Involvement of Rho GTPases in the Transcriptional Inhibition of Preproendothelin-1 Gene Expression by Simvastatin in Vascular Endothelial Cells

Octavio Hernández-Perera, Dolores Pérez-Sala, Estrella Soria, Santiago Lamas

Abstract—Endothelial dysfunction is characterized by an impaired vasodilatory response to endothelial agonists as well as by alterations in adhesion and coagulation processes. 3-Hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) have been shown to be useful in the reversal of endothelial dysfunction, an effect that may be independent of the reduction in cholesterol levels. Both the L-arginine–nitric oxide–cGMP and endothelin pathways are involved in the regulation of vascular tone. Here, we show that the basal transcription rate of the preproendothelin-1 gene was decreased by simvastatin (10 μmol/L) in bovine aortic endothelial cells. Transfection studies with the preproendothelin-1 gene promoter showed that mevalonate (100 μmol/L) was able to prevent the inhibitory effect mediated by simvastatin. Protein geranylgeranylation, but not farnesylation, proved to be crucial for a correct expression of the preproendothelin-1 gene. The C3 exotoxin from Clostridium botulinum that selectively inactivates Rho GTPases, the processing of which involves geranylgeranylation, reproduced the inhibitory effect of simvastatin on the expression of preproendothelin-1. Overexpression of dominant-negative mutants of RhoA and RhoB led to a significant reduction in the preproendothelin-1 promoter activity, whereas the expression of wild-type and constitutively active forms of these proteins resulted in an increase, in support that Rho proteins are required for the basal expression of the preproendothelin-1 gene. Finally, we show that the Rho-dependent activation of the preproendothelin-1 gene transcription was inhibited by simvastatin. Thus, the control of vascular tone and proliferative response mediated by endothelin-1 is regulated at multiple levels, among which the Rho proteins play an essential role. (Circ Res. 2000;87:616-622.)

Key Words: cholesterol ■ endothelium ■ endothelin ■ GTP-binding proteins ■ statins

Endothelial dysfunction is an ill-defined pathophysiological entity in which the normal physiological role of the endothelium is disturbed. In particular, the ability of endothelial cells to promote relaxation responses to endothelial agonists such as acetylcholine or bradykinin is fundamentally impaired.1 Treatment with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) is associated with a partial reversion of endothelial dysfunction, as documented with noninvasive techniques.2-5 This improvement is not necessarily related to a detectable descent in serum cholesterol levels. We and others have described that inhibition of the mevalonate–cholesterol biosynthetic pathway by statins results in the regulation of the expression of vasoactive factors.6,7 Exposure of endothelial cells to statins is associated with reduced levels of endothelin-1 (ET-1) peptide, a powerful vasoconstrictor, and a decrement in the expression of the preproET-1 gene.

The interruption of the cholesterol biosynthetic pathway at the step immediately upstream of mevalonate may have profound consequences for the cell. Prenylation of GTP-binding proteins is dependent on the formation of mevalonate-derived isoprenoid compounds as farnesyldiphosphate (FPP) and geranylglycerolphosphate (GGPP).8,9 Farnesylation and geranylgeranylation are important posttranslational modifications for the cellular functions of GTPases, including Ras, Rac, and Rho. Ras proteins are important mediators of proliferative responses, having fostered the development of farnesyltransferase inhibitors as potential therapeutic tools to inhibit the activity of oncogenic forms of Ras.10 In the case of Rho proteins, inhibition of the prenyltransferases involved in their maturation may lead to alterations in a panoply of cellular functions, such as organization of the actin cytoskeleton, gene expression, membrane trafficking, growth, transformation, or programmed cell death.11,12

The regulation of vascular tone is a complex process in which the autonomic nervous system, physical adaptive forces, and endocrine, paracrine, and autocrine functions act in a concerted fashion. Among the paracrine vasoconstrictive...
factors generated by the endothelium is the peptide ET-1, which elicits a contractile and proliferative action in the vascular smooth muscle layer. Previously published data from our group demonstrated that statins were able to decrease the expression of the ET-1 transcript. We now show that simvastatin is able to depress ET-1 levels in bovine aortic endothelial cells (BAECs) by acting at the level of transcription. Furthermore, our data indicate that Rho proteins are essentially involved in the basal expression mechanisms of the preproET-1 gene.

Materials and Methods

Cell culture media and supplies were obtained from BioWhittaker. Simvastatin was kindly supplied by Merck Sharp & Dohme. Farnesyltransferase and geranylgeranyltransferase inhibitors were obtained from Calbiochem. Recombinant C3 exoenzyme was a kind gift of Michel Popoff (Institut Pasteur). FPP and GGPP were obtained from Sigma Chemical Co.

Cell Culture

BAECs were isolated and cultured until passage 8 as previously described. They were kept in serum-free medium during incubations.

Nuclear Run-On

Nuclei were isolated through Dounce homogenization, and transcription assays were performed according to published protocols. Radiolabeled RNA was hybridized to denatured plasmids that contained cDNAs of bovine preproET-1 and bovine endothelial NO synthase (eNOS) [both in pBS(+) (1 pmol)] and rat GAPDH in pUC18 (2 pmol) immobilized onto nylon membranes. The density of autoradiographic signals was quantified with an AGFA image scanner and appropriate software. The specificity of the transcription assay was determined according to the lack of hybridization to the empty vector pBS(+).

Plasmids

A 7-kb XbaI-XbaI fragment of the human preproET-1 gene inserted in the pGEM3 vector was kindly provided by Kenneth Bloch (Massachusetts General Hospital). Reporter fusion plasmids that contained preproET-1 promoter −0.65-pET1-LUC, −1.5-pET1-LUC, and −5.2-pET1-LUC were generated by subcloning 0.8-kb SacI-BglII, 1.6-kb EcoRI-BglII, and 5.3-kb XbaI-BglII human preproET-1 genomic fragments into the proper cloning site upstream of the firefly luciferase gene in the pGL3-Basic vector (Promega). Renilla luciferase reporter vectors with Tfx-50 or LipofectAMINE reagent (GIBCO BRL) were a kind gift of Michel Popoff (Institut Pasteur). FPP and GGPP were obtained from Sigma Chemical Co. to assess the effect of simvastatin on the promoter activity of the preproET-1 gene, BAECs were transfected with a 7-kb XbaI-XbaI fragment of the human preproET-1 gene inserted in the pGEM3 vector was kindly provided by Kenneth Bloch (Massachusetts General Hospital). Reporter fusion plasmids that contained preproET-1 promoter −0.65-pET1-LUC, −1.5-pET1-LUC, and −5.2-pET1-LUC were generated by subcloning 0.8-kb SacI-BglII, 1.6-kb EcoRI-BglII, and 5.3-kb XbaI-BglII human preproET-1 genomic fragments into the proper cloning site upstream of the firefly luciferase gene in the pGL3-Basic vector (Promega). Renilla luciferase reporter vectors with Tfx-50 or LipofectAMINE reagent (GIBCO BRL) were a kind gift of Michel Popoff (Institut Pasteur). FPP and GGPP were obtained from Sigma Chemical Co.

Figure 1. Effect of simvastatin on preproET-1 (ppET-1) gene transcription in BAECs. Cells were treated with 10 μmol/L simvastatin (SV) for the indicated times. Nascent transcripts for preproET-1 and GAPDH were evaluated with nuclear run-on. Densitometric band intensities of the preproET-1 autoradiographic signals are expressed as a fraction of those of GAPDH (bottom).

Northern Blotting

The isolations of total RNA and Northern analysis were performed as previously described. Full-length bovine preproET-1 and β-actin probes were labeled with the Rediprime DNA labeling system (Amersham International).

Immunoblot Analysis

Cellular proteins were isolated and immunoblotting was performed as described previously. Blots were probed with anti-pan-Ras antibody (Oncogene Research Products).

Data Analysis

Data are expressed as mean±SEM. Comparisons were made with ANOVA or Student’s t test as previously described.

Results

Simvastatin Downregulates the Expression of PreproET-1 at the Transcriptional Level

Previous results demonstrated that the preproET-1 gene downregulation in the presence of statins was not due to destabilization of the preproET-1 transcript, because comparable half-lives were observed in the presence or absence of simvastatin. Run-on transcription studies in isolated nuclei from BAECs (Figure 1) showed that 10 μmol/L simvastatin decreased the transcriptional rate of the preproET-1 gene in a time-dependent fashion (32%, 67%, and 69% reduction after 8-, 16-, and 24-hour treatment, respectively). Simvastatin did not modify the transcriptional rate of the eNOS gene (data not shown), thus suggesting that the effect on the preproET-1 gene transcription is specific.

To assess the effect of simvastatin on the promoter activity of the preproET-1 gene, BAECs were transfected with a
Mevalonate Prevents the Inhibitory Effect of Simvastatin on PreproET-1 Gene Transcription

We had previously shown that mevalonate, but not cholesterol, prevented the effect of simvastatin on preproET-1 mRNA expression. To determine whether the effect of mevalonate was taking place at the transcriptional level, BAECs were transfected with the −0.65-pET1-LUC construct and exposed to simvastatin in the presence or absence of mevalonate. As shown in Figure 2B, mevalonate almost completely prevented the inhibitory effect of simvastatin (80±10% prevention, n=4). This suggested that isoprenoids from the cholesterol biosynthetic pathway downstream of mevalonate could be important for the expression of the preproET-1 gene.

GGPP but Not FPP Prevents the Inhibitory Effect of Simvastatin on PreproET-1 Expression

To evaluate the capacity of FPP and GGPP to prevent the inhibitory action of simvastatin, BAECs were treated with simvastatin in the presence or absence of these isoprenoid compounds. As shown in Figure 3A, GGPP but not FPP was able to prevent the reduction of the preproET-1 mRNA expression elicited by simvastatin. The effect of GGPP was concentration dependent (Figure 3B), with a maximal effect observed at 5 μmol/L (70±10% reversion, n=6).

To rule out the possibility that the failure of FPP to prevent the inhibitory effect of simvastatin could be due to a defective cellular uptake, we analyzed the capability of FPP (5 μmol/L) and GGPP (5 μmol/L) to prevent simvastatin-mediated inhibition of the processing of Ras GTPases, which preferentially involves farnesylation. Inhibition of Ras processing can be detected with an electrophoretic mobility shift from a lower to an upper band, as previously shown. FPP prevented the decrement in the abundance of the processed form of Ras elicited by simvastatin (10 μmol/L, 24 hours), whereas GGPP had no significant effect (simvastatin 49%, FPP plus simvastatin 107%, GGPP plus simvastatin: 48% of control values, n=2). Hence, the inability of FPP to prevent simvastatin-mediated downregulation of the preproET-1 gene cannot be attributed to low cellular uptake. Altogether, these results suggest that a reduction in the pool of GGPP is involved in the inhibition of the basal expression of preproET-1 by simvastatin.

Inhibition of Geranylgeranyltransferase I but Not of Farnesytransferase Mimics the Effect of Simvastatin on PreproET-1 Expression

Consistent with previous results, the inhibition of geranylgeranyltransferase I with the specific inhibitor GGTI-286 resulted in a downregulation of the preproET-1 message (Figure 4A), which was dose dependent and significant from a concentration of 5 μmol/L, reaching a maximum at 10 μmol/L. In contrast, a slight increase in preproET-1 mRNA levels was observed in cells treated with the farnesyltransferase inhibitor FTI-277 (Figure 4B).

Inhibition of the Activity of Rho Proteins Results in Downregulation of PreproET-1 Gene Expression

These results led us to hypothesize that geranylgeranylation of proteins involved in the coupling of signal transduction to

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**Figure 2.** A, Time course of preproET-1 (ppET-1) promoter activity in simvastatin-treated BAECs. Cells were transfected with −0.65-pET1-LUC plus pRL-CMV, treated with vehicle (control) or 10 μmol/L simvastatin, and harvested at the indicated treatment times. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed relative to control. Results are mean±SEM from triplicate determinations of 3 independent experiments. B, Effect of mevalonate on simvastatin inhibition of preproET-1 gene transcription. Transfected cells were treated with vehicle or 10 μmol/L simvastatin in the absence or presence of 100 μmol/L mevalonate for 24 hours. The histogram represents mean±SEM from triplicate determinations of 4 independent experiments. *P<0.05 vs control.

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reporter construct that contained 0.65 kb of the preproET-1 5′ regulatory region (−0.65-pET1-LUC). As shown in Figure 2A, simvastatin induced a time-dependent inhibition of preproET-1 promoter activity. This inhibition was marked at 24 hours (40±8%) and maximal at 48 hours (66±13%). Transfections with the −1.5-pET1-LUC and −5.2-pET1-LUC plasmids, containing longer upstream fragments of the 5′ regulatory region, resulted in similar levels of both basal promoter activity (not shown) and response to simvastatin treatment (10 μmol/L, 24 hours): −1.5-pET1-LUC 42±6%, −5.2-pET1-LUC 40±4% inhibition (n=5). Taken together, these results indicate that simvastatin inhibits preproET-1 mRNA expression at the transcriptional level and that no critical cis-regulatory elements involved in this inhibition are located in regions of the promoter upstream of that contained in the −0.65-pET1-LUC construct.
transcription could be important for the regulation of preproET-1 expression. Small GTP-binding proteins represent potential candidates to mediate simvastatin effects. Of these, the Rho family of proteins are known to be geranylgeranylated, in contrast with Ras proteins, which preferentially undergo farnesylation. Rho proteins are targets for the ADP-ribosyltransferase C3 toxin from \textit{Clostridium botulinum}, which selectively modifies Rho (RhoA, RhoB, and RhoC) over Rac or Cdc42 proteins, at the site (Asn41) located in the effector region of the GTPase, causing protein inactivation.\textsuperscript{24} As shown in Figure 5, C3 exoenzyme inhibited preproET-1 mRNA expression in a time- and concentration-dependent manner, reaching the most pronounced effect at 0.1 \textmu g/mL after 24-hour treatment (20-fold decrease). This suggests that Rho proteins regulate the expression of the preproET-1 gene.

To confirm the involvement of Rho proteins in the control of preproET-1 gene expression, we performed transient transfections with the pET1-LUC plasmid in the presence of different expression vectors that coded for wild-
type and dominant-negative forms of the RhoA and RhoB proteins. As shown in Figure 6, transient expression of the wild-type rhoA and rhoB genes resulted in significantly increased promoter activity (200±20% and 169±5%, respectively; n=3). Transfection experiments with the dominant-negative mutants (N19RhoA and N19RhoB) led to a significant reduction in promoter activity (39±9% and 45±9% inhibition for RhoA and RhoB mutants, respectively; n=3). A typical experiment with the RhoA constructs would offer the following pattern in relative luciferase units: insertless vector 0.99±0.15, N19RhoA 0.53±0.05, and wild-type RhoA 2.26±0.46; triplicate determinations). In contrast, in transient transfection experiments performed with the dominant-negative mutant of the ras gene, a stimulation of preproET-1 promoter activity was observed (data not shown).

On the whole, these data support that Rho proteins are specifically involved in the control of basal expression of the preproET-1 gene.

Simvastatin Inhibits Rho Protein–Mediated Induction of PreproET-1 Promoter Activity

To provide a link between the effects of simvastatin and the role of Rho proteins on the expression of the preproET-1 gene, we explored whether simvastatin could inhibit the stimulatory effects of RhoA and RhoB overexpression on preproET-1 promoter activity. Simvastatin significantly reduced the strong stimulation of preproET-1 promoter activity induced by a constitutively active RhoA mutant (L63-RhoA, Figure 7A). Likewise, the addition of simvastatin for 24 hours after BAECs were transiently transfected with −0.65-pET1-LUC and different Rho expression vectors resulted in a significant inhibition of the stimulatory effects of wild-type RhoA and RhoB proteins (Figure 7B). The degree of inhibition achieved in RhoA-transfected cells was similar or even higher than that observed in cells transfected with the empty vector (control in Figure 7), thus suggesting that the inhibitory action of simvastatin on preproET-1 gene expression is mediated in part by an effect on the Rho proteins, most probably related to an interference with their correct processing.

Effect of Simvastatin on the Expression of PreproET-1 Is Independent of NO

Because NO has been shown to affect the production of ET-1 and because simvastatin may regulate NO synthesis in some endothelial cell types, it was important to rule out the potential role of NO on the effects herein described. We previously reported that statins did not affect basal eNOS expression or activity in BAECs. In addition, a competitive NOS antagonist, Nω-nitro-l-arginine methyl ester (L-NAME), did not revert the inhibitory effect of simvastatin on either preproET-1 mRNA expression (Figure 8A) or preproET-1 promoter activity (Figure 8B).
Discussion

In the present work, the data were consistent with an inhibitory effect of statins on preproET-1 gene transcription. Moreover, we identified geranylgeranylation as an essential requirement for the basal expression of the preproET-1 gene. Finally, we propose that Rho GTPases regulate the expression of the preproET-1 gene, establishing a link between the function of Rho GTPases and the expression of a vasoconstrictor peptide that plays an essential regulatory role in vascular tone and cell proliferation.

Both nuclear run-on experiments and transfection studies with the preproET-1 promoter indicate a transcriptional downregulatory effect of statins. The requirement of an intermediate product modified by geranylgeranylation is supported by experiments in which GGPP was able to prevent statin-mediated repression of the preproET-1 gene and by the fact that a geranylgeranyltransferase I inhibitor recapitulated the effect of simvastatin. In contrast, the failure of FPP to prevent the effect of simvastatin and the slight increase in preproET-1 expression observed in the presence of FTI-277 render unlikely the possibility that a farnesylated product is responsible for statin-mediated changes in preproET-1 expression. The downregulation of preproET-1 gene expression observed in the presence of low concentrations of Clostridium botulinum C3 exotoxin, which selectively inhibit Rho but not Ras or Rac, strongly suggests a parallelism between the simvastatin-mediated effect and Rho inhibition. Studies with pharmacological inhibitors of the prenyltransferases, as well as transfection experiments with different functional versions of RhoA and RhoB GTPases, are consistent with the participation of Rho in the preproET-1 expression mechanism. Dominant-negative mutants of both proteins clearly inhibited the basal activity of the preproET-1 promoter, whereas wild-type or constitutively active isoforms were markedly stimulated.

One of the questions that remains to be addressed is the potential mechanism by which Rho proteins regulate preproET-1 promoter activity. The transcription factors activator protein-1 and GATA-2 play a crucial role in the expression of preproET-1 through a cooperative interaction. Some members of the Rho family of GTPases, RhoA, Rac1, and CDC42Hs, have been shown to regulate transcription through c-Fos serum response elements. A complete canonical sequence for serum response elements is not present within the functional 5′ regulatory region used in our experiments. However, it is possible that a decrement in c-Fos expression mediated by simvastatin could depress intracellular activator protein-1 levels, thus decreasing the expression of the preproET-1 gene. In this regard, an inhibitory effect of statins on the expression of c-Fos and c-Jun mRNA and protein has been reported in proliferating renal epithelial tubular cells.

The downstream pathways by which ET-1 promotes cell proliferation and tumor growth have been intensely studied. Rho proteins have been identified as part of this route in cardiac myocytes. It is therefore plausible that the inhibition of Rho proteins participates in the growth inhibitory action of statins, germane to the ET-1 proliferative response, as has been suggested for platelet-derived growth factor on vascular smooth muscle cell proliferation.

In the context of endothelial dysfunction, Rho proteins have also been proposed to downregulate eNOS expression, thus accounting for the upregulatory effect of statins on eNOS levels. In proliferating endothelial cells, a reciprocal regulation between NO and ET-1 has been observed. Therefore, it is tempting to speculate that this interdependence may rely on a common set of mediators or cellular switches, among which Rho GTPases are strong candidates. In this setting, signals that trigger the activation of Rho GTPases within the vascular wall would enhance vasoconstrictive and proliferative responses while simultaneously depressing vasodilating and growth-inhibitory molecules.

Figure 8. Effect of L-NAME on simvastatin-mediated inhibition of the preproET-1 (ppET1) gene. A, Northern analysis of preproET-1 mRNA in BAECs treated with vehicle, simvastatin (10 μmol/L), or L-NAME (0.5 mmol/L) for 24 hours. Shown is the densitometric analysis of preproET-1 levels corrected with β-actin expression. Data are mean±SEM of 4 independent experiments. B, BAECs were transfected with −0.65-pET1-LUC, pRL-CMV, and L63-RhoA plasmids. After 24 hours, they were treated as described in A. Firefly luciferase activity was corrected with Renilla luciferase activity. Data are expressed relative to values obtained in cells transfected in the absence of L63-RhoA and treated with vehicle. Shown is the mean of a representative experiment in duplicate, from a total of 2.
such as NO. Of interest, the overexpression of RhoA in cardiac muscle has been shown to contribute to heart contractile failure.\(^3\) Thus, it seems sound to consider Rho GTPases as potential pharmacological targets for endothelial dysfunction and vascular wall proliferative disease.

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References

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