Upregulation of Endothelial Nitric Oxide Synthase by HMG CoA Reductase Inhibitors

Ulrich Laufs, MD; Vito La Fata, BA; Jorge Plutzky, MD; James K. Liao, MD

***Background*—**Oxidized low-density lipoprotein (ox-LDL) causes endothelial dysfunction in part by decreasing the availability of endothelial nitric oxide (NO). Although HMG CoA reductase inhibitors restore endothelial function by reducing serum cholesterol levels, it is not known whether they can also directly upregulate endothelial NO synthase (ecNOS) activity.

***Methods and Results*—**Human saphenous vein endothelial cells were treated with ox-LDL (50 μg/mL thiobarbituric acid reactive substances 12 to 16 nmol/mg) in the presence of HMG CoA reductase inhibitors simvastatin and lovastatin. In a time-dependent manner, ox-LDL decreased ecNOS mRNA and protein levels (91±4% and 67±8% reduction after 72 hours, respectively). Both simvastatin (1 μmol/L) and lovastatin (10 μmol/L) upregulated ecNOS expression by 3.8-fold and 3.6-fold, respectively, and completely prevented its downregulation by ox-LDL. These effects of simvastatin on ecNOS expression correlated with changes in ecNOS activity. Although L-mevalonate alone did not affect ecNOS expression, cotreatment with L-mevalonate completely reversed ecNOS upregulation by simvastatin. Actinomycin D studies revealed that simvastatin stabilized ecNOS mRNA ($\tau_{1/2}$, 43 versus 35 hours). Nuclear run-on assays and transient transfection studies with a −1.6 kb ecNOS promoter construct showed that simvastatin did not affect ecNOS gene transcription.

***Conclusions*—**Inhibition of endothelial HMG CoA reductase upregulates ecNOS expression predominantly by posttranscriptional mechanisms. These findings suggest that HMG CoA reductase inhibitors may have beneficial effects in atherosclerosis beyond that attributed to the lowering of serum cholesterol by increasing ecNOS activity. **(*Circulation*. 1998;97:1129-1135.)**

Key Words: atherosclerosis ■ endothelium-derived factors ■ lipoproteins ■ genes

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Endothelial dysfunction is an early marker of atherosclerosis and is often defined as the impaired release or activity of endothelium-derived relaxing factor (EDRF).[1] NO or closely related molecules account for most of the activities of EDRF.[2] Recent studies suggest that a loss of endothelium-derived NO activity may contribute to the atherogenic process.[3] For example, endothelium-derived NO inhibits several components of the atherogenic process including monocyte adhesion to the endothelial surface,[4] platelet aggregation,[5] vascular smooth muscle cell proliferation,[6] and vasoconstriction.[7] In addition, NO can prevent oxidative modification of LDL, which is a major contributor to atherosclerosis, particularly in its oxidized form.[8] Thus endothelial dysfunction is not only a marker of atherosclerosis but also may serve as an important regulator of the atherogenic process.

Clinical trials with HMG CoA reductase inhibitors have shown that a reduction in serum cholesterol level is correlated with improved survival in patients with coronary artery disease.[9,10] In fact, one of the earliest recognizable benefits after treatment with HMG CoA reductase inhibitors is the restoration of endothelium-dependent relaxation.[11,12] Studies in animals and humans have indicated a strong correlation between elevated serum cholesterol levels and abnormal endothelium-dependent relaxation.[13,14] We have previously shown that ox-LDL inhibits EDRF release by downregulating the expression and activity of type III ecNOS.[15] Consequently, improvement in endothelial function by HMG CoA reductase inhibitors is often attributed to the reduction in serum cholesterol levels through inhibition of hepatic HMG CoA reductase. Indeed, a recent study demonstrated that a single treatment of LDL apheresis is sufficient to significantly improve endothelium-dependent relaxations in hypercholesterolemic humans.[16]

Although the mechanism by which HMG CoA reductase inhibitors restore endothelial function is primarily attributed to the inhibition of hepatic HMG CoA reductase and the subsequent lowering of serum cholesterol levels, little is known whether inhibition of endothelial HMG CoA reductase has additional beneficial effects on endothelial function. We hypothesize that an additional mechanism by which

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Selected Abbreviations and Acronyms

ecNOS = endothelial cell NO synthase
NO = nitric oxide
ox-LDL = oxidized LDL
TBARS = thiobarbituric acid reactive substances

HMG CoA reductase inhibitors can be beneficial in atherosclerosis is through their direct effects on the vascular wall. Thus the purpose of this study is to determine whether inhibition of endothelial HMG CoA reductase can upregulate and restore ecNOS expression in the presence of ox-LDL.

Methods

Materials

All standard culture reagents were obtained from JRH Bioscience. Unless indicated otherwise, all reagents were purchased from Sigma Chemical Co. [α-^{32}P]CTP (3000 Ci/mmol) was supplied by New England Nuclear. Purified human LDL was obtained from Calbiochem and Biomedical Technologies Inc. The level of endotoxin was determined by the chromogenic Limulus amebocyte assay. Simvastatin and lovastatin were obtained from Merck, Sharp, and Dohme, Inc. Because endothelial cells lack lactonases to process simvastatin and lovastatin to their active forms, these agents were chemically activated before their use as previously described.[17,18]

Cell Culture

Human saphenous vein endothelial cells were harvested from saphenous veins and cultured as described.[15] For transfection studies, bovine aortic endothelial cells of less than three passages were used. In all experiments, the endothelial cells were placed in 10% lipoprotein-deficient serum for 48 hours before treatment conditions. Endothelial cells were pretreated with actinomycin D (5 μg/mL) for 1 hour before treatment with ox-LDL and/or simvastatin. Cellular viability was determined by cell count, morphology, and Trypan blue exclusion.

Preparation of LDL

The LDL from a single donor was prepared by discontinuous ultracentrifugation of freshly isolated plasma according to the method of Chung et al.[19] The purity of the LDL samples was confirmed by SDS/polyacrylamide and cellulose acetate gel electrophoresis. Cholesterol and triglyceride contents were determined as previously described.[15] The LDL protein concentration was determined by the method of Lowry.[20] For comparison, commercially available LDL (Biomedical Technologies Inc and Calbiochem) were characterized and used in selected experiments. Oxidized LDL was prepared by exposing freshly isolated LDL to $CuSO_4$ (5 to 10 μmol/L) at 37°C for various durations (6 to 24 hours). The extent of LDL modification was expressed as nanomoles of malondialdehyde per milligram of LDL protein (TBARS).[21] Only mild to moderate ox-LDL with TBARS values between 12 and 16 nmol/mg LDL protein (ie, 3 to 4 nmol/mg LDL cholesterol) were used in this study.

Northern Blotting

Equal amounts of total RNA (10 to 20 μg/lane) were separated by 1% formaldehyde–agarose gel electrophoresis, and hybridization and washing were performed as described.[15] The full-length human endothelial ecNOS DNA[8] was labeled with random hexamer priming, [α-^{32}P]CTP (3000 Ci/mmol), and Klenow (Pharmacia). Loading conditions were determined by ethidium bromide staining of 28S ribosomal RNA on the nylon membranes.

Western Blotting

Cellular proteins were prepared and separated on SDS/PAGE as described.[15] Immunoblotting was performed with a murine monoclonal antibody to human ecNOS (1:400 dilution, Tansduction Laboratories, Lexington, Ky). Immunodetection was accomplished with a sheep anti-mouse secondary antibody (1:4000 dilution) and the enhanced chemiluminescence kit (Amersham Corp).

Assay for ecNOS Activity

The ecNOS activity was determined by a modified nitrite assay with freshly prepared 2,3-diaminonaphthalene (1.5 mmol/L DAN in 1 mol/L HCL) as previously described.[22,23] Fluorescence of 1-(H)-naphthotriazole was measured with excitation and emission wavelengths of 365 and 450 nm, respectively. Standard curves were constructed with known amounts of sodium nitrite. Nonspecific fluorescence was determined in the presence of N^{ω}-monomethyl-L-arginine (5 mmol/L).

Nuclear Run-on Assay

Confluent endothelial cells ($\approx 5\times 10^7$ cells) grown in lipoprotein-deficient serum were treated with simvastatin (1 μmol/L) or 95% O_2 for 24 hours. Nuclei were isolated and in vitro transcription was performed as previously described.[23] Equal amounts (1 μg) of full-length human ecNOS, β-tubulin (ATCC #37855), and pGEM-3z cDNA were vacuum-transferred onto nitrocellulose membranes with a slot blot apparatus (Schleicher & Schuell). The relative intensity of ecNOS band was determined as the ratio of ecNOS to β-tubulin intensity and for each corresponding set of experiments (ie, simvastatin or hyperoxia) was divided by the relative intensity of the control condition.

Transfection Assays

For transient transfections, bovine rather than human endothelial cells were used because of their higher transfection efficiency by the calcium-phosphate precipitation method (12% versus <4%).[24] We used the human ecNOS promoter construct F1.LUC, which contains a −1.6 kb 5′-upstream sequence linked to the luciferase reporter gene as described by Zhang et al.[25] Bovine endothelial cells (60% to 70% confluent) were cotransfected with 30 μg of the indicated constructs and CMV.β-Gal. Endothelial cells were placed in lipoprotein-deficient serum for 48 hours after transfection and treated with ox-LDL (50 μg/mL, TBARS 12.4 nmol/mg) in the presence of simvastatin (1 μmol/L) for an additional 24 hours. The luciferase and β-galactosidase activities were determined by chemiluminescence (Dual-Light, Tropix) with a Berthold L9501 luminometer.

Data Analysis

Band intensities were analyzed densitometrically by the National Institutes of Health Image program.[26] All values are expressed as mean±SEM compared with controls and among separate experiments. ANOVA and paired and unpaired Student's *t* tests were used to determine any significant changes in densitometric values, nitrite production, and promoter activities. A significant difference was taken for probability values <.05.

Results

Cell Culture

For all experimental conditions, there were no observable adverse effects of ox-LDL or HMG CoA reductase inhibitors on cellular morphology, cell number, immunofluorescent staining, and Trypan blue exclusion (>95%). Higher concentrations of ox-LDL (>100 μg/mL) with greater oxidative modification (ie, TBARS values of >30 nmol/mg) caused vacuolization and some detachment of endothelial cells after 24 hours. Neither simvastatin (0.01 to 0.1 μmol/L) nor lovastatin (10 μmol/L) produced any noticeable adverse effects on human endothelial cell for up to 96 hours. How-

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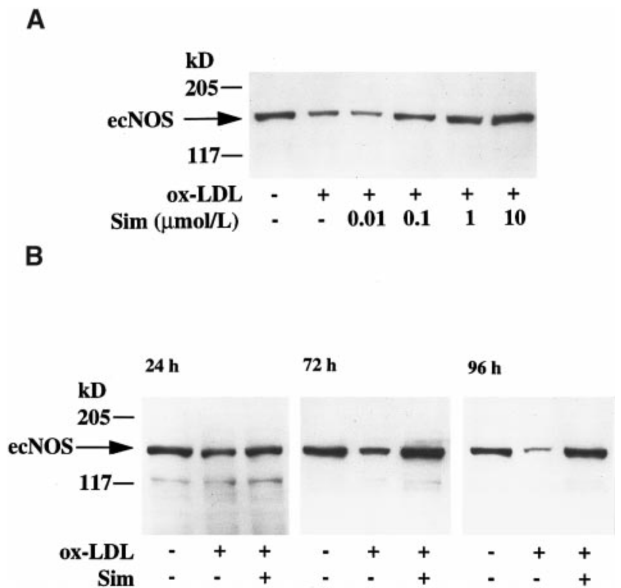

Figure 1. Western blots (40 μg protein/lane) showing the effects of ox-LDL (50 μg/mL, TBARS 12.2 nmol/mg) on ecNOS protein levels in the presence and absence of simvastatin (Sim). A, Concentration-dependent effects of simvastatin (0.01 to 10 μmol/L) at 48 hours. B, Time-dependent effects of simvastatin (0.1 μmol/L). Blots are representative of four separate experiments.

ever, higher concentrations of simvastatin (>15 μmol/L) or lovastatin (>50 μmol/L) caused cytotoxicity after 36 hours and therefore were not used.

Characterization of LDL

The LDL had a protein, cholesterol, and triglyceride concentration of 6.3±0.2, 2.5±0.1, and 0.5±0.1 mg/mL, respectively. In contrast, lipoprotein-deficient serum was devoid of both apolipoprotein B-100 protein and low-density lipid bands and had nondetectable levels of cholesterol. There was no detectable level of endotoxin (<0.10 EU/mL) in the lipoprotein-deficient serum or ox-LDL samples by the chromogenic Limulus amebocyte assay. In addition, there was no apparent difference between our own preparation and commercially obtained LDL samples in terms of electrophoretic mobility. Copper-oxidized LDL had TBARS values ranging from 4.6±0.5 to 33.1±5.2 nmol/mg. The degree of ox-LDL used in this study was mild to moderate, with TBARS value ranging from 12 to 16 nmol/mg LDL protein (ie, 3 to 4 nmol/mg LDL cholesterol).

Effect of ox-LDL and HMG CoA Reductase Inhibitors on ecNOS Protein

We have previously shown that ox-LDL (50 μg/mL) downregulates ecNOS expression.[15] Compared with untreated cells, treatment with ox-LDL (50 μg/mL, TBARS 12.2 nmol/mg) caused a 54±6% decrease in ecNOS protein after 48 hours (*P*<.01, n=4) (Fig 1A). There was no difference between our preparation of ox-LDL and commercially available ox-LDL with similar TBARS values in terms of the degree of ecNOS downregulation. Addition of simvastatin (0.01 μmol/L) did not significantly affect the downregulation of ecNOS protein by ox-LDL (57±8% decrease, *P*>.05, n=4). However, in the presence of 0.1 μmol/L of simvastatin, ox-LDL no longer produced any significant decrease in ecNOS protein levels (4±7% decrease, *P*<.01, n=4). Higher concentrations of simvastatin (1 and 10 μmol/L) resulted in not only a reversal of ecNOS downregulation by ox-LDL but also significant increases in ecNOS protein levels above baseline (146±9% and 210±12%, respectively, *P*<.05, n=4). Simvastatin or lovastatin that were not chemically activated had no effect on ecNOS expression (data not shown).

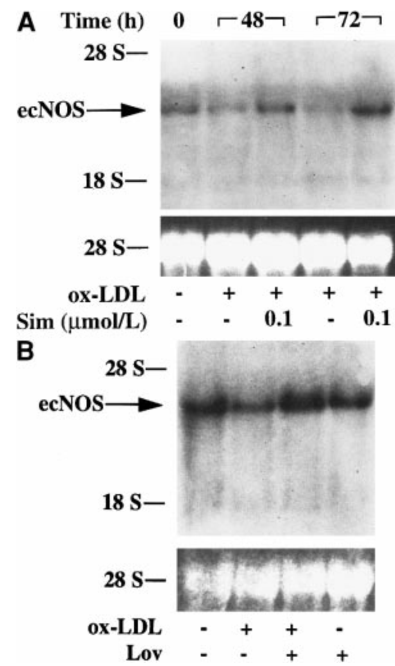

Figure 2. Northern blots (20 μg total RNA/lane) showing the effects of ox-LDL (50 μg/mL, TBARS 15.1 nmol/mg) on ecNOS mRNA levels in the presence and absence of HMG CoA reductase inhibitors. A, Time-dependent effects of simvastatin (Sim, 0.1 μmol/L). B, Effects of lovastatin (Lov, 10 μmol/L) after 24 hours. Each experiment was performed three times with comparable results. The corresponding ethidium bromide-stained 28S band intensities were used to standardize loading conditions.

In a time-dependent manner, treatment with ox-LDL (50 μg/mL, TBARS 12.2 nmol/mg) decreased ecNOS protein expression by 34±5%, 67±8%, and 86±5% after 24 hours, 72 hours, and 96 hours, respectively (*P*<.05 for all values, n=4,) (Fig 1B). Compared with ox-LDL alone, cotreatment with simvastatin (0.1 μmol/L) attenuated the decrease in ecNOS protein level after 24 hours (15±2% versus 34±5%, *P*<.05, n=4). Longer incubation with simvastatin (0.1 μmol/L) for 72 hours and 96 hours not only reversed the inhibitory effects of ox-LDL on ecNOS expression but also increased ecNOS protein levels by 110±6% and 124±6% above basal expression (*P*<.05, n=4). Thus compared with ox-LDL alone, cotreatment with simvastatin produced a 1.3-fold, 3.3-fold, and 8.9-fold increase in ecNOS protein levels after 24 hours, 72 hours, and 96 hours, respectively.

Effect of ox-LDL and HMG CoA Reductase Inhibitors on ecNOS mRNA

The effect of simvastatin on ecNOS mRNA levels occurred in a time-dependent manner and correlated with its effect on

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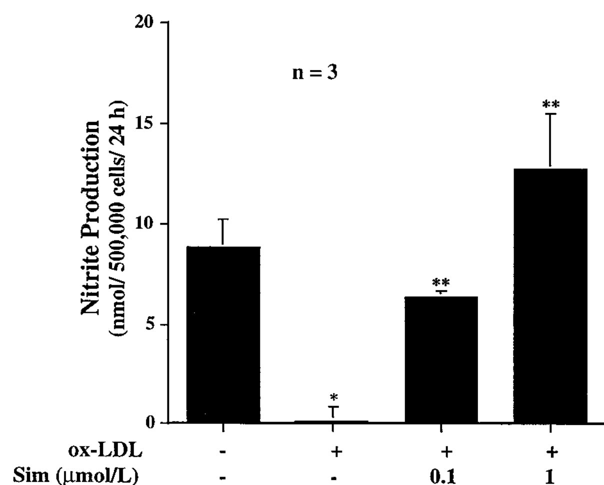

Figure 3. Effect of ox-LDL alone or in combination with the indicated concentrations of simvastatin (Sim) on LNMA-inhibitable nitrite production from human endothelial cells. Experiments were performed three times in duplicate. *$P<.05$ compared with control, **$P<.05$ compared with treatment with ox-LDL.

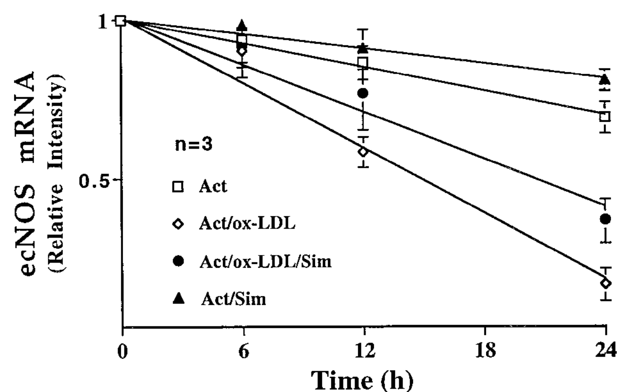

Figure 4. Densitometric analyses of Northern blots from actinomycin D (Act) studies showing the effects of ox-LDL (50 μg/mL, TBARS 12.2 nmol/mg) or simvastatin (Sim, 0.1 μmol/L), alone or in combination, on ecNOS mRNA levels. Band intensities of ecNOS mRNA (relative intensity) were plotted as a semi–log function of time (hours). Data points represent mean±SEM of three separate experiments.

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ecNOS protein levels (Fig 2A). Northern analyses showed that ox-LDL (50 μg/mL, TBARS 15.1 nmol/mg) produced a time-dependent 65±5% and 91±4% decrease in ecNOS mRNA levels after 48 hours and 72 hours, respectively ($P<.01$, n=3). Compared with ox-LDL at the indicated time points, cotreatment with simvastatin 0.1 μmol/L) increased ecNOS mRNA levels by 6.3-fold after 48 hours and 14.5-fold after 72 hours ($P<.01$ for all values, n=3).

To determine whether treatment with another HMG CoA reductase inhibitor has similar effects as simvastatin, we treated endothelial cells with lovastatin. Again, ox-LDL decreased steady-state ecNOS mRNA by 52±5% after 24 hours ($P<.01$, n=3) (Fig 2B). Treatment with lovastatin (10 μmol/L) not only reversed the inhibitory effects of ox-LDL on ecNOS mRNA but also caused a 40±9% increase in ecNOS mRNA level compared with that of untreated cells. Compared with ox-LDL alone, cotreatment with lovastatin caused a 3.6-fold increase in ecNOS mRNA levels after 24 hours. Treatment with lovastatin alone, however, produced 36% increase in ecNOS mRNA levels compared with untreated cells ($P<.05$, n=3).

Effect of ox-LDL and Simvastatin on ecNOS Activity

The activity of ecNOS was assessed by measuring the LNMA-inhibitable nitrite production from human endothelial cells.[23] Basal ecNOS activity was 8.8±1.4 nmol/500 000 cells/24 hours. Treatment with ox-LDL (50 μg/mL, TBARS 16 nmol/mg) for 48 hours decreased ecNOS-dependent nitrite production by 94±3% (0.6±0.5 nmol/500 000 cells/24 hours, $P<.001$) (Fig 3). Cotreatment with simvastatin (0.1 μmol/L) significantly attenuated this downregulation, resulting in a 28±3% decrease in ecNOS activity compared with untreated cells (6.4±0.3 nmol/500 000 cells/24 hours, $P<.05$). Cotreatment with a higher concentration of simvastatin (1 μmol/L) not only completely reversed the downregulation of ecNOS by ox-LDL but also resulted in a 45±6% increase in ecNOS activity compared with baseline (12.8±2.7 nmol/500 000 cells/24 hours, $P<.05$).

Effect of Simvastatin on ecNOS mRNA Stability

The posttranscriptional regulation of ecNOS mRNA was determined in the presence of the transcriptional inhibitor actinomy-

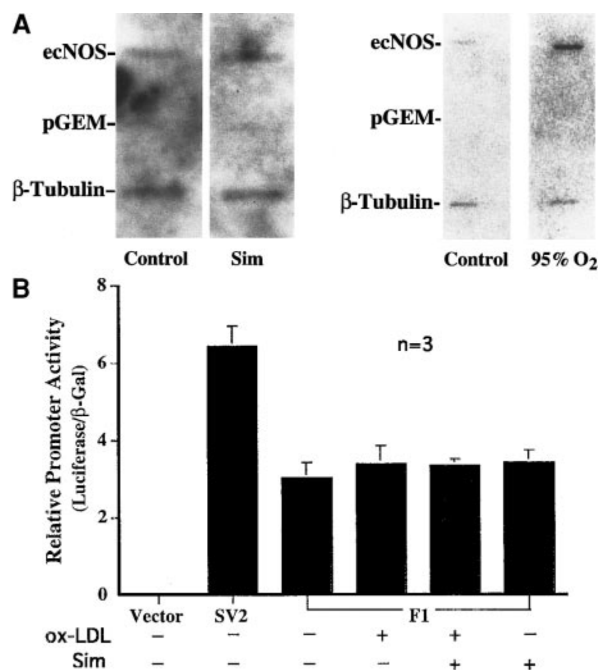

Figure 5. A, Nuclear run-on assay showing the effects of simvastatin (Sim, 1 μmol/L) or 95% O_2 on ecNOS gene transcription at 24 hours. The β-tubulin gene transcription and lack of pGEM band served as internal controls for standardization and nonspecific binding. Blots shown are representative of four separate experiments. B, Effects of ox-LDL (50 μg/mL, TBARS 15.1 nmol/mg) or simvastatin (1 μmol/L), alone or in combination, on ecNOS gene transcription. Bovine aortic endothelial cells were transiently-transfected with plasmid vectors containing no promoter (vector), the SV40 early promoter (SV_2), and the F1 ecNOS promoter construct. For control of transfection efficiency, F1 luciferase activity was standardized to the corresponding β-galactosidase activity (relative promoter activity).

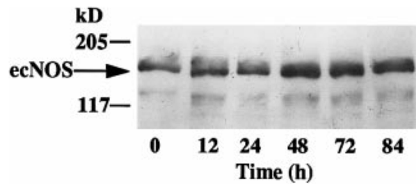

Figure 6. Western blots (40 μg protein/lane) showing the time-dependent effects of simvastatin (0.1 μmol/L) on ecNOS protein levels. The blot is representative of four separate experiments.

cin D (5 μg/mL) (Fig 4). Oxidized LDL (50 μg/mL, TBARS 13.1 nmol/mg) shortened the half-life of ecNOS mRNA ($\tau_{1/2}$, 35±3 to 14±2 hours, $P<.05$, n=3). Cotreatment with simvastatin (0.1 μmol/L) prolonged the half-life of ecNOS mRNA by 1.6-fold ($\tau_{1/2}$, 22±3 hours, $P<.05$, n=3). Treatment with simvastatin alone prolonged ecNOS mRNA half-life by 1.3-fold over baseline ($\tau_{1/2}$, 43±4 hours, $P<.05$, n=3).

Effect of Simvastatin on ecNOS Gene Transcription

To determine whether the effects of simvastatin on ecNOS expression occurs at the level of ecNOS gene transcription, we performed nuclear run-on assays using endothelial cells treated with simvastatin (1 μmol/L) for 24 hours (Fig 5A). Preliminary studies using different amounts of radiolabeled RNA transcripts demonstrate that under our experimental conditions, hybridization was linear and nonsaturable. The density of each ecNOS band was standardized to the density of its corresponding β-tubulin. The specificity of each band was determined by the lack of hybridization to the nonspecific pGEM cDNA vector. For studies with simvastatin, in untreated endothelial cells (control), there was constitutive ecNOS transcriptional activity (relative index of 1.0±0.2). Treatment with simvastatin (1 μmol/L) did not significantly affect ecNOS gene transcription compared with that of untreated cells (relative index of 1.2±0.3, $P>.05$, n=4). However, treatment of endothelial cells with hyperoxia (95% O_2) significantly increased ecNOS gene expression from control conditions (relative index of 2.6±0.5 versus 1.0±0.3, $P<.05$, n=4).

To further confirm the effects of simvastatin on ecNOS gene transcription by a different method, we transfected bovine aortic endothelial cells with a −1600 to +22 nucleotide ecNOS 5′-promoter construct linked to a luciferase reporter gene (F1).[25] This promoter construct contains putative *cis*-acting elements for activator protein (AP)-1 and -2, sterol regulatory element-1, retinoblastoma control element, shear stress response element (SSRE), nuclear factor-1 (NF-1), and cAMP response element (CRE). Treatment with ox-LDL (50 μg/mL, TBARS 14.5 nmol/mg) or simvastatin (1 μmol/L), alone or in combination, did not significantly affect basal F1 promoter activity (Fig 5B). However, laminar fluid shear-stress (12 dyne/cm^2 for 24 hours) was able to induce F1 promoter activity by 16-fold after 24 hours (data not shown), indicating that the F1 promoter construct is functionally responsive if presented with the appropriate stimulus.

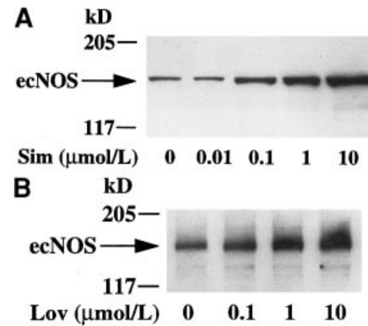

Figure 7. Western blots (40 μg protein/lane) showing the concentration-dependent effects of (A) simvastatin (Sim, 0.01 to 10 μmol/L) and (B) lovastatin (Lov, 0.1 to 10 μmol/L) on ecNOS protein levels after 48 hours. Blots are representative of three separate experiments.

Effect of Simvastatin and Lovastatin on ecNOS Expression

To further characterize the effects of HMG CoA reductase inhibitors on the upregulation ecNOS expression, we treated endothelial cells with simvastatin (0.1 μmol/L) for various durations (0 to 84 hours). Treatment with simvastatin (0.1 μmol/L) increased ecNOS protein levels by 4±6%, 21±9%, 80±8%, 90±12%, and 95±16% after 12 hours, 24 hours, 48 hours, 72 hours, and 84 hours, respectively ($P<.05$ for all time points after 12 hours, n=4) (Fig 6). Higher concentrations of simvastatin similarly increased ecNOS protein levels but in significantly less time compared with lower concentrations of simvastatin (data not shown).

In a concentration-dependent manner, treatment with simvastatin (0.01 to 10 μmol/L, 48 hours) increased ecNOS expression by 1±6%, 80±8%, 190±10%, and 310±20%, respectively ($P<.05$ for concentrations ≥0.1 μmol/L, n=4) (Fig 7A). The upregulation of ecNOS expression by simvastatin, therefore, is dependent on both the concentration and duration of simvastatin treatment. For comparison, treatment with lovastatin (0.1 to 10 μmol/L, 48 hours) also increased ecNOS expression in a concentration-dependent manner (10±6%, 105±8%, and 180±11%, respectively, $P<.05$ for concentrations >0.1 μmol/L, n=3) (Fig 7B) but significantly less effectively than simvastatin at comparable concentrations. Therefore at the same concentration, simvastatin had greater effects on ecNOS expression compared with lovastatin. These results are consistent with reported IC_{50} values for simvastatin and lovastatin.[27]

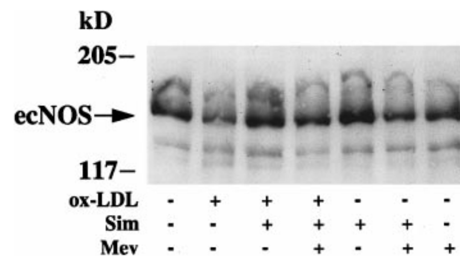

Figure 8. Western blots (40 μg protein/lane) showing the effects of ox-LDL (50 μg/mL, TBARS 15.1 nmol/mg), simvastatin (Sim, 1 μmol/L), or L-mevalonate (Mev, 0.1 μmol/L), alone or in combination, on ecNOS protein levels after 48 hours. Three separate experiments yielded similar results.

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Effect of L-Mevalonate on ecNOS Expression

To confirm that the effects of simvastatin on ecNOS expression were due to the inhibition of endothelial HMG CoA reductase, endothelial cells were treated with ox-LDL (50 μg/mL, TBARS 15.1 nmol/mg), or simvastatin (1 μmol/L), alone or in combination, in the presence of L-mevalonate (100 μmol/L) (Fig 8). Treatment with ox-LDL decreased ecNOS expression by 55%±6% after 48 hours, which was completely reversed and slightly upregulated in the presence of simvastatin (1 μmol/L) (150±8% above basal expression) ($P<.05$ for both, n=3).

Compared with endothelial cells treated with ox-LDL and simvastatin, addition of L-mevalonate reduced ecNOS protein by 50±5% ($P<.05$, n=3) (Fig 8). Furthermore, the upregulation of ecNOS expression by simvastatin alone (2.9-fold increase, $P<.05$, n=3) was completely reversed by cotreatment with L-mevalonate. Treatment with L-mevalonate alone did not have any appreciable effects on basal ecNOS expression ($P>.05$, n=3). Similar findings were also observed with L-mevalonate and lovastatin (data not shown).

Discussion

We have shown that inhibition of HMG CoA reductase in vascular endothelial cells upregulates the expression and activity of ecNOS and prevents their downregulation by ox-LDL. The inhibitory effects of simvastatin or lovastatin on endothelial HMG CoA reductase were concentration-dependent and specific since their effects on ecNOS corresponded to their respective IC_{50}s and could be bypassed and reversed with L-mevalonate.[27] The mechanisms by which HMG CoA reductase inhibitors increase ecNOS expression occurs through an increase in ecNOS mRNA stability. Our findings, therefore, provide important counterregulatory mechanisms by which HMG CoA reductase inhibitors can preserve ecNOS expression in the presence of ox-LDL. This novel effect of HMG CoA reductase inhibitors on ecNOS expression could contribute to the restoration of endothelial function beyond that achieved by reduction in serum cholesterol levels.

Although hyperoxic conditions (ie, 95% O_2) increase ecNOS gene transcription as we have previously reported,[23] we did not find any significant effects of simvastatin on ecNOS gene transcription. Furthermore, the effect of simvastatin on ecNOS mRNA stability was rather specific because simvastatin did not prolong the half-life of other constitutively expressed genes such as GAPDH and the G-protein α_s subunit (data not shown). The mechanism by which simvastatin upregulates ecNOS expression most likely occurs through inhibition of endothelial HMG CoA reductase because the effects of simvastatin on ecNOS expression were reversed in the presence of L-mevalonate. Interestingly, L-mevalonate alone did not produce any change in ecNOS expression, indicating that basal intracellular L-mevalonate levels may be sufficient to maximally inhibit ecNOS expression.

In our experimental design, the effects of HMG CoA reductase inhibitors on ecNOS expression were independent of extracellular cholesterol concentration because all of the cells were treated with the same concentration of ox-LDL. Furthermore, in contrast to in vivo studies, our study focuses on the inhibition of endothelial rather than hepatic HMG CoA reductase. Such direct beneficial effects of HMG CoA reductase inhibitor therapy on the vessel wall are supported by lipid-lowering studies showing that although similar levels of serum LDL reductions were achieved with HMG CoA reductase inhibitors and other modalities such as partial ileal loop bypass or treatment with cholestyramine, the clinical benefits were significantly higher with HMG CoA reductase inhibitors.[28,29] Furthermore, a recent study demonstrated that improvement of endothelial function after 4 weeks of simvastatin treatment did not correlate with significant decreases in serum cholesterol levels.[3] These observations suggest that the inhibition of endothelial as well as hepatic HMG CoA reductase can both contribute to the restoration of endothelial function in atherosclerosis.

The inhibitory effects of ox-LDL on ecNOS expression were directly related to the concentration and the degree of oxidative modification of the LDL particle.[15] The concentration of ox-LDL used in this study (ie, 50 μg/mL) was >200 times lower than the normal serum LDL cholesterol level and is comparable to ox-LDL concentrations used in previous studies.[8] Indeed, ox-LDL concentrations of <10 μg/mL also caused a decrease in ecNOS expression, albeit to a lesser extent than higher ox-LDL concentrations.[15] The degree of oxidative modification of LDL used in our study was mild to moderate in terms of TBARS values when compared with previous studies using minimally modified LDL.[8] In addition, the lower concentrations of simvastatin (0.01 to 1 μmol/L) used in this study are within range of the expected tissue levels derived from prescribed pharmacological dosages.[30,31] Consistent with the reported IC_{50} values for simvastatin and lovastatin, simvastatin upregulated ecNOS expression almost 10-fold higher than lovastatin at equimolar concentrations.[27]

In summary, we have identified an important mechanism by which HMG CoA reductase inhibitors could enhance endothelial NO production by directly upregulating ecNOS expression and activity. By reversing the inhibitory effects of ox-LDL on ecNOS expression, HMG CoA reductase inhibitors may increase the availability of endothelium-derived NO, which is known to mediate vasodilation, inhibit platelet aggregation and smooth muscle proliferation, and attenuate endothelium-leukocyte interactions.[4–8] It remains to be determined how L-mevalonate or its downstream lipid metabolites can lead to the stabilization of ecNOS mRNA.

Acknowledgments

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