3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibition Prevents Endothelial NO Synthase Downregulation by Atherogenic Levels of Native LDLs

Balance Between Transcriptional and Posttranscriptional Regulation

José Martínez-González, Berta Raposo, Cristina Rodríguez, Lina Badimon

Abstract—Atherogenic levels of native low density lipoproteins (nLDLs) decrease the bioavailability of endothelium-derived NO and downregulate endothelial NO synthase (eNOS) expression in cultured human endothelial cells. Here, we show that simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, within the therapeutic range (0.01 to 1 μmol/L) prevented the downregulation of eNOS mRNA and protein promoted by nLDL (180 mg cholesterol/dL, 48 hours) in human umbilical vein endothelial cells. This effect of simvastatin was completely reversed by mevalonate, the product of the reaction, and to a lesser extent by farnesol and geranyl geraniol. Simvastatin significantly stabilized eNOS mRNA in cells treated with nLDL during 48 hours (eNOS mRNA half-life = 11 hours in controls versus >24 hours in nLDL per 0.1 μmol/L simvastatin–treated cells). The downregulation of eNOS by nLDL was abrogated by cycloheximide, an inhibitor of protein synthesis, and by N-acetyl-leucyl-leucyl-norleucinal, a protease inhibitor that reduces the catabolism of sterol regulatory element binding proteins. Sterol deprivation increased the downregulation produced by nLDL on eNOS and sterol regulatory element binding protein-2 expression levels. However, no differential modulation of the retardation bands corresponding to the putative sterol-responsive element present in the eNOS promoter was detected by electrophoretic mobility shift assay. Our results suggest that nLDL promote eNOS downregulation operating at a transcriptional level, whereas simvastatin prevents such an effect through a posttranscriptional mechanism. (Arterioscler Thromb Vasc Biol. 2001;21:804-809.)

Key Words: endothelial cells ■ low density lipoproteins ■ NO synthase ■ HMG-CoA reductase inhibitors ■ simvastatin

Hypercholesterolemia produces endothelial dysfunction and early impairment of endothelium-dependent vasodilation associated with a reduced bioavailability of NO.1 Conversely, one of the earliest benefits of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), drugs that reduce cardiac morbidity and mortality,2,3 is the improvement of endothelium-dependent relaxation.4 Because NO is involved in virtually all endothelium-dependent protective effects,5 statins could indirectly modulate all these events through a regulation of NO production.

Cholesterol lowering could contribute to the improvement of endothelium-dependent vasodilatation. It has been suggested that treatment of elevated plasma cholesterol levels reduces the oxidative stress within the vessel wall, subsequently preventing inactivation of NO by free radicals.6 In addition, the NO-dependent improvement in forearm blood flow observed in fluvastatin-treated patients was weakly but significant related to a reduction in the level of plasma LDL cholesterol.7 However, the restoration of endothelial function sometimes occurs before significant reduction in serum cholesterol levels.4,8 In addition, Williams et al9 have shown that the response to acetylcholine of coronary arteries from pravastatin-treated monkeys was better than that of untreated animals with similar plasma cholesterol levels. Recent evidences suggest a direct effect of statins on endothelial NO synthase (eNOS), the key enzyme in the biosynthesis of NO in endothelial cells.10–12 Thus, statins have the ability to increase NO bioavailability, an effect that appears to be mediated by an indirect effect related to the reduction of LDL cholesterol level per se and by a direct effect on NO production.

Recently, we have shown that atherogenic levels of native LDL (nLDL, >160 mg/dL) downregulate eNOS mRNA and protein expression.13,14 These results emphasize the role that nLDL could play in the early NO deficit in hypercholesterolemic patients. Our present results indicate that simvastatin, an HMG-CoA reductase inhibitor, at concentrations within the range of those used in clinical practice, prevent the downregulation of eNOS mRNA and protein levels produced by atherogenic levels of nLDL (180 mg cholesterol/dL). In

Received November 27, 2000; revision accepted December 22, 2000.
From the Cardiovascular Research Center, IIBB/CSIC-Institut de Recerca del Hospital de la Santa Creu i Sant Pau-UAB, Barcelona, Spain.
Correspondence to Prof Lina Badimon, Laboratorio de Investigación Cardiovascular, Hospital de la Santa Creu i Sant Pau, Avda. Sant Antonio Maria Claret #167, 08025 Barcelona, Spain. E-mail lbmucv@cid.csic.es

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addition, we show that nLDLs promote eNOS downregulation operating at a transcriptional level, whereas simvastatin is able to compensate such an effect through a posttranscriptional mechanism.

Methods

Isolation and Characterization of LDLs

Human LDLs from pooled plasma of normocholesterolemic donors of the Barcelona area were obtained and characterized as described previously.\textsuperscript{13,14} The thiobarbituric acid–reactive substance (TBARS) content of LDL was used as an indirect evaluation of lipid peroxidation. TBARS levels, determined as described previously,\textsuperscript{13,14} were <1.0 nmol malonaldehyde (MDA) per milligram of LDL protein. LDL did not contain detectable levels of endotoxin (<0.10 U/mL) by the chromogenic limulus amebocyte assay. nLDLs used in the experiments were <48 hours old. Oxidized LDLs (oxLDLs) were prepared by exposing freshly isolated nLDLs to 10\textsuperscript{–6} mol/L hydrogen peroxide. nLDLs (10 mg/mL) were incubated with 10\textsuperscript{–6} mol/L 1,10-phenanthroline, and 10\textsuperscript{–3} mol/L LDL did not contain detectable levels of endotoxin (<0.10 U/mL) before treatment.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were extracted by collagenase digestion and were characterized as described.\textsuperscript{13,14} Cells were cultured in medium 199 (GIBCO) supplemented with 20 mmol/L HEPES, pH 7.4 (GIBCO), 30 μg/mL endothelial cell growth supplement (Sigma), 100 μg/mL heparin (Sigma), 20% FCS (Biological Industries), and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). HUVECs were used between passages 2 and 90%. Before treatment, HUVECs were exposed to increasing concentrations of simvastatin (20 μg/mL heparin (Sigma), 20% FCS (Biological Industries), and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). HUVECs were used between passages 2 and 90% before treatment. Human LDLs from pooled plasma of normocholesterolemic donors of the Barcelona area were obtained and characterized as described previously.\textsuperscript{13,14} The thiobarbituric acid–reactive substance (TBARS) content of LDL was used as an indirect evaluation of lipid peroxidation. TBARS levels, determined as described previously,\textsuperscript{13,14} were <1.0 nmol malonaldehyde (MDA) per milligram of LDL protein.

Dynamics). To determine the effect of nLDL and simvastatin on eNOS mRNA half-life, 5,6-dichlorobenzimidazole (DRB) was used as an inhibitor of mRNA synthesis.

Analysis of SREBP-2 mRNA levels by RT-PCR

Total RNA (1 μg) from HUVECs treated with atorvastatin of LDL and control cells, in either 20% FCS or 10% lipoprotein-deficient serum (LPDS), was reverse-transcribed.\textsuperscript{15} SREBP-1, SREBP-2, and GAPDH (used to normalize results) mRNA levels were analyzed by reverse transcriptase (RT)–polymerase chain reaction (PCR). PCR products were labeled with digoxigenin.\textsuperscript{11} The oligonucleotides used were as follows: SREBP-1 (5′-ATGTAATGC- GATGCGCGCCG-3′ and 5′-TGGATCCTCGGACATCAG-3′), SREBP-2 (5′-TGGGACATTCTGACCAAA-3′ and 5′-GCCAGGAGGAAGTCG-3′), and GAPDH (5′-TTGACACCA-TGGAGAAGGG-3′ and 5′-GCAGGTGATGTTCTGCGG-3′). Amplification was carried out by 19 (GAPDH), 27 (SREBP-1), and 21 (SREBP-2) cycles of 94°C for 1 minute, 61°C for 1 minute, and 72°C for 2 minutes. PCR products were resolved by electrophoresis in agarose gels, transferred onto nylon membranes, and UV–cross-linked. Finally, digoxigenin-labeled products were detected by antidigoxigenin antibody linked to alkaline phosphatase.\textsuperscript{11}

Electrophoretic Mobility Shift Assay

A double-stranded probe (−1393 to −1374) containing the putative sterol-responsive element (SRE)-1 present in the eNOS promoter was used as a probe in electrophoretic mobility shift assay (EMSA) analysis. Nuclear extracts (10 μg) from HUVECs, obtained as previously described,\textsuperscript{16} were incubated for 15 minutes on ice in a final volume of 20 μL with 1 μg of poly(dI-dC) in 25 mmol/L Tris-HCl (pH 8), 4 mmol/L MgCl\textsubscript{2}, 5% glycerol, 0.5 mmol/L dithiothreitol, 0.5 mmol/L EDTA, and 60 mmol/L KCl. Then 30 000 cpm of probe, labeled with [γ\textsuperscript{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase, was added, and incubation proceeded for an additional 30 minutes. DNA-protein complexes were resolved on a 5% polyacrylamide gels at 4°C in 0.5× Tris-borate-EDTA buffer. Free probe and shifted bands were detected by autoradiography.

Other Methods

To assess the possible cytotoxic effect of LDL and simvastatin treatment, lactate dehydrogenase activity in the media from LDL-treated cells was determined as previously described.\textsuperscript{11} Cell viability was analyzed by trypan blue exclusion test.

Internucleosomal DNA fragmentation was evaluated as an index of cell apoptosis. Total DNA was extracted, with use of the Kristal kit (Cambridge Molecular Technology), and was fractionated on 1% agarose/Tris-acetate-EDTA/ethidium bromide gels.

Statistical Analysis

Results are expressed as mean±SEM, unless otherwise stated. A Statview II (Abacus Concepts) statistical package for the Macintosh computer system was used for all the analyses. Multiple groups were compared by 1-factor ANOVA, followed by Fisher PLSD and Scheffé F test to assess specific group differences. Differences between any 2 groups were evaluated by the 2-tailed t test.

Effect of Simvastatin on eNOS Protein Levels

HUVEC cultures were incubated with atorvastatin of LDL concentrations (180 mg cholesterol/dL) and increasing concentrations of simvastatin (0.01 to 1 μmol/L) for 48 hours. The incubation time and atherogenic nLDL concentration for the present study were selected according to previous results.\textsuperscript{13} A significant downregulation of eNOS protein levels was observed in nLDL-treated cells (Figure 1A and 1B). Simvastatin prevents, in a dose-dependent manner, the downregulation of eNOS protein produced by atherogenic levels of nLDL. The effect was evident at simvastatin concentrations as low as 0.01 to 0.1 μmol/L. The iNOS isoform was undetectable by Western blot analysis, and the expression pattern of Cox-1, the main regulatory isoform of prosta-
cyclin biosynthesis, was not modified by either nLDL or simvastatin treatment (Figure 1C). No significant differences in TBARS values in supernatants of the cells incubated with nLDL or nLDL/simvastatin were observed (data not shown). Interestingly, under our experimental conditions, simvastatin alone did not affect eNOS protein levels, but it induced eNOS protein levels in cells incubated in LPDS (Figure 1D). Neither nLDL nor simvastatin produced a significant effect on cell toxicity (analyzed by cell morphology, trypan blue exclusion, and lactate dehydrogenase activity) or apoptotic cell death. The typical apoptotic telltale ladder pattern was not observed in any treatment (Figure 1E).

Dependence of eNOS expression of Mevalonate Derivatives

The effect of simvastatin was completely reversed by mevalonate (200 μmol/L), the product of the enzymatic reaction inhibited by simvastatin, showing that the effect of simvastatin was specifically derived from the inhibition of HMG-CoA reductase activity (Figure 2A). The addition of farnesol (10 μmol/L) and geranyl geraniol (10 μmol/L) produced only a partial effect. In the absence of simvastatin, the effect of these products on eNOS protein levels was negligible. Isoprenylation is essential for the posttranslational modification of a variety of proteins.21 Because farnesol partially reversed the effect of simvastatin on eNOS mRNA levels, we studied Ras, a farnesylated protein, to analyze whether the simvastatin doses used in the present study could affect the isoprenylation of HUVEC proteins. As Figure 2B shows, 0.1 μmol/L simvastatin significantly inhibited Ras posttranslational processing, shown by the appearance of a slower migrating band corresponding to nonprenylated unprocessed Ras molecules. Normal Ras processing was restored by RS-mevalonic acid or farnesol but not by geranyl geraniol.

Effect of Simvastatin on eNOS mRNA Levels

Northern blot analyses were performed to determine whether eNOS mRNA levels were regulated by simvastatin. nLDL (180 mg cholesterol/dL, 48 hours) produced a decrease in eNOS mRNA levels similar to that produced by highly oxidized LDLs (50 μg/mL, 48 hours; Figure 3). Simvastatin prevented, in a dose-dependent manner, the downregulation of eNOS mRNA levels produced by atherogenic levels of nLDL, and mevalonate abrogated such an effect (Figure 3). Simvastatin alone did not produce a significant effect on eNOS mRNA levels after 48 hours of treatment. Stripped membranes were reprobed by using a ribosomal cDNA as a loading control and a murine iNOS cDNA. In concordance with Western experiments, no iNOS mRNA was detected.

Figure 1. Dose-response effect of simvastatin. HUVECs were incubated with nLDL (180 mg cholesterol/dL) in the presence of increasing concentrations of simvastatin (sim). Total protein was extracted from cell monolayers and analyzed by Western blot (30 μg per lane). Membranes were sequentially incubated with eNOS and Cox-1 antibodies. A, Representative blot corresponding to eNOS. B, Bar graph showing the effect of sim on eNOS protein levels (relative to controls). Values represent mean±SEM of 5 independent experiments performed in duplicate. *P<0.05 vs control cells; #P<0.05 vs nLDL-treated cells. C, Representative blot corresponding to Cox-1. D, Effect of increasing concentrations of sim alone on eNOS protein levels in cells incubated in LPDS (10%). E, Effect of nLDL and sim (μmol/L) on apoptotic cell death. Ethidium bromide staining of a representative experiment (n=3) is shown. M indicates molecular weight markers.

Figure 2. Effect of mevalonate derivatives on eNOS protein levels and Ras processing. HUVECs were incubated with nLDL (180 mg cholesterol/dL) in the presence of 0.1 μmol/L sim and 200 μmol/L mevalonate (MEV), 10 μmol/L farnesol (FAR), or 10 μmol/L geranyl geraniol (GER). Total protein was extracted from cell monolayers and analyzed by Western blot (30 μg per lane). Membranes were sequentially incubated with eNOS and Ras antibodies. A, Representative blot (n=3) corresponding to eNOS. B, Representative blot corresponding to Ras. P indicates processed; U, unprocessed.
Effect of Simvastatin on eNOS mRNA Half-Life

The effect of simvastatin on eNOS mRNA stability was assessed. HUVECs were incubated with nLDL (180 mg cholesterol/dL), simvastatin alone (0.1 μmol/L), or nLDL plus simvastatin or without any treatment (control) for 48 hours. Then, DRB (50 μmol/L) was added to inhibit further transcription, and total RNA was isolated and examined by Northern blot at various time periods (8, 16, and 24 hours). The decay of eNOS mRNA from control cells and from cells incubated with nLDL or simvastatin alone was similar (Figure 4). In contrast, simvastatin significantly increased eNOS mRNA half-life in cells incubated with nLDL (≈11 hours in control cells versus >24 hours in nLDL/simvastatin-treated cells).

Analysis of the Mechanism Involved in eNOS Downregulation by nLDL

Because nLDL did not reduce eNOS mRNA half-life, a transcriptional regulation seemed to be taking place. To better characterize the effect of nLDL on eNOS expression, the effect of cycloheximide, a protein synthesis inhibitor, was analyzed. In agreement with previous results,22 cycloheximide (1 μg/mL) produced an increase in eNOS mRNA expression, suggesting that eNOS is negatively regulated under basal conditions (Figure 5A). Cycloheximide also abolished the downregulation of eNOS mRNA levels produced by nLDL, suggesting that the effect of LDL requires the participation of newly synthesized protein(s). Because SREBPs have been involved in the regulation of several genes by LDL,23 we analyzed the effect of ALLN, an inhibitor of neutral cysteine protease that reduces catabolism of the soluble fragment SREBP.16 ALLN abolished the eNOS protein downregulation induced by nLDL in a dose-dependent manner (Figure 5B).

The downregulation of eNOS by nLDL was also observed in adult vessel (porcine aortic) endothelial cells and was better evidenced when the cells were deprived of lipoproteins (incubated with LPDS, Figure 5C). nLDL induced the downregulation of SREBP-2 mRNA levels in parallel with eNOS downregulation in cells incubated with FCS or LPDS (Figure 5D). In these conditions, SREBP-1 mRNA levels did not change (data not shown).

EMSA analyses detected specific DNA-protein complexes between nuclear extracts from HUVECs and probes containing the putative SRE in the eNOS promoter (Figure 6). However, the retardation band was not differentially modulated by atherogenic nLDL levels.

Discussion

Recently, we provided biochemical and functional evidence that atherogenic levels of nLDL impair NO release by endothelial cells through a downregulation of eNOS mRNA and protein levels.13,14 In the present study, we show that nLDLs downregulate eNOS mRNA levels acting at a transcriptional level, whereas simvastatin prevents nLDL-
**Figure 6.** Analysis of the putative SRE-1 of the eNOS promoter by EMSA. NE indicates nuclear extracts. Representative EMSA performed with a 32P-end-labeled probe containing this element and nuclear extracts (+NE, 10 μg; −NE, without nuclear extracts) from HUVECs incubated with or without nLDL (180 mg/dL, 24 hours). A, Competition with the unlabeled probe (1:20). B, Nuclear extracts incubated with the mentioned probe and in parallel with an unrelated Oct-1 probe used to normalize results.17

induced eNOS downregulation posttranscriptionally by stabilizing eNOS mRNA.

Inhibition of HMG-CoA reductase with simvastatin (at concentrations as low as 0.01 to 0.1 μmol/L, similar to those used clinically) prevented nLDL-induced downregulation of eNOS through an increase in eNOS mRNA and protein levels. Because a coordinate regulation between the NO and cyclooxygenase pathways has been observed, we analyzed the effect of nLDL/simvastatin on Cox-1, the main regulatory isoenzyme of prostaglandin I2 biosynthesis. In the present study, Cox-1 protein expression pattern was neither affected by nLDL nor by treatment with simvastatin. Thus, the mevalonate pathway specifically regulates eNOS. The effect of simvastatin was specifically reversed by mevalonate and, to a lesser extent, by farnesol and geranyl geraniol. Interestingly, none of these products alone reversed the eNOS downregulation induced by nLDL through an increase in eNOS mRNA half-life. A similar mechanism has been reported for the effects of statins on oxLDL-induced eNOS downregulation.11 These authors showed that geranylated proteins, in particular Rho, are involved in such an effect. Rho could act as a negative regulator of eNOS by decreasing eNOS mRNA translation and stability via effects on the cytoskeletal location of eNOS mRNA.29 However, our results show that geranyl geraniol only mildly reduced the simvastatin-induced increase of eNOS mRNA levels downregulated by nLDL. It is noteworthy that oxLDLs decrease eNOS stability (whereas nLDLs do not) and that the increase of eNOS mRNA half-life produced by simvastatin with nLDLs is higher than that observed with oxLDLs.30 A sequence involved in transcript destabilization has been described in the 3′-untranslated region of eNOS mRNA,11 and the stabilization of eNOS mRNA by simvastatin has been related to an increase in the binding of certain cytoplasmic proteins that recognize a cytidine-rich region within the 3′-untranslated region of eNOS mRNA.31

Our experiments with DRB indicate that nLDLs do not modify mRNA half-life. These results, together with the results of incubations with cycloheximide, in which the nLDL effect was completely abrogated when protein synthesis was inhibited, strongly suggest that the nLDL effects on eNOS regulation work at a transcriptional level. Several DNA regulatory binding sequences have been described in the 5′-flanking region of the human eNOS, including SRE-1.20 This element could mediate the activation of eNOS gene transcription on the binding of SREBPs. We observed that ALLN (inhibitor of neutral cysteine proteases), which reduces the catabolism of the soluble fragment (active form) of SREBP,16 abolishes the effects of nLDL. Because free cholesterol inhibits the cleavage of SREBPs, the increase in eNOS protein levels by ALLN suggests an active role for SREBPs on the regulation of eNOS gene transcription by atherogenic concentrations of nLDL. The downregulation of eNOS by nLDL was parallel to a decrease of SREBP-2 mRNA levels, and the effect was enhanced in both genes when the cells were deprived of sterols. EMSA analyses detected specific DNA-protein complexes between nuclear extracts from HUVECs and probes containing the putative SRE present in the eNOS promoter; however, the retardation bands were not differentially modulated by nLDL. SREBPs, mainly SREBP-2, are sensitive sensors of cell sterol levels. In fact, they act quickly when a unbalance in cell sterol is detected,32 and SRE functions in many genes as a conditionally positive element, activating expression only when sterol levels are low. These SRE/SREBP characteristics, the long incubation times required to observe the downregulation of eNOS by nLDL, and the apparent absence of modulation of the SRE-1 present in the eNOS promoter suggest that SREBP binding to this element would not be sufficient to downregulate eNOS transcription. The downregulation of eNOS by nLDL could involve additional regulatory proteins; in fact, this regulation was abrogated when de novo protein synthesis was inhibited. Taken together, these results suggest that eNOS downregulation by nLDL could be the result of the coordinate actions of several regulatory proteins. In this regard, we have found that the eNOS SRE-1 is flanked by putative Yin Yang-1 (YY1)-like binding sequences. YY1 is a zing finger protein that possesses the unusual property of regulating transcription in 3 ways (initiation, activation, and repression), depending on the...
gene contexts. Recently, YY1 binding sites have been identified as negative regulators of transcription of SREBP-responsive genes, and recombinant YY1 seems to bind to the eNOS promoter. Thus, one may hypothesize a role for this protein in the modulation of eNOS by LDL. In fact, Karantouzis-Fegaras et al., by trans-factor binding and functional expression studies, revealed a surprising degree of cooperativity and complexity of the eNOS promoter. However, more experiments are needed to elucidate the specific cis elements involved in the downregulation of eNOS by sterols.

In summary, evidence supports a crucial role for nLDL in hypercholesterolemia-related endothelial dysfunction, which is strongly linked to the decrease of NO production by endothelium. Atherogenic nLDL levels could downregulate eNOS acting at transcriptional level. In this context, the inhibition of HMG-CoA reductase by simvastatin, at doses used in clinical practice, abolishes the deleterious effect of nLDL. Statins, acting at a posttranscriptional level, increase eNOS mRNA half-life, providing a compensatory mechanism that might balance the NO-dependent endothelial functions impaired by hypercholesterolemia. Elucidation of the specific proteins involved in the regulation of eNOS at transcriptional and posttranscriptional levels will shed more light on the role of the mevalonate pathway in lipid-mediated endothelial dysfunction.

Acknowledgments
This study has been possible thanks to funds partially provided by Fondo de Investigación Sanitaria (FIS) 98-715, FIS 98/0641, Merck Sharp & Dohme, Spain, and Catalana-Occidente. We thank Dr de Castellarnau and Olga Bell for their technical assistance. Berta Raposo is a predoctoral fellow of the Fundación de Investigación Cardiovascular.

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doi: 10.1161/01.ATV.21.5.804
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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