Effect of HMG-CoA Reductase Inhibition on Endothelial Dysfunction-Inducing Protein in Hypercholesterolemic Rabbits

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Introduction and objectives. In our laboratory, we recently obtained evidence 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 the level of eNOS expression in hypercholesterolemic rabbits as an in vivo model of endothelial dysfunction.

Methods and results. Endothelium-dependent relaxation in response to acetylcholine was reduced in aortic segments from hypercholesterolemic rabbits compared with controls. Treatment of hypercholesterolemic rabbits with simvastatin (25 mg/kg body weight/day) 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. Simvastatin treatment upregulated eNOS expression and reduced the interaction of cytosolic protein with the 3'-untranslated region of eNOS mRNA.

Conclusions. These results demonstrate the presence of a 60-KDa protein that binds to eNOS mRNA and reduces eNOS expression in the vascular wall.

Key words: Endothelium. Nitric oxide. mRNA. Hypercholesterolemia.

INTRODUCTION

Nitric oxide (NO) is a gas generated in the endothelial cells by means of the enzyme activity of endothelial nitric oxide synthase (eNOS). Nitric oxide generated by eNOS activity is responsible for the endothelium-dependent vasodilatory response, which is lower in animal models and in hypercholesterolemic patients.
Although initially eNOS protein was defined as a constitutive enzyme, it was later shown that both cytokines and oxidized low density lipoproteins (LDL) can inhibit the expression of eNOS by destabilizing its mRNA.\textsuperscript{7,8} There is in vitro evidence showing that 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase inhibitors preserve the expression of eNOS by stabilizing its mRNA, including in conditions in which cholesterol values remain unchanged, although the mechanism by which these drugs preserve the expression of eNOS has not been determined.\textsuperscript{9}

One of the control mechanisms of mRNA stability occurs by means of its interaction with cytosolic proteins. There are specific sequences to which these proteins can bind within different mRNA. Some of these sequences have been identified within the 3’untranslated region (3’-UTR) of mRNA.\textsuperscript{10,11} Our laboratory has recently shown that bovine endothelial cells in culture and isolated neutrophils contain cytosolic proteins that form complexes with the 3’UTR of the mRNA of eNOS.\textsuperscript{12,13} The interaction of these proteins with the mRNA of eNOS is associated with destabilization of the mRNA of the eNOS enzyme.\textsuperscript{12} Therefore, these cytosolic proteins may be directly involved in initiating endothelial dysfunction. Nevertheless, there are no studies of the values of these cytosolic proteins in an in vivo model of endothelial dysfunction.

The goal of our study was to determine the expression of eNOS and the presence of the cytosolic proteins that specifically bind to the 3’UTR of the RNA of eNOS in the arterial wall of hypercholesterolemic rabbits. As we have already mentioned, statins have been shown to be useful in preventing endothelial dysfunction during hypercholesterolemia and preserve the expression of eNOS in endothelial cells in culture.\textsuperscript{9}

Subsequently, in the second leg of this study, we analyzed the effect of treatment with simvastatin, a new HMG-CoA reductase inhibitor,\textsuperscript{14,15} on these parameters.

**ABBREVIATIONS**
- Ach: acetylcholine
- RNA: ribonucleic acid
- mRNA: messenger RNA
- HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
- LDL: low density lipoproteins
- kDa: kilodalton
- NO: nitric oxide
- eNOS: endothelial nitric oxide synthase
- SNP: sodium nitroprussiate
- 3’-UTR: 3’ untranslated region

**MATERIALS AND METHODS**

**Study protocol**

The study protocol was approved by the Institutional Ethics Committee on Animal Experimentation. The experiments were performed on New Zealand white rabbits, with a weight of 2.5 kg±0.3 kg. The rabbits were individually housed at 20°C±3°C with alternating cycles of 12 hours of light and 12 hours of darkness. A group of 15 rabbits were fed a standard diet for their species for 6 weeks (normal cholesterol control group). Another group of 15 rabbits were fed a standard diet for 3 weeks and then a standard diet supplemented with simvastatin (25 mg/kg body weight/day) (C+Simv) for 3 more weeks. A group of 45 rabbits was fed a standard diet for rabbits enriched with cholesterol that contained 0.5% cholesterol and 6% coconut oil for 3 weeks (Epinay’s, France). After the 3 weeks had elapsed, the hypercholesterolemic rabbits were randomly divided into 3 experimental groups of 15 rabbits each. One of these groups was fed a standard diet for rabbits enriched with cholesterol for 3 weeks (HC). The second group was fed a standard diet enriched with cholesterol and simvastatin (25 mg/kg body weight/day) that was administered with food for the last 3 weeks (H+Simv). The last group of 15 rabbits, after 3 weeks of a diet enriched with cholesterol, was subjected to dietetic regression; that is, they were fed a standard diet for the last 3 weeks (Reg). The diet was restricted to 120 g/day. At the end of 6 weeks, the animals were anesthetized with pentobarbital (30 mg/kg), and the thoracic aorta was removed.

**Vasodilatation studies**

The thoracic aorta segments were divided into 3 parts, one of which was immediately frozen in liquid nitrogen for molecular biological testing. The other 2 parts of the thoracic aorta were suspended in an organ bath of 5% O\textsubscript{2} and 95% CO\textsubscript{2} that contained a Kreb’s Henseleit solution (in mol/L: NaCl, 115; KCl, 4.6; KH\textsubscript{2}PO\textsubscript{4}, 1.2; CaCl\textsubscript{2}, 2.5; NaHCO\textsubscript{3}; glucose, 11.1, EDTA, 0.02, and pH 7.4), as described previously.\textsuperscript{16} The aortic segments were connected to an isometric force transducer connected to a computer (Power Lab 400, AD Instruments, Casterhill, NSW, Australia). The segments were tensed at a resistance force of 2 g and equalized for 1 hour. Dependent relaxation of the endothelium was determined by the addition of incremental doses of acetylcholine (Ach) from 109 to 104 mmol/L, and independent relaxation of the endothelium was determined by adding incremental doses of sodium nitroprussiate (SNP) of 109 to 105 mmol/L. The aortas were previously contracted with Phenylephrine (105 mmol/L).\textsuperscript{15} The dose response curves were calculated cumulatively. All the experiments...
were performed with the use of indomethacin (105 mol/L), thus preventing possible cyclooxygenase activation.

**Determination of protein eNOS expression**

The levels of protein eNOS expression were analyzed by Western blot analysis, as described previously. The frozen aortas were homogenized and solubilized in Laemmli buffer containing 2-mercaptoethanol. The proteins were separated in a denaturizing gel of SDS/10% polyacrylamide. Western blot analysis was performed using a monoclonal antibody against eNOS protein (Transduction Laboratories, Lexington, Ky., USA) at a concentration of 1:2500. The eNOS protein was detected by using luminescence (ECL, Amersham, Buckinghamshire, England) and evaluated by densitometry (Molecular Dynamics, Sunnyvale, Calif, USA). To determine the molecular weight, we used color markers (Sigma Chemicals, St. Louis, Miss, USA). To provide a control, we incubated membranes with antibeta-actine antibodies (Calbiochem, Germany).

**Probe of the 3’ NO codifying region**

Complementary oligonucleotides of the cRNA of bovine eNOS (Genbank accession number BTNIOXSY) were obtained from Bio-synthesis, Inc. (Lewisville, Tex, USA) as described previously. We used oligonucleotide 1 (5’-GGATCTAGAACGCTA T CACGAGGACATT-3’) and oligonucleotide 2 (5’-AGGAAGCTTAGTTGTCCTCAACTTCTG-3’) to obtain the pNOS-UTR-L probe. This probe covered 166 bases of the codifying region of the mRNA of the eNOS and 393 bases of the 3’-UTR region (from nucleotide 3485 to 4012). The polymerase chain reaction (PCR) amplified products, after using 2 oligonucleotides as primers, were purified by electrophoresis in agarose gel and digested with Xba I and Hind III for later binding to pGEM4Z (Promega Biotech, Madison, Wis, USA).

In order to produce single-chain RNA, the plasmids were matched with the corresponding restriction enzymes and transcribed with T7 polymerase RNA. The RNA was radioactively marked with 32P-CTP (Amersham Iberica, Madrid, Spain; 109 cpm/µg) in accordance with the specifications of the equipment used (Promega Biotech, Madison; Wis, USA).

**Northern blot analysis**

In order to determine the level of stability of the mRNA of the eNOS, the aortic segments obtained from the various groups of rabbits were incubated ex vivo during varying periods of time in RPMI medium with actinomycin D (10 µg/mL), a transcription inhibitor, for 1 hour. After 1 hour, they were washed with fresh RPMI medium and incubated in the same medium for varying periods of time. The total RNA was isolated according to the Chomazkuski and Sachi method. Northern blot analysis was performed using as complementary ADN the UTR-L-RNA marked radioactively (5±10 3 cpm/mL). To control gel load, we used ethidium bromide tincture to determine the RNA of 28S and 18S.

**Electrophoresis mobility assay**

We prepared cytoplasmatic extracts from the various aortas. To this end, the aortas were homogenized and resuspended in hypotonic solution (25 mol/L Tris-HCl [pH, 7.9], 0.5 mmol/L EDTA, and 1 mmol/L PMSF) and then subjected to 4 cycles of heat and cold followed by centrifugation at 12 000 g 4°C for 15 minutes. The supernatants were collected and frozen at −80°C until they were used.

As described previously, the cytoplasmic lysates (10 µg) were incubated with 5-10×10^4 cpm radioactivity of the UTR-L probe in 15 mmol/L HEPES (pH, 7.9), 10 mmol/L KCl, 5 mmol/L Cl2 Mg, 1 mmol/L DTT, 1 µg/ml yeast tRNA, 40 units of Sin-RNA (Promega Biotech, Madison, Wis, USA), and 10% glycerol at a total volume of 15 µl for 10 minutes at 25°C. We then added 20 units of RNA-asa T1 (Gibco- BRL, Dieselstrasse, Germany), which was incubated for 30 minutes at 37°C. The samples underwent electrophoresis in a 4% polyacrylamide gel in 0.25×E (Tris-Borate-EDTA). The gel was dried and developed with Kodak X-OMAT-S film.

**Protein-RNA cross-linking assay**

We incubated 10 µg of the cytoplasmic lysates from the various groups studied, obtained from the aortic segments with 105 cpm of RNA in the medium described previously at a total volume of 20 µL for 10 minutes at 25°C. The samples were irradiated with ultraviolet light in ice in a Stratalinker (Stratagene LTD, Cambridge, England) for 20 minutes. We then performed digestion by RNA-asa (20 µg of RNA-asa A and 20 units of RNA-asa T1 for 30 minutes) at 37°C. The samples were heated for 10 minutes at 70°C in Laemmli buffer without 2-mercaptoethanol and underwent electrophoresis in an SDS-PAGE denaturizing gel. The gels were exposed for 3 to 5 days on Kodak film with 2 intensifiers.

**Statistical methods**

The results are expressed as mean±standard deviation (SD). Unless otherwise specified, each of the studies was performed on a minimum of 10 rabbits. The comparison was made by using the ANOVA test or the Student t test for paired and unpaired data, as appro-
Appropriate. The Bonferroni correction for multiple comparisons was used to determine the level of significance of the P values. A P<.05 was considered significant.

RESULTS

Vasodilatation studies

The Ach (acetylcholine) produced a dependent relaxation of the dose in the segments of the aorta precontracted with phenylephrine obtained from the rabbits fed a standard diet (control group) (Figure 1). The acetylcholine-dependent relaxation was significantly reduced in the aortic segments of rabbits fed a diet rich in cholesterol (Figure 1). Therefore, the EC50 for the segments from the control rabbits was $2 \times 10^7$ mol/L, and that of the rabbits fed a diet rich in cholesterol was $0.9 \times 10^6$ mol/L ($P<.05$). In addition, there was a greater response to Ach in the segments of the aortas obtained from control rabbits than in those from the rabbits fed a diet rich in cholesterol (Figure 1). Treatment with simvastatin of the rabbits fed a diet rich in cholesterol normalized the relaxation induced by acetylcholine (Figure 1). Treatment with simvastatin of rabbits fed a standard diet did not change the vasodilatory response to Ach (EC50 107 mol/L) with respect to the control rabbits. We did not observe significant differences in the independent vasorelaxant endothelial response to SN between the aortic segments obtained from control rabbits and from those rabbits fed with cholesterol and treated with simvastatin (Figure 2).

The plasma concentrations of cholesterol in the animals fed cholesterol were greater than that in the control animals (control, 37 mg/dL±3 mg/dL; fed with cholesterol, 905 mg/dL±28 mg/dL; $P<.05$). Treatment with simvastatin reduced the plasma cholesterol values, although these values remained significantly higher than those of the control rabbits (570 mg/dL±20 mg/dL; $P<.05$). We later analyzed the changes in cholesterol observed in the rabbits treated with simvastatin on dependent endothelial relaxation. In order to do this, a group of rabbits was fed a hypercholesterolemic diet for 3 weeks followed by 3 weeks of a normal diet. Plasma concentrations of cholesterol in these rabbits were significantly reduced in comparison with the rabbits fed cholesterol for 6 weeks (600 mg/dL±23 mg/dL; $P<.05$). This effect was accompanied by an improvement in the dependent endothelial relaxation in response to Ach, although this improvement was less than that observed in the rabbits treated with simvastatin (Figure 1).

Expression of eNOS in the vascular wall

In the aortic segments of the rabbits fed cholesterol, we observed a significant reduction in the expression of eNOS protein compared with that found in the control animals and in the control animals treated with simvastatin (Figure 3). Treatment with simvastatin preserved expression of eNOS in the vascular wall (Figure 3).

The expression of eNOS was also improved in the rabbits subjected to diet regression, although this was significantly less ($P<.05$) than that in hypercholesterolemic rabbits treated with simvastatin (Figure 3).

Stability of mRNA of ex vivo eNOS

To determine the half-life of the mRNA of the eNOS, we measured the mRNA values of eNOS in aorta segments incubated ex vivo with actinomycin D.

The half life of mRNA of the eNOS in the vascular...
The wall of rabbits fed a standard diet was greater than that of the rabbits fed a diet rich in cholesterol (Figure 4). Treatment with simvastatin prolonged the half life of mRNA of the eNOS in the rabbits fed cholesterol (Figure 4).

It is of interest that the baseline mRNA values of eNOS were greater in the walls of the control rabbits than that in the rabbits fed a diet rich in cholesterol (Figure 4). The presence of cytosolic proteins that cross-link with the 3′ untranslated region of mRNA of the eNOS

The addition of cytoplasmic aortic extracts obtained from control rabbits to the probe marked radioactively containing the extreme 3′-UTR of the mRNA of the eNOS, probe UTR-L, resulted in the appearance of a delayed band, which also appeared in the cytosolic extracts of the aortas of the control rabbits treated with simvastatin (Figure 5). The cross-linking of the probe between UTR-L and the cytosolic aorta proteins increased when the cytosolic aorta extracts were obtained from hypercholesterolemic rabbits (Figure 5). Treatment with simvastatin significantly reduced the interaction of cytosolic proteins with the radioactively marked UTR-L probe. Similarly, the control rabbits treated with simvastatin reduced the cross-link compared with control rabbits. The cross-link of the cytosolic extracts to UTR-L was also reduced in the rabbits who were returned to a standard diet, although it continued to be greater than that observed in the rabbits treated with simvastatin \((P<.05)\) (Figure 5).

Treatment of the cytosolic aorta extracts with proteinase K (87 µg/mL) prior to incubation with probe UTR-L impedes the formation of the complex, which indicates the involvement of proteins in the interaction of these cytosolic extracts with the 3′-UTR region of the mRNA of the eNOS.

Identification of the cytosolic protein cross-linked to the 3′ untranslated region of mRNA of the eNOS

The identification of the cytosolic proteins involved in the cross-link with the end 3′-UTR of mRNA of the eNOS was performed by prior fixation of the cross-link of the cytosolic proteins to mRNA with ultraviolet light, with a specific band appearing with a molecular weight that appeared to be 60 kDa when incubated with aortic cytosolic extracts with the UTR-L probe. The intensity of the 60 kDa band was greater in the cytosolic extracts obtained from the aortic segments of rabbits fed a diet rich in cholesterol (Figure 6). Treatment of the hypercholesterolemic rabbits with simvastatin reduced the level of cross-link of the 60 kDa cytoplasmic protein (Figure 6). These results indicate that the rabbit aortas express EDIP proteins.

DISCUSSION

Our study shows a new mechanism associated with the dysfunction of the NO system in hypercholesterolemic rabbits. These animals showed dysfunction of
the dependent vasodilatory response of the endothelium, which was associated with a lesser expression of eNOS protein in the endothelium. On the other hand, cytosolic extracts from the vascular wall contain proteins, (particularly a cytosolic protein with an apparent molecular weight of 60 kDa) that interact with the 3´-UTR region of mRNA of the eNOS. The reduction in the union activity of 60-kDa cytosolic protein was associated with greater stability of eNOS mRNA and a greater expression of eNOS protein, in addition to an improved dependent endothelial vasodilatory response.

Regulation of mRNA of the eNOS by EDIP

The eNOS values in endothelial cells in culture may be regulated by cytokines and oxidized LDL by means of the destabilization of the mRNA of the eNOS. In a previous study, we showed that in the cytosol of bovine endothelial cells in culture there are proteins that interact with the 3´-untranslated region of mRNA of the eNOS, and are associated with a reduced stability of mRNA of eNOS. In this study, we show that in a setting of in vitro endothelial dysfunction, these cytosolic proteins also appear. The increase in the cross-link activity of these cytosolic proteins was inversely related to the value of the eNOS protein, the ex vivo mRNA eNOS stability, and the dependent endothelial vasodilatory response.

Studies of the union of the RNA-protein showed that cytosolic protein has an apparent molecular weight of 60 kDa. This molecular weight is within the range observed in other cytosolic proteins that cross-link with the 3´-UTR region of other mRNA, as it happens with the transferrine receptor. We called the 60 kDa cytosolic protein an inducing protein of endothelial dysfunction (EDIP in English).

The improvement in endothelial function observed in the rabbits treated with simvastatin was accompanied by a reduction in the cross-link activity of the cytosolic proteins in the vascular wall to mRNA of the eNOS; in other words, a decrease in the value of the 60 kDa cytosolic protein, an increase in the stability of...
mRNA of the ex vivo, and a marked increase in the expression of eNOS protein in the vascular wall. These results suggest an association between the cross-link activity of the EDIP cytosolic protein and Rho level of expression of the eNOS protein.

The role of cholesterol in the effect of simvastatin

Simvastatin completely improves cholesterol, although the concentrations of cholesterol in serum remained elevated in the hypercholesterolemic control rabbits. In this sense, in the rabbits subjected to a regression in their diet, we also observed an equal reduction in cholesterol to those rabbits that received simvastatin. Nevertheless, the improvement in endothelial function, the upregulation of eNOS expression, and the level of interaction of cytosolic extract to mRNA of the eNOS improved more after treatment with the HMG-CoA reductase inhibitor. Taken together, these results suggest an effect of simvastatin beyond it hypcholesteremic action. It has recently been shown that the improvement in endothelial function that results from simvastatin treatment is not related to a decrease in cholesterol. In addition, an in vitro study by our group has shown that simvastatin prevents in vitro expression of eNOS and the presence of EDIP protein in bovine endothelial cells stimulated by tumor necrosis factor-α in conditions in which the cholesterol in not modified.

Therefore, this would mean that there is both a dependent and independent component of cholesterol in preserving expression of sNOS by simvastatin in the vascular wall.

Relationship of the EDIP protein with the protective mechanisms of statins

The results of this study do not rule others factors that may be involved in the induction of the endothelial dysfunction. In this regard, Rho proteins have recently been associated with the protective effect of statins in endothelial function. This is due to the fact that Rho proteins have an important role in the regulation of protein activity through the cellular cytoskeleton, so that the Rho factor may be involved in the location of EDIP protein with the eNOS messenger. Along these same lines, statins have been shown to protect the bioavailability of NO, inhibiting the formation of vascular wall free radicals. Nevertheless, we do not know whether other mechanisms are involved in the regulation of EDIP protein. Future studies on the topic will be surely be undertaken.

In summary, our study demonstrates for the first time the existence of EDIP protein in an in vivo setting of endothelial dysfunction; this 60 kDa EDIP protein may be responsible for the destabilization of mRNA of the eNOS and, as a result, destabilization of endothelial dysfunction associated with hypercholesterolemia. This EDIP protein may be a therapeutic target for the protection of endothelial function.

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