Regulation of Endothelial Nitric Oxide Synthase Expression in the Vascular Wall and in Mononuclear Cells From Hypercholesterolemic Rabbits

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Background—We recently obtained evidence demonstrating that cultured bovine endothelial cells contain cytosolic proteins that form complexes with the 3′-untranslated region of endothelial nitric oxide synthase (eNOS) mRNA and are associated with its destabilization. The aim of this study was to determine the presence of such proteins and eNOS expression in hypercholesterolemic rabbits as an in vivo model of endothelial dysfunction.

Methods and Results—Endothelium-dependent relaxation to acetylcholine and the calcium ionophore A23187 was reduced in aortic segments from hypercholesterolemic rabbits compared with controls. Treatment of hypercholesterolemic rabbits with cerivastatin (0.1 mg · kg body wt⁻¹ · d⁻¹) restored endothelium-dependent relaxation. Aortic eNOS expression was reduced in hypercholesterolemic rabbits and was accompanied by enhanced binding activity of a 60-kDa cytosolic protein and reduced stability of eNOS mRNA. Cerivastatin treatment upregulated eNOS expression and reduced the interaction of the cytosolic protein with the 3′-untranslated region of eNOS mRNA. Mononuclear cells from hypercholesterolemic rabbits also showed a marked reduction of eNOS expression and eNOS mRNA stability and an increase in binding activity of the cytosolic protein, which were also prevented by cerivastatin treatment.

Conclusions—These results demonstrate the presence of a 60-kDa protein that binds to eNOS mRNA and reductions in eNOS expression in both vascular wall and mononuclear cells that are prevented by cerivastatin. (Circulation. 2001; 104:1822-1830.)

Key Words: hypercholesterolemia ■ endothelium ■ leukocytes ■ nitric oxide synthase

Nitric oxide (NO) is generated in endothelial cells by endothelial NO synthase (eNOS)¹² and is responsible for the endothelium-dependent vasorelaxation, which is reduced in arteries from hypercholesterolemic animals and humans.⁴,⁵

Although eNOS was initially considered to be constitutive, it was later demonstrated that cytokines and oxidized LDLs downregulate eNOS expression by destabilizing eNOS mRNA.⁶,⁷ In vitro evidence shows that HMG-CoA reductase inhibitors reverse this downregulation under cholesterol-clamped conditions by stabilizing eNOS mRNA via unknown mechanisms.⁸

Sequences controlling mRNA stability have been identified within its 3′-untranslated region (3′-UTR).⁹ We recently demonstrated that cultured bovine endothelial cells contain cytosolic proteins that form complexes with the 3′-UTR of eNOS mRNA¹⁰ and cause its destabilization,¹⁰ thus potentially leading to endothelial dysfunction. Whether the level of these proteins is modified in in vivo situations with demonstrated endothelial dysfunction, ie, hypercholesterolemia, however, is not established.

In this study, we have determined the presence of such proteins and eNOS expression in arteries of hypercholesterolemic rabbits. Because statins prevent endothelial dysfunction and protect eNOS expression in cultured endothelium,⁸,¹¹ we used cerivastatin, a newly synthesized statin,¹² to analyze its actions on these parameters.

Mononuclear cells also produce NO by an eNOS-like NOS,¹³ thus opening the possibility that changes in eNOS could be correlated with modifications in eNOS expression in mononuclear cells.

Methods

Animal Procedures
The protocol was approved by the Institutional Ethics Committee for animals and performed according to international conventions on animal experimentation.
Male New Zealand White rabbits (2.5 ± 0.3 kg) were housed individually at 20 ± 3°C with a 12-hour light/dark cycle and free access to water. Fifteen control rabbits were fed with regular rabbit chow for 6 weeks, and 45 rabbits were fed a diet containing 0.5% cholesterol plus 6% coconut oil for 3 weeks (UAR). After week 3, cholesterol-fed rabbits were randomly divided into 3 groups of 15 rabbits: (1) rabbits fed the hypercholesterolemic diet for 3 additional weeks, (2) rabbits fed the hypercholesterolemic diet plus cerivastatin (0.1 mg · kg body wt −1 · d −1) administered with the diet for 3 additional weeks, and (3) rabbits fed with standard chow for 3 additional weeks. Feeding was restricted to 120 g/d.

Next, the rabbits were anesthetized with pentobarbital (30 mg/kg IM), and the aorta, from the aortic arch to the diaphragm, was removed. Blood (40 mL) was collected from the central ear artery for mononuclear cell isolation just before the rabbits were anesthetized.

### Isometric Tension Measurement

Aortic segments were cut into 3 portions. One portion (from the aortic arch to the second intercostal arteries) was immediately frozen in liquid nitrogen for molecular biology determinations. The remaining 2 segments (from the second intercostal arteries to the celiac artery) were suspended in an organ bath gassed with 95% O2/5% CO2 containing Krebs-Henseleit solution (in mmol/L: NaCl 115, KCl 4.6, KH2PO4 1.2, MgSO4 1.2, CaCl2 2.5, NaHCO3 25, glucose 11.1, and calcium-sodium EDTA 0.02, pH 7.4) and connected to isometric force displacement transducers coupled to a computer system (Power Laboratory 400, AD Instruments), as reported.14 Endothelium-dependent relaxation to acetylcholine (ACh) and endothelium-independent relaxation to sodium nitroprusside were tested on arteries precontracted with 10 −5 mol/L phenylephrine as reported.14 Dose-response curves were determined with the p[NOS]-UTR-S by PCR as described.10,18 Oligo 1 (5′-GTGGATCCCTGGCTACTATCCCTACCC-3′) and oligo 2 (5′-AGGAGCTTTAGTGTCCTCTACATTCTG-3′) were used to produce plasmid NOS (pNOS)-UTR-L by reverse transcription-polymerase chain reaction (PCR) (from bovine aortic endothelial cell total RNA). UTR-L covers a fragment of the coding region (214 bases) and 3′-UTR (448 bases) of eNOS cDNA.

Oligonucleotides complementary to bovine eNOS cDNA (GenBank accession number BT030821) were purchased from Biosynthesis Inc. eNOS-UTR plasmids were made as previously reported.10,18 Oligonucleotide probes were UV-irradiated in ice in a Stratalinker (Stratagene Ltd) for 20 minutes of film exposure and evaluated by densitometry (Molecular Labs).

### Mononuclear Cell Isolation

Mononuclear cells were isolated and manipulated under sterile conditions. Cells were isolated by Ficoll-Hypaque centrifugation and immediately frozen at −80°C for molecular biology determinations.

### Determination of eNOS Expression

eNOS expression was analyzed by Western blot as described.15 In brief, the frozen aortas were pulverized and solubilized in Laemmli buffer containing 2-mercaptoethanol. Mononuclear cells were also lysed and solubilized in this solution. From the total extracted proteins (~50 μg from aortic segments and 80 μg from mononuclear cells), 15 μg/lane was separated in denaturing SDS/10%polyacrylamide gels as described.15 Western blot analysis was performed with a monoclonal antibody against eNOS protein (Transduction Laboratories) at a concentration of 1:2500. Specific eNOS protein was detected by enhanced chemiluminescence (ECL, Amersham) after 5 minutes of film exposure and evaluated by densitometry (Molecular Dynamics). A Western blot analysis of a parallel gel with identical samples was performed with a monoclonal antibody against a constitutively expressed protein, β-actin (1:2000). Prestained protein markers (Sigma Chemical Co) were used for molecular mass determinations.

### Immunohistochemistry

Three rabbits from each experimental group were perfused with 100 mL fixative solution containing 4% paraformaldehyde in serum saline (1:1 vol/vol). The aortas were embedded in paraffin wax and sectioned as previously reported.15,17 eNOS protein was identified by use of a polyclonal antibody against eNOS protein (Santa Cruz) at a dilution of 1:1500 by incubating reactions with biotinylated antiserum to goat IgG (1:300 dilution, Vector Laboratories) for 1 hour and in a solution of peroxidase-linked ABC (ABC kit, Vector Laboratories) for another hour, as reported.15,17 To reveal peroxidase activity, a nickel-enhanced diaminobenzidine procedure was used.

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of RNAse T1 for 30 minutes at 37°C. Then they were heated for 10 minutes at 70°C in Laemmli buffer without 2-mercaptoethanol and electrophoresed on denaturing SDS-PAGE. Gels were exposed for 3 to 5 days to Kodak film with 2 intensifying screens.

Statistical Methods
Results are expressed as mean±SEM. Unless otherwise stated, each study was performed in a minimum of 10 rabbits. Comparisons were done by ANOVA or paired and unpaired Student’s t test when appropriate. Bonferroni’s correction for multiple comparisons was used to determine the level of significance of the P value. A value of P<0.05 was considered statistically significant.

Results
Vasorelaxation Studies
Relaxations to ACh were significantly reduced in phenylephrine-precontracted aortic segments from hypercholesterolemic rabbits (Figure 2A). EC50 was 2×10−7 mol/L for control segments and 0.9×10−6 mol/L for hypercholesterolemic rabbit segments (P<0.05). Moreover, a greater maximum response to ACh was observed in control aortic rings than in hypercholesterolemic rabbit rings (Figure 2A).

Cerivastatin normalized relaxation to ACh in hypercholesterolemic rabbits (Figure 2A) and did not modify it in standard chow–fed rabbits compared with untreated control rabbits (EC50 10−7 mol/L; Figure 2A). Deendothelialization of the aorta by gentle rubbing completely prevented relaxation to ACh in all experimental groups (data not shown).

No significant differences in vasorelaxation to sodium nitroprusside were found between control, cerivastatin-treated, and cerivastatin-untreated hypercholesterolemic rabbits (Figure 2A).

Plasma cholesterol was higher in cholesterol-fed than in control animals (control 37±3 mg/dL, cholesterol-fed 905±28 mg/dL, P<0.05). Cerivastatin treatment and regressed diet decreased plasma cholesterol to 620±10 and 600±23 mg/dL, respectively (P<0.05), although it remained significantly higher than in control rabbits (P<0.05).

Relaxation to ACh was improved in regressed-diet rabbits, although significantly greater than in cerivastatin-treated cholesterol-fed rabbits (Figure 2B).

The vasodilating response to A23187, an endothelium-dependent but receptor-independent agent, was also significantly reduced in aortic segments from hypercholesterolemic rabbits (Figure 2B). Cerivastatin treatment improved A23187-induced relaxation (Figure 2B).

eNOS Expression in the Vascular Wall
Aortic segments from hypercholesterolemic rabbits showed a significant reduction of eNOS protein with respect to control animals (Figure 3A). Cerivastatin fully prevented this reduction (Figure 3A). Cerivastatin treatment of standard chow–fed rabbits tended to increase eNOS expression in the vascular wall (Figure 3A).

eNOS expression was also upregulated in regressed-diet rabbits, although it was significantly lower (P<0.05) than in cerivastatin-treated cholesterol-fed rabbits (Figure 3A). eNOS protein was immunolocalized only at the vessel wall endothelium (Figure 3B). Aortic segments from cholesterol-fed rabbits showed a reduced eNOS expression, which was normalized by cerivastatin treatment (Figure 3B). The autopsies of the rabbits revealed that all the animals were free of infections and tumors.

Ex Vivo eNOS mRNA Stability
eNOS mRNA stability was determined in aortic segments incubated ex vivo with actinomycin D. eNOS mRNA half-life was longer in standard chow–fed rabbits than in hypercho-
Cholesterolemic rabbits (Figure 4). Cerivastatin treatment prolonged eNOS mRNA half-life in hypercholesterolemic rabbits (Figure 4). It is noteworthy that the basal level of eNOS mRNA was higher in control and cerivastatin-treated hypercholesterolemic rabbits than in untreated hypercholesterolemic rabbits (Figure 4).

Aortic Cytosolic Proteins and 3'-UTR of eNOS mRNA

Addition of aortic cytoplasmic extracts from control rabbits to a labeled probe containing the entire 3'-UTR of eNOS mRNA, the UTR-L probe, produced a gel-shifted band that was enhanced when the cytosolic extracts came from hypercholesterolemic rabbits (Figure 5A). Cerivastatin treatment prevented binding of the cytosolic extracts to labeled UTR-L even consistently below the controls (Figure 5A). Binding of the cytosolic extract to UTR-L was also reduced in regressed-diet rabbits, although it was greater ($P < 0.05$) than in cerivastatin-treated cholesterol-fed and control rabbits (Figure 5A). Cerivastatin treatment of Chow-fed rabbits tended to consistently reduce binding of the cytosolic extracts to even below the level observed in untreated control rabbits (Figure 5A).

Proteinase-K treatment (87 μg/mL) of the cytosolic extracts before their incubation with UTR-L abolished complex formation, indicating the involvement of cytosolic proteins (Figure 5B). Aortic cytosolic extracts from hypercholesterolemic rabbits in which the endothelium was mechanically removed by gentle rubbing showed a significant reduction of the gel-shifted band after incubation with labeled 3'-UTR-L (Figure 5B) (endothelialized 180±13, deendothelialized 50±10; n=8; $P < 0.05$), suggesting that in the vascular wall, these proteins are expressed primarily in the endothelium. Absence of endothelium in the rubbed vessels was confirmed by hematoxylin-eosin staining.

Specificity of the Binding Activity of the Cytosolic Proteins

Unlabeled UTR-L prevented binding of cytosolic extracts to labeled UTR-L in a concentration-dependent manner (Figure 5C). Computer analysis of the nucleotide sequence of the 3'-UTR of eNOS mRNA shows 2 differentiated regions: an adenine-uridine (AU)-rich region with 2 AUUUA pentamers in the 3'-half of the UTR-S probe and a uridine-cytidine (UC)-rich region in the 5'-half of the UTR-S probe (Figure 1). Binding between labeled UTR-L and aortic extracts from hypercholesterolemic rabbits was dose-dependently prevented by unlabeled UTR-UC but not by unlabeled UTR-AU (Figure 5C, middle). Binding prevention was very similar with unlabeled UTR-L and unlabeled UTR-UC (Figure 5C, left and middle), suggesting that no additional binding site was present in the...
3’-UTR of eNOS mRNA outside the UTR-UC fragment. In this regard, a gel-shifted band similar to that with labeled UTR-L was observed when labeled UTR-UC was used as probe and was also prevented by an excess (1000 ng) of unlabeled UTR-UC (Figure 5C, right).

Although the nucleotide sequence of rabbit RNA is yet unidentified, we amplified rabbit 3’-UTR by use of oligo 2 and oligo 3, described in “Methods,” as primers to produce the pNOS-UTR-SR probe by reverse transcription–PCR (with rabbit aortic endothelial cell total RNA as template). The PCR product (the UTR-SR probe) was a 448-nucleotide fragment, longer than but highly (>87%) homologous with bovine UTR-S (see Figure 1). Computer analysis of the amplified nucleotide sequence of rabbit 3’-UTR of eNOS mRNA also showed a C-rich region. Aortic cytosolic extracts from hypercholesterolemic rabbits showed a higher binding to labeled UTR-SR than those from normal chow-fed rabbits (Figure 6A, right). Binding to rabbit UTR-SR probe was prevented by unlabeled rabbit UTR-SR (1000 ng) and bovine UTR-UC (1000 ng) but not by bovine UTR-AU (1000 ng) (Figure 6A, right). Binding prevention was very similar with unlabeled rabbit UTR-SR and bovine UTR-UC, again suggesting that cytosolic proteins bind to a 3’-UTR C-rich region of rabbit eNOS mRNA. Sequentiation of the rabbit 3’-UTR C-rich region, shown in Figure 6A (left), was 100% homologous with the bovine C-rich region. Figure 6A (left) shows the predicted 2D configuration of the 3’-UTR C-rich region of rabbit eNOS mRNA.

**UV Cross-Linking of RNA-Protein Complex**

Mixtures of 3’-UTR-L and UV light–exposed aortic cytosolic extracts showed a band with an apparent molecular weight of 60 kDa (Figure 6B), whose intensity was markedly enhanced in extracts from hypercholesterolemic rabbits (Figure 6B). Cerivastatin treatment of hypercholesterolemic rabbits reduced the intensity of the band (Figure 6B). The band was absent when excess unlabeled UTR-L (1000 ng) was used (Figure 6B), indicating that only this band bound specifically to the 3’-UTR of eNOS mRNA.

**eNOS Expression in Mononuclear Cells**

eNOS expression by mononuclear cells was markedly reduced in hypercholesterolemic rabbits compared with control

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** A, Top, Representative gel mobility shift assay of cytosolic proteins that bind to 3’-UTR of eNOS mRNA in aortic segments from control (C), cholesterol-fed (HC), cerivastatin-treated chow-fed (C+CERI), cerivastatin-treated cholesterol-fed (CERI), and regressed-diet (REG) rabbits. Bottom, Densitometric scanning of gel mobility shift assay. Results are mean±SEM of segments from 12 different rabbits for each experimental group. 

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**eNOS Expression in Mononuclear Cells**

eNOS expression by mononuclear cells was markedly reduced in hypercholesterolemic rabbits compared with control
animals (Figure 7A). Furthermore, in 8 of the 12 tested hypercholesterolemic rabbits, eNOS expression by mononuclear cells was almost undetectable, suggesting that it could be even more sensitive to modification than endothelial eNOS expression. This diminished eNOS expression was associated with increased mononuclear cytosolic extract binding to labeled UTR-L probe (Figure 7B). Binding was prevented by proteinase K, unlabeled UTR-L, and unlabeled UTR-UC (data not shown).

Cerivastatin treatment reversed hypercholesterolemia-induced eNOS protein downregulation (Figure 7A) and was associated with a diminished cytosolic extract binding to labeled UTR-L probe (Figure 7B). Binding was prevented by proteinase K, unlabeled UTR-L, and unlabeled UTR-UC (data not shown).

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Discussion
These results provide new evidence about the mechanisms involved in endothelial NO dysfunction in hypercholesterolemia. Hypercholesterolemic rabbits showed dysfunction of endothelium-dependent vasorelaxation associated with reduced endothelial eNOS expression. Moreover, cytosolic extracts from the vascular wall contain a 60-kDa protein...
that binds to a specific U+C-rich region within the 3'-UTR of eNOS mRNA. Binding enhancement was associated with reduced eNOS mRNA stability and decreased eNOS expression. HMG-CoA reductase inhibition reduced the binding and was associated with protection of eNOS expression in the vascular wall and restoration of endothelium-dependent vasorelaxation.

Previously, we have demonstrated that the cytosol of cultured bovine endothelial cells contains proteins that bind to the 3'-UTR of eNOS mRNA and are associated with reduced eNOS mRNA stability. We now demonstrate such proteins in vascular wall endothelium, which is suggested by their marked reduction in deendothelialized vessels. The residual band observed in deendothelialized segments can be explained by the presence of these protein-binding activities.
attributed to nonendothelial cells, including infiltrated monocytes. Increased binding was inversely correlated with eNOS expression, ex vivo eNOS mRNA stability, and endothelium-dependent vasorelaxation.

The cytosolic proteins bind to a U+C-rich region within the 3'-UTR of eNOS mRNA. UV cross-linking and SDS-PAGE identified a 60-kDa protein that interacts with the 3'-UTR of eNOS mRNA and is within the range of other 3'-UTR–binding proteins.\(^9\) Recently, we also demonstrated such protein in cultured endothelial cells.\(^8\)

Because rabbit eNOS mRNA has not yet been cloned, in most of the experiments we used 3'-UTR probes from bovine eNOS mRNA. Numerous studies also use bovine eNOS cDNA to transfer eNOS to rabbit blood vessels.\(^22\)\(^23\) In an attempt to determine whether the C-rich region in rabbit 3'-UTR of eNOS mRNA binds the cytosolic proteins, however, we developed a rabbit RNA probe using primers from the flanking regions of the C- and A-rich regions of bovine 3'-UTR of eNOS mRNA and adding rabbit RNA as template for the PCR. Binding of aortic or mononuclear cell cytosolic extracts to rabbit 3'-UTR RNA probe was greater in hypercholesterolemic than in control rabbits. Moreover, competition experiments supported binding to a specific C-rich region within the 3'-UTR of eNOS mRNA. Sequentiation of the rabbit UTR probe used demonstrated a C-rich region 100% homologous to the bovine C-rich region. Human 3'-UTR of eNOS mRNA also contains the C-rich region, suggesting that it could be conserved in different species. As a hypothesis, binding of the protein could modify the spatial configuration of this region, which shows an RNAse active site, as has been reported for iron-regulated transferrin-receptor mRNA stability.\(^3\)

Cerivastatin treatment restored the impaired response to ACh in hypercholesterolemic rabbits, reduced protein binding to eNOS mRNA, reduced the cytosolic protein, increased ex vivo eNOS mRNA stability, and markedly increased eNOS expression in the vascular wall. These results suggest an association between protein binding and eNOS expression.

Cerivastatin completely preserved endothelial function, although serum cholesterol remained elevated. Moreover, although cholesterol reduction was very similar in regressed-diet and cerivastatin-treated rabbits, endothelial parameters were much more improved by cerivastatin, suggesting an effect beyond its hypocholesterolemic action. In this regard, improvement of endothelial function by simvastatin also does not correlate with decreases in serum cholesterol.\(^24\)

Our results did not rule out the recently demonstrated involvement of the L-mevalonate–derived isoprenoid intermediate geranylgeranyl pyrophosphate and its posttranslationally modified protein, Rho, in vivo statin regulation of eNOS expression.\(^25\) Inhibition of Rho levels may prevent anchoring of eNOS mRNA to the cytoskeleton and its colocalization with the cytosolic protein. Superoxide production has also been involved in hypercholesterolemic endothelial dysfunction.\(^26\) In addition to oxidizing NO, free radicals could alter the cytosolic protein. These hypotheses were not addressed in this study.

eNOS-like NOS has been identified in mononuclear cells.\(^13\)\(^27\) We found a correlation between eNOS expression, eNOS mRNA stability, and cytosolic protein binding in mononuclear cells and in the endothelium.

In both vascular wall and mononuclear cells, cerivastatin treatment not only reversed the hypercholesterolemic inhibition of eNOS expression but also tended to cause a decrease in cytosolic protein binding compared with untreated control rabbits. Vasodilation to ACh was not potentiated, however, which suggests that other factors (vasoconstrictor factors and/or eNOS activity) could play a role.

Our results show the presence of a cytosolic protein and eNOS expression in the vascular wall and mononuclear cells. Further studies will be necessary to test the activity of this protein on eNOS mRNA stability and to verify whether it is causally related to the reduction in eNOS expression.

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