Posttranscriptional Regulation of Endothelial Nitric Oxide Synthase During Cell Growth

Charles D. Searles, Yoichi Miwa, David G. Harrison, Santhini Ramasamy

Abstract—The expression of the endothelial NO synthase (eNOS) is dramatically influenced by the state of cell growth. In proliferating cells, mRNA levels are increased 4-fold compared with postconfluent, nonproliferating cells. Nuclear run-on analysis indicated that there is no difference in the transcriptional rate of eNOS in proliferating versus postconfluent cells. The half-life of eNOS mRNA, measured after actinomycin D transcriptional arrest, was 3-fold greater in preconfluent compared with confluent endothelial cells. Using UV–cross-linking analysis, a cytoplasmic protein with an apparent molecular mass of 51 kDa was found to bind to terminal 5′-end eNOS mRNA 3-fold more in confluent cells than in proliferating cells. Further characterization of the eNOS mRNA indicated that a 43-nt sequence at the origin of the 3′-untranslated region (UTR) is critical in binding of this protein. Endothelial cells were stably transfected with a chimeric cDNA plasmid containing chloramphenicol acetyl transferase (CAT) ligated to the eNOS coding region and either the wild-type 3′-UTR (pcDNA CAT/eNOSwtUTR) or a mutant 3′-UTR lacking the 43 nt found to bind the 51-kDa protein (pcDNA CAT/eNOSmutUTR). The CAT/eNOS mRNA half-life was dramatically stabilized in these latter cells as compared with cells transfected with pcDNA CAT/eNOSwtUTR). Thus, this 43-nt region plays a critical role in destabilizing eNOS mRNA. These studies demonstrate a mechanism for modulation of eNOS expression during cell growth via interactions between the proximal 3′-UTR and a novel 51-kDa cytosolic protein. (Circ Res. 1999;85:588-595.)

Key Words: mRNA stability ■ proliferation ■ RNA binding protein ■ 3′-untranslated region ■ gene regulation

Nitric oxide produced by endothelial NO synthase (eNOS) promotes vasodilation, inhibits smooth muscle cell growth, diminishes platelet aggregation, and decreases leukocyte adhesion. Although eNOS is constitutively expressed, it has become clear that its expression is subject to modest, but likely important, degrees of regulation. In cultured cells, eNOS expression is increased by shear stress, cyclic strain, hypophosphatidylcholine, low concentrations of oxidized LDL, oxidized linoleic acid, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. In vivo, exercise training in dogs increases eNOS expression and the ability of blood vessels to dilate in response to agonists that release endogenous nitric oxide (NO).

In contrast, exposure of cultured endothelial cells to tumor necrosis factor (TNF)-α, hypoxia, and high concentrations of oxidized LDL decreases eNOS levels. In these latter conditions, posttranscriptional changes in mRNA half-life play an important role in downregulation of eNOS.

Earlier, our laboratory found that eNOS expression is rather dramatically influenced by the state of endothelial cell proliferation. The mRNA for eNOS was 4- to 6-fold greater in proliferating cells as compared with cells several days after confluence. In addition, protein expression (by Western analysis) and enzyme activity (by arginine-to-citrulline conversion) were similarly increased in proliferating versus confluent cells. In this study, the mechanisms responsible for the increase in eNOS mRNA expression during cell growth were not defined. Thus, the purpose of the present study was to examine factors responsible for changes in eNOS levels during endothelial cell proliferation. Nuclear run-on assays and studies of mRNA stability were used to examine both transcriptional and posttranscriptional mechanisms, respectively. On the basis of these results, we further examined the role of protein/RNA interactions and the role of specific portions of the 3′-untranslated region (UTR) of the eNOS mRNA in regulation of eNOS expression during cell growth. Our findings demonstrate a novel mechanism for control of eNOS levels in endothelial cells.

Materials and Methods

Cell Culture

Bovine aorta endothelial cells (BAECs) were harvested and cultured in medium (M199; Gibco Laboratories) containing 10% FCS (HyClone Laboratories) as described earlier. Experiments were conducted on cells between passages 3 and 9. Cells were split at a ratio of 1:6. Preconfluent cells were studied 24 hours after splitting. Cells generally reached confluence 3 to 4 days after splitting and were studied at least 3 days after the cells made uniform cell-to-cell contact as judged by visual analysis (≈6 to 7 days after splitting).
containing, in mmol/L, Tris HCl (pH 8.0) 5; MgCl₂ 2.5; KCl 150; and ATP, GTP, and CTP 2 each, as well as 100 m
imaged as described for Northern analysis.

Nuclear Run-On Assay

Nuclear run-on assays were performed using a method described by Greenberg16 with modifications. Identical numbers of nuclei from

Preparation of RNA probes

The 3′-UTR of eNOS was excised from the full-length cDNA using

The plasmid was linearized with EcoRI, and a 545-nt riboprobe was generated using a T7 polymerase that represented the entire 3′-UTR and 96 nt of the coding region. To obtain a 35-nt probe corresponding to the AU-rich region of the eNOS 3′-UTR (3968 to 4002), oligonucleotides (sense, 5′-CATTTCAATATTATTATTAGAG - ATTTACCATAAG-3′, and antisense, 5′-CTTATGGTTAAATCTCTCAATATTTGGAATG-3′) were synthesized and used to produce a double-stranded DNA sequence with Kpn I and EcoRI restriction sequences added to allow directional insertion into pBluescript. The ligated plasmid was linearized with EcoRI, and T7 polymerase was used to create this AU-rich element-containing riboprobe. To generate other riboprobes from both the 3′-UTR and the coding region of eNOS, polymerase chain reaction–generated templates were used as shown in Tables 1 and 2, respectively. The template for the 43-nt sense probe was obtained by synthesizing an oligonucleotide corresponding to eNOS 3648 to 3690 with T7 promoter linked at the 5′ end. In vitro transcription reactions were performed using the T7 polymerase in the presence of [32P]UTP with a commercially available kit (MAXIscript, Ambion Inc). In general, ∼1 μg of linearized plasmid or polymerase chain reaction–generated template was incubated with 50 μCi [32P]UTP; 0.5 mmol/L each of ATP, GTP, and CTP; and 2.5 mmol/L of cold UTP, in 1× Ambion transcription buffer, with 10 units of the T7 polymerase. Reactions were allowed to proceed at 37°C for 30 to 60 minutes. Specific unlabeled RNA probes for use as competitors were synthesized by replacing labeled UTP with 0.5 mmol/L of cold UTP in the transcription reaction. After in vitro transcription, the probes were

### Table 1. Primers Used for 3′-UTR Riboprobe Construction

<table>
<thead>
<tr>
<th>Riboprobe Length</th>
<th>Primers</th>
<th>Amplified Region in eNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>349 nt</td>
<td>5′-AACACAAAAGGCTGACTGAGGCT-3′</td>
<td>3552–3900</td>
</tr>
<tr>
<td>303 nt</td>
<td>5′-GGGTCTCCAGTAGCTGAGTGGCTG-3′</td>
<td>3552–3854</td>
</tr>
<tr>
<td>229 nt</td>
<td>5′-AGCTCTGGAACTCCTTGAGGCT-3′</td>
<td>3552–3780</td>
</tr>
<tr>
<td>139 nt</td>
<td>5′-CAAGCCCTCTTCCTGGG-3′</td>
<td>3552–3690</td>
</tr>
<tr>
<td>96 nt</td>
<td>5′-TCAGGGGCGGGGGGTTGCTG-3′</td>
<td>3552–3647</td>
</tr>
<tr>
<td>76 nt</td>
<td>5′-CAACAGAGCAAGCGGTAAGGC-3′</td>
<td>3648–3723</td>
</tr>
<tr>
<td>316 nt</td>
<td>5′-GCTCTAGACTGAGTGGATCCCCGGGCTGC-3′</td>
<td>3781–4096</td>
</tr>
</tbody>
</table>

*Sequence from the pBluescript plasmid.
†Underlined sequences in sense primers refer to T7 promoter.

### Table 2. Primers Used for Coding Region Riboprobe Construction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Riboprobe Length</th>
<th>Amplified Region in eNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>GAATTGTAATACGACTCATTAGGCG</td>
<td>613 nt 1–613</td>
</tr>
<tr>
<td>Antisense</td>
<td>AGCTCCACCACGTATGGTCG</td>
<td>614–1546</td>
</tr>
<tr>
<td>Sense</td>
<td>GAATTGTAATACGACTCATTAGGCGCAGTTGTGTGATGCCG</td>
<td>933 nt</td>
</tr>
<tr>
<td>Antisense</td>
<td>TTACCGCGGTGGCCACTTGGG</td>
<td>742 nt 1547–2288</td>
</tr>
<tr>
<td>Sense</td>
<td>GAATTGTAATACGACTCATTAGGCGTCTGCTCCTCATGG</td>
<td>1263 nt 2289–3551</td>
</tr>
<tr>
<td>Antisense</td>
<td>AGCGTCTGTCACTCCTCTGGG</td>
<td></td>
</tr>
</tbody>
</table>

*Underlined sequences refer to T7 promoter.
purified by size separation in 5% acrylamide. Riboprobes were either gel purified or extracted with phenol/chloroform.

UV–Cross-Linking Assay
Nuclear and cytosolic protein fractions were prepared according to the protocol by Dignam et al.17 Radiolabeled riboprobes (10^5 to 10^6 cpm) were incubated with 10 to 20 μg of either cytosolic or nuclear proteins for 10 minutes at 4°C in a binding buffer of the following composition (in mmol/L): HEPES (pH 7.9) 10, KCl 40, MgCl₂ 3, and DTT 2; 7.5% glycerol; 5 mg/mL heparin; and 200 μg/mL yeast tRNA in a total volume of 24 μL. Subsequently, the reaction mixture was irradiated for 7 minutes in a UV–cross-linker, model 2400 (Stratagene). RNase A (20 μg) was then added to the reaction mixture for 30 minutes at 37°C. The samples were boiled for 3 minutes in Laemmli buffer and separated by electrophoresis on a 12% SDS-polyacrylamide gel. The gels were dried and exposed to a phosphor imager. Specific bands were visualized using a phosphor imager. Non-specific binding was negligible as assessed by binding to vector DNA. It should be noted that the apparent decrease in hybridization of nascent transcripts to eNOS cDNA in the blot representing confluent cells is caused by reduced background.

Western Blotting
Twenty μg of cellular protein was electrophoresed using 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. AU-1 protein was detected using a 1:5000 dilution of immune serum against AU-1 (a gift from Dr Gary Brewer, Department of Microbiology and Immunology, Wake Forest School of Medicine, Winston-Salem, NC) and secondary goat anti-rabbit antibody. Immunoreactive bands were detected using the enhanced chemiluminescence detection system (Amersham Corp) and quantified using densitometry.

Stable Transfection of Endothelial Cells
BAECs (passage 1) in 100-mm plates were transfected with 10 to 20 μg of either the plasmid pcDNACAT/eNOS9UTR, or the plasmid pcDNACAT/eNOS9UTR using Lipofectin (Gibco) according to the manufacturer's directions. Stably transfected cells were selected by addition of 500 μg/mL G418 to the culture medium and expanded in the presence of G418.

Statistical Analysis
Statistical significance was assessed by unpaired t test. Data are expressed as mean ± SEM. The mean data between different treatments were compared. Differences between the groups were considered significant when P < 0.05.

Results
eNOS mRNA Steady-State, Transcription Rate, and Half-Life in Proliferating Versus Confluent Endothelial Cells
As reported previously, steady-state eNOS mRNA levels were 4-fold higher in proliferating as compared with confluent endothelial cells (Figure 1A and 1B). To determine whether this was due to a change in eNOS transcription, nuclear run-on analyses were performed (Figure 2A). After correcting for background, the intensity of bands for eNOS nascent transcripts was identical between proliferating and confluent endothelial cells (Figure 2B). In contrast, eNOS mRNA half-life was substantially different in the 2 growth states. In confluent cells, eNOS mRNA half-life was 9 hours, whereas in proliferating cells it was ~3-fold longer (Figure 3A and 3B).

eNOS mRNA/Protein Interactions in Preconfluent and Confluent Cells
Because mRNA stability is regulated by protein/mRNA interactions, UV–cross-linking analysis was used to study RNA-protein interactions from endothelial cells of different growth states. A 32P-labeled 545-nt riboprobe corresponding to the full-length eNOS 3'-UTR and 96 bases of the terminal eNOS coding region (bases 3552 to 4096, Table 1) was used to study binding with either nuclear or cytosolic proteins between different growth states. Two proteins were identified to bind to the 545-nt probe. The larger of these, a ~75-kDa protein, was predominantly nuclear in location, and binding...
of this protein to 545-nt riboprobe did not differ between confluent and proliferating cells (Figure 4A). Interestingly, a protein with an apparent molecular mass of 51 kDa, present predominantly in the cytoplasm, bound to the 545-nt riboprobe more in confluent cells than in proliferating cells. Densitometric analysis of repeated experiments showed that the binding of this ~51-kDa protein to the 545-nt probe was 3-fold higher in confluent as compared with proliferating cells (Figure 4B). Binding of the ~75 and ~51 kDa was inhibited by preincubation of cell extracts with 100x unlabeled probe (Figure 4C).

In other experiments, 4 large riboprobes corresponding to the coding region of eNOS were used in UV–cross-linking assays but were not observed to specifically bind to proteins from either confluent or proliferating endothelial cells (data not shown).

Characterization of the cis Element in the eNOS 3′-UTR Involved in Binding of the 51-kDa Protein

A series of riboprobes corresponding to truncations of the eNOS 3′-UTR were created and used in UV–cross-linking experiments (Table 1 and Figure 5). Binding to the ~51-kDa protein was observed for riboprobes corresponding to 3552 to 3900 (349 nt), 3552 to 3854 (303 nt), and 3552 to 3780 (229 nt) of eNOS and not for riboprobe corresponding to 3781 to 4096 (316 nt) (Figure 5). In each of these cases of interaction with the ~51-kDa protein, binding was specific, as it was competed by preincubation of the cytoplasmic extract with excess respective unlabeled cold probes. Binding of the last of these, a 229-base riboprobe, to the ~51-kDa protein, was easily demonstrated (Figure 6A). Further division of this 229-nt region into a proximal 96-nt probe (corresponding to the terminal coding region) revealed no binding of the ~51-kDa protein, although binding to the larger ~75-kDa protein present in the nucleus persisted (Figure 6A). A riboprobe corresponding to the proximal portion of the 3′-UTR (a 76-nt riboprobe representing nucleotides 3648 to 3723 of eNOS) also demonstrated binding (Figure 6B) that could be competed with unlabeled probe and prevented by preincubation of the cell extract with proteinase K (data not shown). A 139-nt riboprobe representing the terminal 96 nt of the coding region and the first 43 nt of the 3′-UTR also demonstrated specific binding of the ~51-kDa protein (Figure 6B).
The above experiments suggest that the proximal 43 nucleotides of the eNOS 3'-UTR, corresponding to the sequence AAC CCC UCU UGC UUC CCA CUG CAG UUC CCG GAG AGA GGG GCU G, are critical in binding of the cytoplasmic 51-kDa protein. To confirm this, additional experiments were performed in which various excess amounts of unlabeled truncated riboprobes were used to compete for binding of the 51-kDa protein to the labeled 545-nt riboprobe. Binding to the 545-nt riboprobe was effectively competed by the unlabeled 229-nt riboprobe, the 76-nt riboprobe, and a 43-nt riboprobe, all of which included structures within the proximal eNOS 3'-UTR. In contrast, the 96-nt riboprobe representing the terminal coding region did not compete for binding of the 51-kDa protein (Figure 7).

AU-rich regions in the 3'-UTR have been shown to modulate mRNA stability in the case of several cytokines and proto-oncogenes via an interaction with AUF-1. In the 3'-UTR of eNOS, there exist 2 AUUUA repeats that could serve as a target for such regulation. However, this does not seem to be the case, as a 35-nt riboprobe encompassing these AU-rich elements did not bind to proteins in extracts from confluent cells (Figure 5). In addition, 25 ng of recombinant AUF-1 (a gift from Dr Gary Brewer) failed to produce a binding complex with the 545-nt eNOS 3'-UTR (Figure 8A). Finally, a Western analysis of proteins from proliferating and confluent BAECs using immune serum raised against AUF-1 (also supplied by Dr Gary Brewer) demonstrated 2 proteins of ~44 to 45 kDa (Figure 8B). The expression of these was predominantly nuclear and was greater in the preconfluent cells than in the confluent cells. This protein doublet corresponding to a 45-kDa protein does not relate, either in size or location, to the proteins we observed binding to the eNOS 3'-UTR.

Examination of Proximal eNOS 3'-UTR in Modulation of Chimeric CAT/eNOS mRNA Stability
To firmly establish a role of the proximal 43 nt of the eNOS 3'-UTR in regulation of mRNA stability, BAECs were stably transfected with 2 chimeric plasmid constructs. One, termed pcDNACAT/eNOS(wtUTR), consisted of the full-length eNOS coding region and 3'-UTR cloned to a cytomegalovirus-driven 270-bp portion of the CAT gene, in pcDNA plasmid. A second, termed pcDNACAT/eNOS(DUTR), was identical, except that the proximal 43 nt of the 3'-UTR had been deleted using splicing-by-overlap extension. Successful deletion of this region was confirmed by sequencing in both directions. Confluent endothelial cells were treated with actinomycin D, and the CAT/eNOS mRNA was followed by Northern analysis using a 32P-labeled 270 bp CAT probe. As shown in Figure 9, the half-life of the mRNA transcribed from
pcDNA CAT/eNOSwtUTR was ≈2 hours. In striking contrast, deletion of the proximal 43 nt of the eNOS 3'-UTR resulted in marked stabilization of the CAT/eNOS message.

Discussion
In previous studies, we demonstrated that steady-state levels of eNOS mRNA, as well as protein and enzymatic activity are markedly increased in proliferating versus confluent cells. In the present experiments, we demonstrated that this regulation of eNOS expression is entirely modulated by posttranscriptional mechanisms. Using UV–cross-linking studies, we found a ≈51-kDa cytoplasmic protein that interacted with the eNOS 3'-UTR to a greater extent in extracts from confluent cells as compared with proliferating cells. Increased binding of this ≈51-kDa cytoplasmic protein corresponded with a decrease in eNOS mRNA levels in confluent cells. Given that RNA/protein interactions are important in regulating mRNA stability, it is likely that the differential binding of this ≈51-kDa protein in the 2 growth states is critical in modulation of eNOS gene expression. In addition, we identified the 43 nt cis element in the proximal portion of eNOS 3'-UTR, responsible for binding of this protein. A transfected chimeric CAT/eNOS mRNA was dramatically stabilized by deletion of the proximal 43 bp of eNOS 3'-UTR.

It has recently become apparent that mRNA decay is a highly regulated process involving interaction of cis-acting sequences and trans-acting factors. A well-defined example of posttranscriptional regulation of eukaryotic genes is that of AU element–mediated mRNA decay. In the case of c-fos, c-myc, granulocyte-macrophage colony-stimulating factor, interleukin (IL)-1, IL-2, and IL-3, AUUUA repeats in the 3'-UTR have been shown to be critical in mRNA destabilization. The trans-acting factor binding to this motif, AUF-1, has been cloned. Interestingly, 2 AUUUA repeats are present in the eNOS 3'-UTR; however, they are unlikely to participate in modulation of eNOS mRNA half-life for the following reasons. First, a riboprobe representing these regions failed to bind proteins derived from BAECs. Second, AUF-1, the protein that recognizes this region, was negligibly expressed in the cytoplasm of confluent endothelial cells. Finally, the recombinant AUF-1 did not bind to the eNOS 3'-UTR (Figure 7). In addition to AUUUA, other 3'-UTR sequences of genes have been shown to influence gene expression. For example, binding of a yet-to-be-identified protein(s) to the pyrimidine-rich motifs has been shown to alter the half-life of mRNAs for α-globin, tyrosine hydroxylase, and GAP-43. Further, proteins interacting with cis elements in the 3'-UTR are responsible for regulating expression mRNA stability of ribonuclease reductases r1 and r2.

In addition to the cytosolic ≈51-kDa protein, a ≈75-kDa protein also bound to the 545- and 229-nt riboprobes, which were predominantly nuclear in location. Because RNA decay occurs in the cytoplasm, and this protein was not differentially expressed in the 2 growth states, it is unlikely to be involved in modulation of eNOS mRNA stability in the 2 growth conditions. For this reason, we proceeded to characterize the cis element involved in binding the cytosolic ≈51-kDa protein.

Using UV–cross-linking studies with truncated riboprobes corresponding to various portions of the eNOS mRNA, we were able to demonstrate that the proximal 43-nt portion of the 3'-UTR was critical for binding of the ≈51-kDa protein. This portion of the 3'-UTR is schematically illustrated in Figure 10, with the critical 43 nt shown in bold. This sequence is unlike previously reported cis-acting sequences involved in mRNA decay. It is quite likely that secondary structure is critical in binding the ≈51-kDa protein. As shown in Figure 10, the predicted structure of the proximal 3'-UTR of eNOS contains a stem loop with adjacent secondary structures (bold region in Figure 10), which could form a stable RNA/protein interactions are important in regulating mRNA stability, it is likely that the differential binding of this ≈51-kDa protein in the 2 growth states is critical in modulation of eNOS gene expression. In addition, we identified the 43 nt cis element in the proximal portion of eNOS 3'-UTR, responsible for binding of this protein. A transfected chimeric CAT/eNOS mRNA was dramatically stabilized by deletion of the proximal 43 bp of eNOS 3'-UTR.

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pocket into which the protein is bound. Of note, the 76- and 139-nt riboprobes seemed to exhibit weaker binding than the 229-nt riboprobe or longer riboprobes encompassing this region, again suggesting that flanking structure may enhance protein recognition. Definitive proof that the proximal 43-nt region of eNOS 3′-UTR modulates mRNA stability was derived from studies using stable transfections of CAT/eNOS constructs. In these studies, deletion of the 43-nt region resulted in an mRNA that was dramatically stabilized as compared with the wild-type CAT/eNOS mRNA.

It has previously been shown that eNOS mRNA is destabilized by the cytokine TNF-α. This likely involves a completely different mechanism as compared with growth modulation of eNOS mRNA stability. Recently, TNF-α treatment has been shown to increase protein binding to a UC-rich region in the midportion of the eNOS 3′-UTR.33 This sequence is quite different from that identified in our present study.

RNA binding proteins may serve dual roles. For example, the iron-regulatory protein that recognizes transferrin and ferritin mRNAs is identical to aconitase, a Krebs cycle enzyme.33 GAPDH has been shown to bind to AU-rich sequences of mRNAs and modulate RNA turnover in addition to its well-described role in glycolysis.34 Likewise, heat shock protein-70 (HSP-70) has been shown to modulate turnover of erythropoietin mRNA in addition to its role in stress responses.35 At present, the ~51-kDa protein that we have observed binding to the proximal 3′-UTR of eNOS has not been characterized in terms its structure or sequence; however, it is conceivable that it may also be a protein with other functions.

Of note, the half-life of eNOS mRNA in confluent cells (9 hours) in this study was substantially lower than that reported previously.12–14 The explanation for this remains unclear; however, it may relate to differences in the state of growth, the origin of the cells, or the cell culture conditions. Of interest, the half-life of eNOS mRNA in proliferating cells in this present study was similar to that previously reported. The use of actinomycin D to study mRNA decay in confluent cells growing over the denuded region was successful in proliferating cells in vivo in rat aortas.38 There has been substantial interest in the role of nitric oxide in modulating vessel growth and development. For example, it has been shown that tube development by growing endothelial cells in 3-dimensional gels in response to transforming growth factor β is dependent on nitric oxide and inhibited by antagonists of NO synthase.39 Although this role of nitric oxide is controversial,40,41 it is possible that increased production of nitric oxide by endothelial cells during the angiogenesis process plays an important role in vessel development. Future studies of interactions of the eNOS mRNA with the ~51-kDa protein identified in the present study and potentially other trans-acting factors may provide mechanistic insight into regulation of eNOS in these various physiological and pathophysiological states.

Acknowledgments

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