induced by oxLDL of eNOS mRNA and protein. No significant effects of either Atorvastatin or Simvastatin on eNOS activity in BAEC homogenates were found at any concentration or time tested (0.1–10 μM, 12–36 h, data not shown). The effects of 10 μM Atorvastatin or 10 μM Simvastatin at 24 h are shown in Table I. Furthermore, treatment with 10 μM Atorvastatin did not modify eNOS enzymatic activity in intact cells as determined by NO_2^- accumulation or A23187-stimulated conversion of L-[^{14}C]arginine (2×10^6 cpm, 10 μM) to L-[^{14}C]citrulline (Table I). Finally, no significant changes in the protein levels of eNOS were detected in immunoblots after treatment with Atorvastatin or Simvastatin. In addition, the subcellular distribution of eNOS, which is related to complex posttranslational modification of the protein and has been clearly shown to be modified in the presence of agonists (35), did not vary after treatment with either drug (data not shown). We then investigated whether the presence of either native or oxidized LDL modi-

Figure 3. Effect of Atorvastatin and Simvastatin on ET-1 content in conditioned medium of BAEC. Cells were treated with vehicle, 10 μM Atorvastatin, or 10 μM Simvastatin for 24 h. The medium was discarded, and fresh medium was added while keeping the same treatments. After a conditioning period of 4 h, medium was collected for quantitation of ET-1 content by ELISA. Data represent mean±SEM of four independent experiments. $*P < 0.01$ versus control.

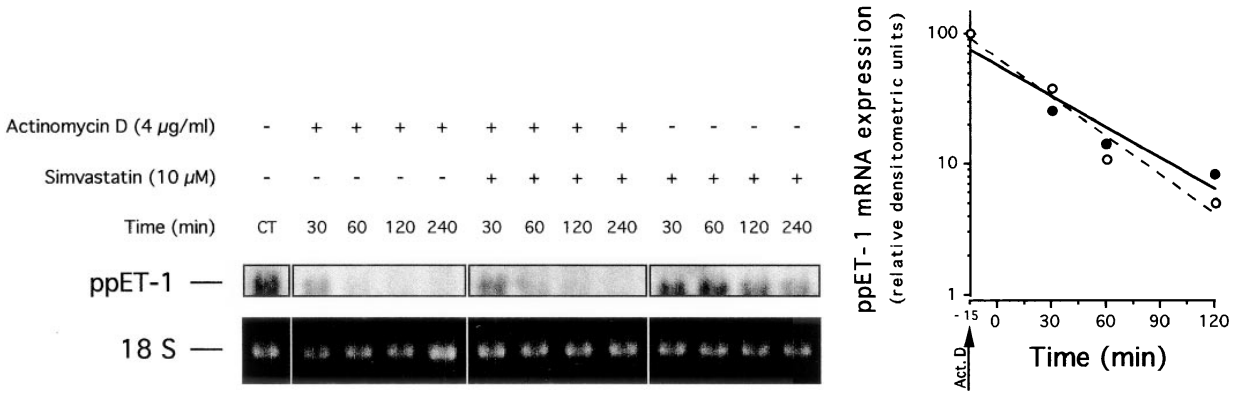


Figure 4. Effect of Simvastatin on pre–proET-1 mRNA stability. After a 15-min preincubation period in the presence or absence of Actinomycin D (*4 μg/ml*), BAEC were treated with vehicle or Simvastatin (*10 μM*). RNA was harvested after the indicated times. *Left*: Northern analysis of pre–proET-1. *Right*: plot of the decay of mRNA levels in BAEC treated with Actinomycin D in the absence (*open circles*, *dashed line*) or presence (*closed circles*, *solid line*) of Simvastatin. Densitometric band intensities were normalized as described in the Methods section. Shown is a representative figure of three independent experiments.

fied the expression of eNOS mRNA and if this process could be influenced by cotreatment with statins. As shown in Fig. 7, oxLDL, but not native LDL, inhibited the expression of eNOS mRNA in BAEC in a concentration-dependent manner (1–50 μg/ml, 24 h), consistent with results reported in human endothelial cells (36). This inhibition was accompanied by a significant decrease in the A23187 ionophore–stimulated activity of eNOS in intact cells (vehicle: 6.7±0.3 versus 50 μg/ml oxLDL: 3.9±0.2 pmol/mg protein/min) as well as a reduction in eNOS protein levels (see Fig. 9). However, in the presence of either Atorvastatin or Simvastatin (10 μM, 24 h), the depressive action elicited by concentrations of oxLDL up to 10 μg/ml on eNOS mRNA steady state levels was clearly reduced as shown in Fig. 8 (43 and 90% reduction for Atorvastatin and Simvastatin, respectively, at 10 μg/ml oxLDL). In addition, Simvastatin also prevented the 10-μg/ml oxLDL–mediated inhibition of eNOS protein expression (64% reduction, Fig. 9).

Discussion

In this work, we provide data consistent with a decreased synthesis of ET-1 in vascular endothelial cells when they are exposed to statins, both in the absence and presence of oxLDL. This decrement is most probably the consequence of an inhibi-

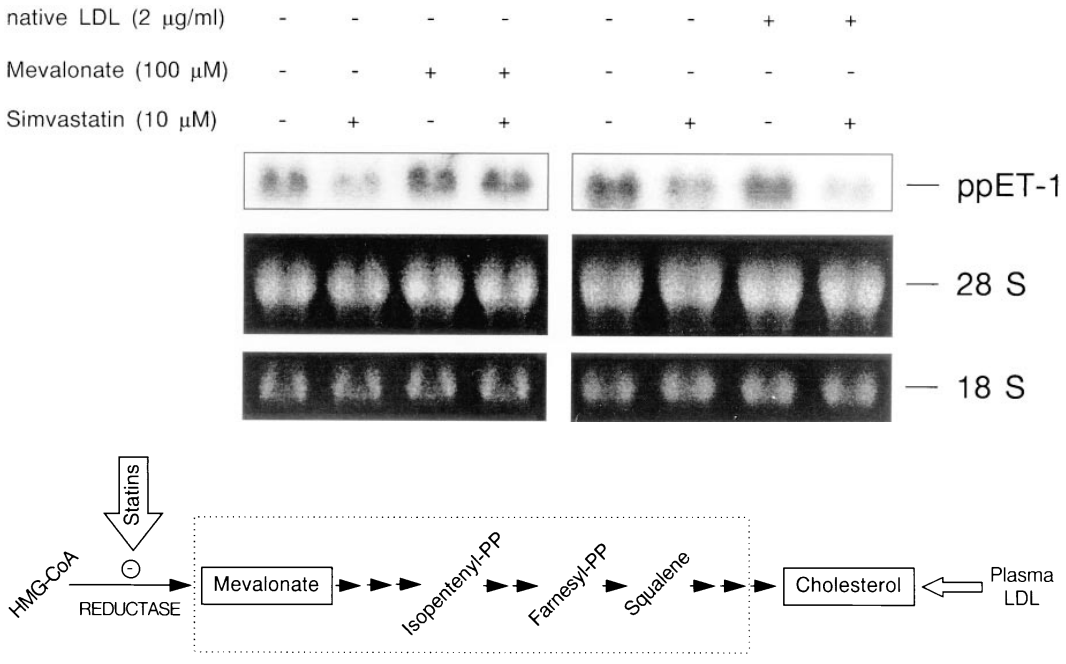
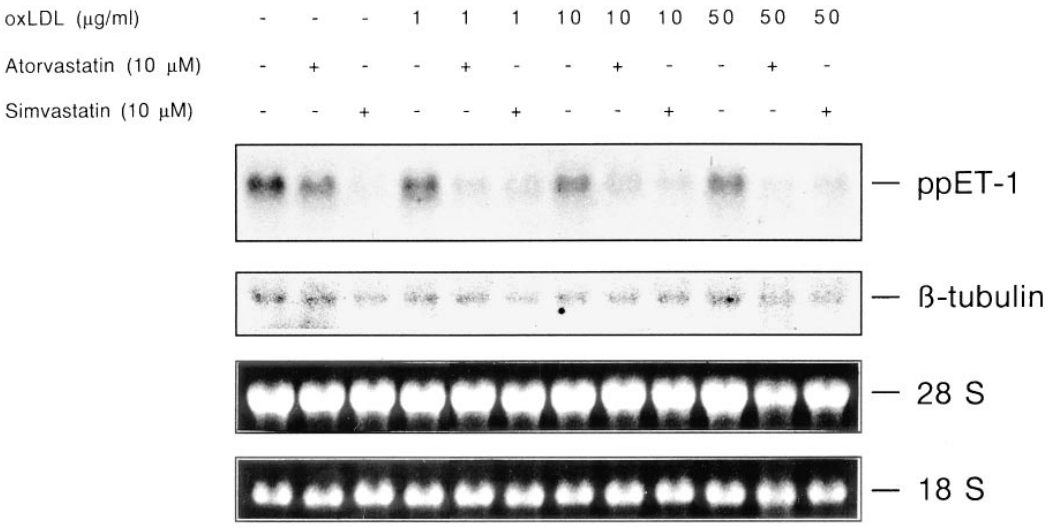


Figure 5. Effects of mevalonate and native LDL on the Simvastatin-mediated inhibition of pre–proET-1 mRNA expression in BAEC. *Top*: a representative Northern blot analysis (10 μg total RNA/lane) showing the pre–proET-1 mRNA expression after 24-h treatment or not with 10 μM Simvastatin in the presence of vehicle, 100 μM mevalonate or 2 μg protein/ml native LDL. Equal amounts of RNA loading per lane was confirmed by ethidium bromide staining of 28 and 18 S ribosomal RNA. *Bottom*: schematic representation of the mevalonate pathway in animal cells. Shown are the steps blocked by statins and the sources of cellular cholesterol, both endogenous, from synthesis within the cell, and exogenous, from receptor-mediated uptake of plasma LDL. The major nonsterol products of mevalonate implied in the regulation of cell function are enclosed within the dotted line.



Treatment	without oxLDL	1 μg/ml oxLDL	10 μg/ml oxLDL	50 μg/ml oxLDL
-	100 (5)	129 (2)	61 ± 5 (3)	83 (2)
Atorvastatin	56 ± 5 (5) **	31 (2)	22 ± 2 (3) **	16 (2)
Simvastatin	30 ± 7 (5) **	28 (2)	27 ± 9 (3) *	34 (2)

Mean ± S.E. (n)

Figure 6. Effects of Atorvastatin and Simvastatin on the pre–proET-1 transcript levels of oxLDL-treated BAEC. *Top*: Northern blot analysis (10 μg total RNA/lane) showing the pre–proET-1 mRNA expression after 24-h treatment with the indicated concentrations of oxLDL in the presence of vehicle, 10 μM Atorvastatin, or 10 μM Simvastatin. Equivalent expression of β-tubulin confirmed that the amount of RNA loaded per lane was similar for each condition. *Bottom*: values correspond to autorradiographic band intensities (relative densitometric units). Data are means±SEM of the indicated number of experiments (*in parenthesis*). $^{**}P < 0.001$, $^{*}P < 0.05$ versus no statin treatment at the corresponding oxLDL concentration.

tion in the expression of pre–proET-1 mRNA at the transcriptional level, as studies with RNA synthesis inhibitors showed that statins did not significantly modify ET-1 transcript stability. The specificity of HMG-CoA reductase inhibition in the genesis of this effect is supported by its reversion by mevalonate. In addition, we found that, although statins per se did not significantly modify the expression of eNOS, they clearly prevented oxLDL-mediated downregulation of its mRNA and protein levels.

Aside from their inhibitory effects on intracellular cholesterol synthesis (3, 4, 37), statins are able to influence other cellular functions such as the regulation of DNA transcription, and both antibody-dependent and natural killer cell cytotoxicity (38). For example, Simvastatin abolished PDGF-induced DNA synthesis in human glomerular mesangial cells (39), Fluvastatin has been shown to inhibit the proliferation of smooth muscle cells (19) and Pravastatin influenced monocyte chemotaxis and transendothelial migration (40). Our observations in cultured endothelial cells, namely abrogation of pre–proET-1 transcript levels in the presence of statins, leading to decreased ET-1 synthesis, suggest the existence of a new regulatory level by which statins may modify cellular homeostasis regarding vasoactive mediators. The mechanisms, whereby this effect is taking place, remain to be elucidated. Our data suggest that changes in mRNA expression take place at the transcriptional level, as has been described previously for other stimuli (23, 41), although further studies are needed to confirm this point. In terms of biochemical events occurring in endothelial cells after prolonged treatment with statins, our data show a recovery of transcript levels with addition of mevalonate. However, cholesterol supply in the form of LDL, at a concentration sufficient to support cell proliferation and other cellular processes (42, 43), was unable to revert these statin-mediated effects, suggesting that a deficit in nonsterol isoprenoids of the mevalonate pathway (1, 2) may be involved in the changes observed in pre–proET-1 mRNA levels. The synthesis of these compounds is mevalonate-dependent but sterol-independent (see Fig. 5). Of interest, a similar requirement has been described for natural killer cell chemotaxis (44).

In vitro studies have shown that oxLDL inhibit the expression of eNOS mRNA, in part through transcriptionally dependent mechanisms (36), although lysophosphatidylcholine, a component of atherogenic lipoproteins and atherosclerotic lesions, has been reported to have an opposite effect (45, 46). In-

Table I. Effects of Atorvastatin and Simvastatin on eNOS Activity

	Control	Atorvastatin	Simvastatin
	pmol/mg protein/min		
Activity in cellular extracts	7.4±0.5	7.2±0.2	8.6±0.2
A23187-stimulated activity*	50±5	56±8	n.d.
NO_2^- accumulation	2.0±0.4	2.3±0.5	n.d.

eNOS activity in BAEC treated with vehicle, Atorvastatin (10 μM) or Simvastatin (10 μM). After 24 h of treatment, experiments were performed as described in the Methods section. In *, samples were processed as described (36). *n.d.*, not determined.

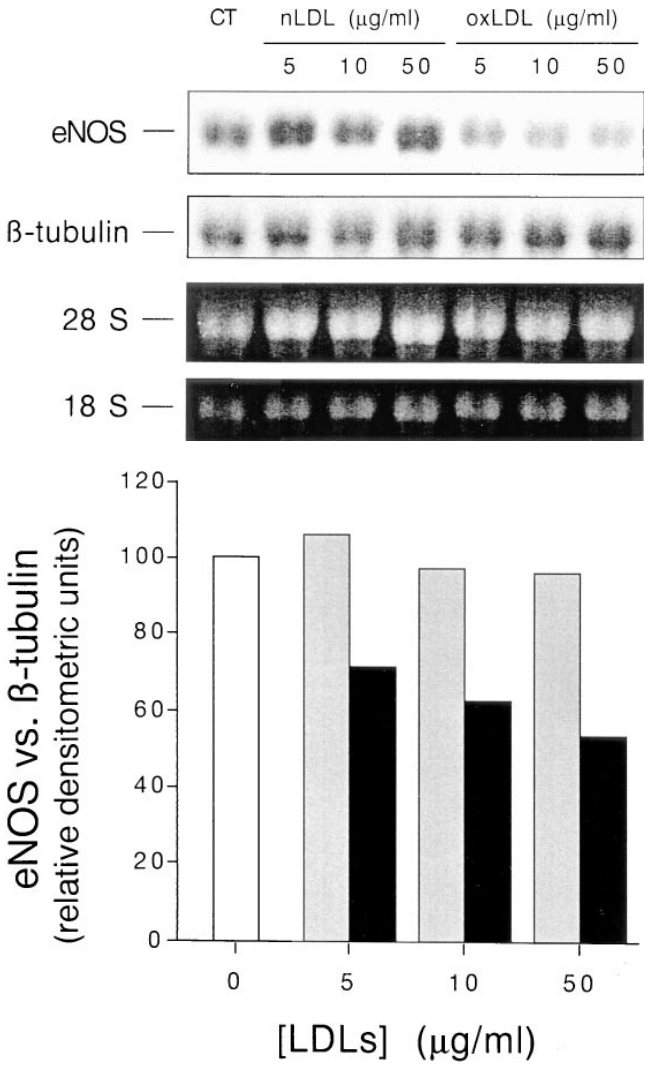


Figure 7. Effects of native and oxidized LDLs on eNOS mRNA expression in BAEC. *Top*: Northern blot analysis (10 μg total RNA/lane) of a concentration-response representative experiment showing the effect of native and oxidized LDLs on eNOS expression after 24 h of treatment. *Bottom*: values derived from a densitometric analysis of the amount of pre–proET-1 mRNA transcript were expressed relative to control levels, corrected for the amount of β-tubulin transcript per lane and plotted as a function of native (*gray bars*) and oxidized (*black bars*) LDLs concentration. Vehicle (*white bar*).

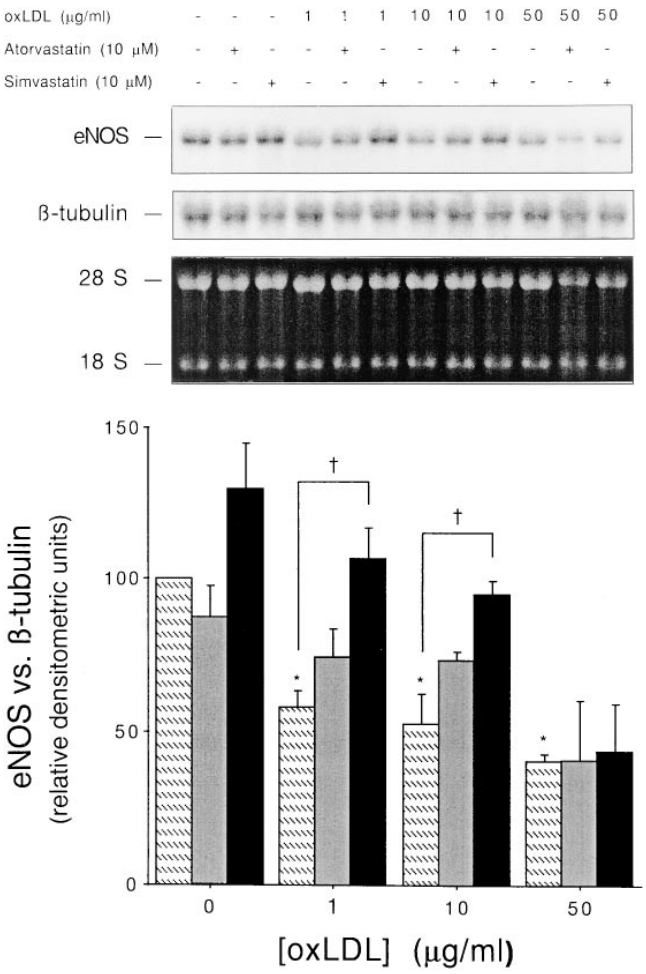
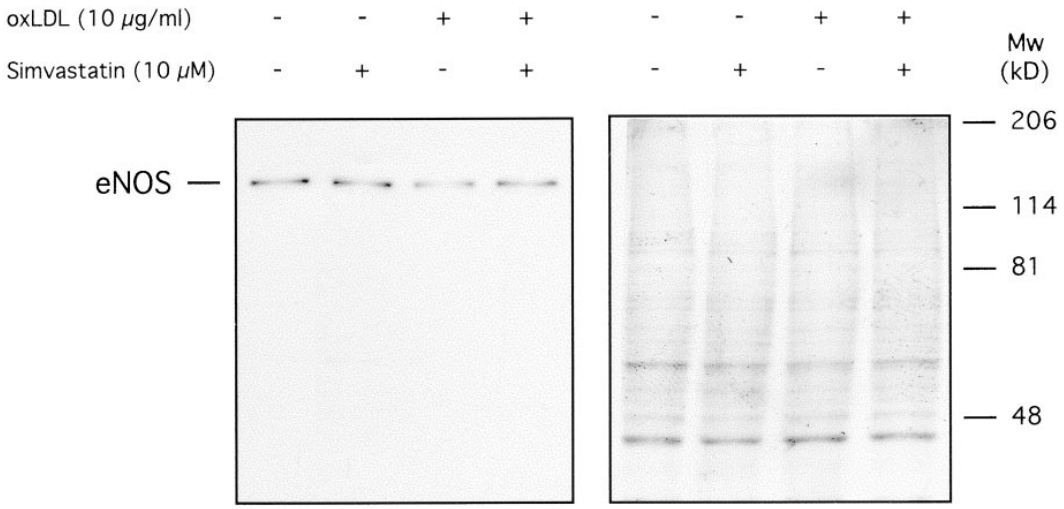


Figure 8. Effects of Atorvastatin and Simvastatin on the oxidized LDL–mediated inhibition of eNOS mRNA expression in BAEC. *Top*: a representative Northern blot (10 μg total RNA/lane) showing eNOS transcript levels after 24-h treatment with the indicated doses of oxLDL in the presence of vehicle, 10 μM Atorvastatin, or 10 μM Simvastatin. *Bottom*: the density of eNOS autorradiographic signals was normalized to the β-tubulin signals, and plotted as a function of oxLDL concentration in the presence of vehicle (*striped bars*), 10 μM Atorvastatin (*gray bars*), or 10 μM Simvastatin (*black bars*). Columns represent mean±SEM of three independent experiments. $^{*}P < 0.01$ versus control of untreated BAEC; $^{\dagger}P < 0.05$ compared with each indicated condition.

hibition of eNOS expression in vivo has been evidenced in hypercholesterolemic porcine and atherosclerotic human vessels (47, 48). However, detailed studies exploring whether intracellular cholesterol levels are important regulators of eNOS expression are lacking, even when sterol-regulatory *cis*-elements are present in the 5′ regulatory region of the eNOS gene (49, 50). Our data confirm previous observations on the capacity of oxLDL to inhibit the eNOS transcript and describe, for the first time, the ability of statins to prevent this effect at the mRNA and protein levels. Interestingly, it has recently been shown that Simvastatin can increase eNOS protein and mRNA expression in human vein endothelial cells and prevent hypoxia-dependent inhibition of transcript and protein levels (51). However, although in our experimental system Simvastatin showed a tendency to augment eNOS catalytic activity and mRNA and protein levels, our data do not support a significant effect per se in the endothelial cell type studied. It is possible that statins counteract the direct inhibitory effect of oxLDL on eNOS expression by modifying intracellular cholesterol synthesis or availability, extremes that need further experimental evidence. Overall, these results may constitute another example of a potential coordinated regulation between ET-1 and nitric oxide systems, as has been shown in bovine and human endothelial cells in diverse experimental conditions (24, 52, 53).

Endothelial dysfunction is a complex phenomenon, one of its main features being an impaired capacity of vascular rings to adequately dilate in response to pharmacological endothe-



eNOS protein expression

Control	Simvastatin	oxLDL	oxLDL + Simvastatin
100 ± 18 (3)	125 (2)	53 ± 10 (3)	83 (2)

% of control levels

Figure 9. Effects of Simvastatin and oxidized LDL on eNOS protein expression in BAEC. *Top*: Western blot analysis (10 μg total protein/lane) showing the eNOS protein levels after 24-h treatment with vehicle or 10 μg/ml oxLDL and in the absence or presence of 10 μM Simvastatin (*left*). Equal amount of protein loading per lane was determined by Coomassie staining of the nylon membrane after the blotting procedure (*right*). *Bottom*: values correspond to eNOS ECL band intensities relative to control levels. Data represent the mean of the indicated number of experiments (*in parenthesis*).

lial agonists. Its presence generally suggests an imbalance in the vasodilatory and vasoconstrictor forces regulating the delicate equilibrium governing physiological vascular tone. Disruption of this equilibrium is present both in vivo and in isolated atherogenic vessels, and largely attributed to alterations in the L-arginine-NO–cGMP pathway (7–9, 18, 54). Also, ET-1 might clearly contribute to perpetuate and amplify cellular responses during atherogenesis, through the interaction with multiple factors which are themselves initiators or maintainers of the arteriosclerotic lesion (10, 47, 55).

Besides their therapeutical role in the management of dyslipemias and atherosclerosis, statins have been proposed to possess additional beneficial clinical effects (19). Several clinical trials have demonstrated an improvement in vasomotor tone and coronary response to endothelial-dependent agonists in patients under statin treatment (56–58). In one study, treatment with statins for a 12-wk period improved coronary perfusion, suggesting that anatomical regression of the atheromatous plaque could not account for this change (59). Notwithstanding the limitations of in vitro studies, it is tempting to speculate that in a proatherogenic scenario, statins might be able to influence the expression of vasoactive factors shifting the balance from vasoconstriction to vasodilatation. These findings at the cellular level should lend some basis to undertake studies in in vivo models.

Note added in proof: While this work was in press, Laufs et al. have reported an upregulation of eNOS by HMG CoA reductase inhibitors in the presence of oxLDL: *Circulation.* 1998. 97:1129–1135.

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