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Endothelial Cytosolic Proteins Bind to the 3′ Untranslated Region of Endothelial Nitric Oxide Synthase mRNA: Regulation by Tumor Necrosis Factor Alpha

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Changes in endothelial nitric oxide synthase (eNOS) expression may be involved in the endothelium-dependent vasorelaxation dysfunction associated with several vascular diseases. In the present work, we demonstrate that eNOS mRNA contains a previously undescribed *cis* element in the 3′ untranslated region (3′ UTR). A U+C-rich segment in the 3′ UTR is critical in complex formation with bovine aortic endothelial cell cytosolic proteins. Tumor necrosis factor alpha (TNF-α), which destabilizes eNOS mRNA, increased the binding activity of the cytosolic proteins in a time-dependent manner. These data suggest that endothelial cytosolic proteins bind to the 3′ UTR of eNOS mRNA. These proteins may play a role in TNF-α-induced eNOS mRNA destabilization.

Nitric oxide (NO) is a gas generated by the metabolic conversion of L-arginine into L-citrulline by the activity of NO-synthesizing enzymes (NO synthases [NOS]) (23). Two major types of NOS activities have been identified in the vessels: an endothelial isoform (eNOS), which is localized in the endothelium under physiological conditions, and an inducible isoenzyme, which requires cytokines or endotoxin activation for its expression (23, 29). Loss of endothelium-derived NO results in vascular abnormalities including vasoconstriction, smooth muscle cell proliferation, and adhesion of blood elements to the vessel walls (12, 16, 23, 26).

Although the eNOS isoform had been initially described as constitutive, in recent years it has been demonstrated that several pathophysiological stimuli, such as hypoxia, chronic exercise, and the subconfluent growth state, upregulate eNOS expression in endothelial cells (13, 18, 30). On the other hand, cytokines, and more particularly tumor necrosis factor alpha (TNF-α), downregulate the expression of eNOS through a to-date undescribed mechanism, although it is well known to occur through destabilization of eNOS mRNA (22, 35).

The regulation of mRNA stability has emerged as an important control mechanism for regulating cellular mRNA levels. Although the mechanisms that control the mRNA stability of different genes have unique features, it appears that in each case specific RNA sequences are required for the recognition of protein factors (11, 36). Some of these specific sequences have been identified within the 3′ untranslated region (3′ UTR). The 3′ UTR interacts with *trans*-acting factors (regulatory proteins) which may affect the half-lives of the mRNAs. In this regard, the iron-responsive element in the 3′ UTR of transferrin receptor mRNA and the corresponding binding protein constitute the best-characterized example of the control of mRNA half-life (28). Other examples are the unstable AUUUA motifs found in the 3′ UTRs of many mRNAs, including those encoding proto-oncogenes, cytokines, and lymphokines (for reviews, see references 27 and 36). The existence of several *trans*-acting factors interacting with these AUUUA motifs has also been reported (27, 36).

eNOS mRNA possesses repeated AUUUA sequences in its 3′ UTR (21). Therefore, these sequences can potentially interact with endothelial proteins, and they may be involved in the regulation of the life span of eNOS mRNA. However, to date there are no reports on possible *trans*-acting factors that interact with the 3′ UTR of eNOS mRNA. In the present study, we demonstrate the existence of endothelial cytosolic proteins which specifically bind to the 3′ UTR of eNOS mRNA.

Decreased expression of eNOS may contribute to the development of vascular disorders in which increased production of TNF-α and impaired endothelium-dependent dilator responses have been reported (3, 19). Accordingly, our aim was to assess whether TNF-α, which decreases the half-life of eNOS mRNA (22, 35) in endothelial cells, changes the binding activity of the already mentioned endothelial cytosolic protein to the 3′ UTR of eNOS mRNA.

MATERIALS AND METHODS

Cell culture and lysate preparation. Bovine aortic endothelial cells (BAEC) were obtained and cultured as previously described (15, 17). To prepare cytoplasmic lysates, confluent monolayers of BAEC were washed twice in ice-cold phosphate-buffered saline, gently scraped in cold detaching buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1 mM EDTA), and transferred to microcentrifuge tubes. After brief centrifugation, the cells were resuspended in hypotonic buffer (25 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and lysed by four cycles of freezing and thawing followed by centrifugation at 12,000 × *g* at 4°C for 15 min. The supernatant was removed, supplemented with glycerol (10% final concentration), and frozen at −70°C until use. The protein content of the cytosolic extracts was determined by the micro-BCA method as described by the manufacturer (Pierce, Rockford, Ill.).

Plasmids and in vitro transcription. Oligonucleotides complementary to bovine eNOS cDNA (GenBank accession no. BTNIOXSY) were purchased from Bio-Synthesis Inc. (Lewisville, Tex). Oligonucleotide 1 (5′-GGATCTAGAACG CTATCACGAGGACATT-3′) and oligonucleotide 2 (5′-AGGAAGCTTAG TAGGTCTCCTAACTTCTG-3′) were used to produce by reverse transcriptase PCR (from BAEC total RNA) a fragment covering 166 bases of the coding region and 393 bases of the 3′ UTR of eNOS cDNA (from 3485 to 4012). Amplification products were purified after agarose gel electrophoresis, subjected to restriction endonuclease digestion with *Xba*I and *Hin*dIII, and ligated to pGEM4Z (Promega, Madison, Wis.) to create plasmid pNOS-UTR-L. Oligonucleotide 3 (5′-GTTGGATCCCCTGTACTATCTCACCCT-3′) and oligonucleotide 2 were used to make by PCR a fragment covering 261 bases of the 3′ middle of the 3′ UTR. The product was subjected to restriction endonuclease digestion with *Bam*HI and *Hin*dIII and inserted in pGEM4Z to create pNOS-UTR-S. To

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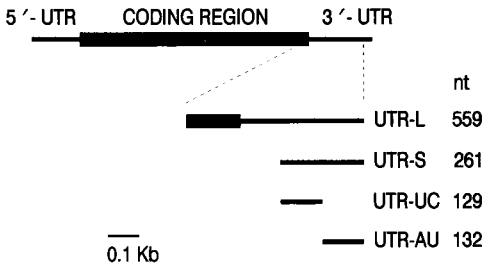

FIG. 1. eNOS RNA probes used in gel mobility shift assays. The diagram shows different segments of the 3′ UTR from eNOS mRNA used in this study. A fragment covering 166 bases of the coding region and 393 bases of 3′ UTR mRNA (UTR-L), the 3′ middle of UTR-L (UTR-S), the adenine–uridine-rich region covering the 3′ half of the UTR-S (UTR-AU), and the uridine–cytidine-rich fragment in the 5′ half of the UTR-S probe (UTR-UC) are shown. The entire eNOS mRNA is not drawn to scale.

make plasmid pNOS-UTR-UC (129 bases from the 5′ end of pNOS-UTR-S), pNOS-UTR-S was cut with *Acs*I and the corresponding fragment was purified by agarose gel electrophoresis and inserted into the *Eco*RI site of pGEM4Z. To make pNOS-UTR-AU (132 bases from the 3′ end of pNOS-UTR-S), *Acs*I-linearized pNOS-UTR-S was purified by agarose gel electrophoresis and religated. To create plasmid pGM-CSF-UTR, two 66-mer oligonucleotides including 53 bases of the AU-rich 3′ UTR of human granulocyte-macrophage colony-stimulating factor (GM-CSF) (34) (GM-CSF 1, 5′-CTAGATCTGTAATATTTTATATATTTATATTTTTAAAATATTATTTATTTATTTATTTAAGATCTG-3′; GM-CSF 2, 5′-TCGACAGATCTTAAATAAATAAATAAATAAATATTTTAAAAATATAAATATATAAATATTACAGAT-3′) were mixed in equimolar amounts, boiled in 10 mM Tris-HCl–500 mM NaCl, and slowly cooled to room temperature for annealing. Duplex oligonucleotides were inserted in the *Xba*I and *Sal*I sites of pGEM4Z.

To produce single-stranded RNA, plasmids were linearized with the corresponding restriction enzyme and transcribed with SP6 or T7 RNA polymerase. Radiolabeled RNA was produced according to the manufacturer's recommendations (Promega) with [^{32}P]UTP or [^{32}P]CTP (10^9 cpm/μg; Amersham Iberica, Madrid, Spain). For competition experiments, unlabeled RNA was synthesized with the RiboMax Large Scale RNA System (Promega).

Band shift assays. Five micrograms of cytoplasmic lysates were incubated with different radiolabeled RNA probes (5×10^4 to 10×10^4 cpm; approximately 1 ng of RNA) in 15 mM HEPES (pH 7.9), 10 mM KCl, 5 mM Cl_2Mg, 1 mM dithiothreitol, 1 μg of yeast tRNA per μl, 40 U of RNasin (Promega), and 10% glycerol in a total volume of 15 μl for 10 min at 25°C. Then, 20 U of RNase T_1 per reaction (Gibco-BRL, Dieselstrasse, Germany) was added and the reaction mixtures were incubated for 30 min at 37°C. Samples were electrophoresed on a 4% native polyacrylamide gel in 0.25× TBE (Tris-borate-EDTA) as running buffer, dried, and autoradiographed with Kodak X-Omat-S film. For competition experiments, different amounts of unlabeled RNAs were preincubated for 10 min with cytosolic lysates prior to the addition of radiolabeled RNA.

RNase T_1 mapping. To determine the RNA sequence protected by the complexes, binding reactions between eNOS-UTR-UC RNA and cytoplasmic proteins were performed as aforementioned (see "Band shift assays"). Following electrophoresis of the RNA-protein complex, the gel was exposed to X-ray film at 4°C overnight. The specific RNA-protein bands were cut from the gel, transferred to a dialysis bag, and electroeluted in 1× TBE buffer at 150 V for 2 h at 4°C. The electroeluted RNA was supplemented with yeast tRNA (30 μg/ml), extracted with phenol-chloroform, and precipitated with ethanol. The intact eNOS-UTR-UC RNA, as well as half of the eluted RNA, was subsequently digested with 20 U of RNase T_1 in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA for 30 min at 37°C. Both reaction mixtures, as well as the nondigested half of the eluted RNA and nondigested intact eNOS-UTR-UC RNA, were electrophoresed on a 15% denaturing polyacrylamide gel. The gel was dried and exposed to X-ray film at −70°C. ^{32}P-RNA size markers were synthesized from *Eco*RI-, *Bam*HI-, and *Hin*dIII-linearized pGEM4Z with SP6 RNA polymerase (11, 32, and 62 nucleotides, respectively) and run in parallel with the samples. A comparison of the sequences of the RNase T_1-digested fragments from the eluted RNA and from the eNOS-UTR-UC, which was not incubated with the cytoplasmic lysates, allowed the identification of sequences protected from RNase T_1 digestion by the bound proteins.

Northern blot analysis. Confluent BAEC between the first and third passages were incubated with TNF-α (10 ng/ml) during different periods of time. Total RNA was isolated according to the methods of Chomczynski and Sacchi (7). Twenty micrograms of RNA was fractionated on 1.3% agarose-formaldehyde gels and transferred by capillarity to Genescreen nylon membranes (Dupont, Boston, Mass.). Membranes were prehybridized at 42°C for 4 h in a solution containing 50% formamide, 1% sodium dodecyl sulfate, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 100 μg of denatured herring sperm DNA per ml, and 50 mM phosphates (pH 6.5) and hybridized for 16 to 18 h in the same solution supplemented with dextran sulfate (10% final concentration) containing 500,000 cpm of radiolabeled eNOS probe per ml. The cDNA probe used was the *Eco*RI-*Bam*HI fragment of pNOS-UTR-L. We used a probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for loading.

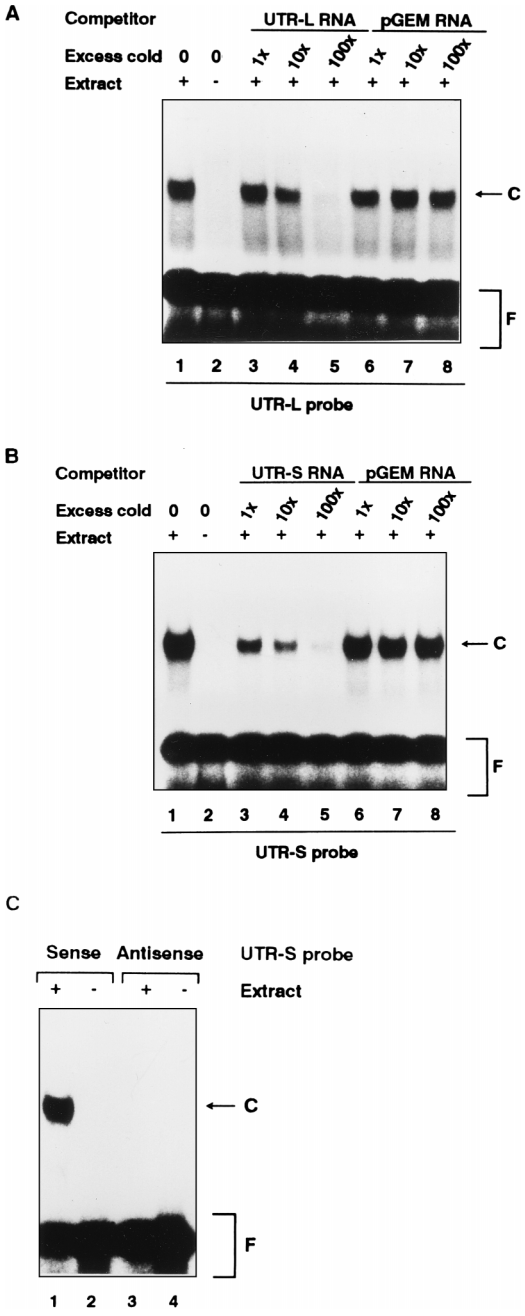

FIG. 2. Gel mobility shift assay with the 3′ UTRs of eNOS mRNA. (A) The autoradiograph shows results of gel mobility shift assays using the UTR-L probe incubated with (lane 1) and without (lane 2) cytosolic extracts. Competition studies were performed with increased amounts of unlabeled UTR-L (specific competitor, lanes 3 to 5) and polylinker pGEM RNA (nonspecific competitor, lanes 6 to 8). (B) Gel mobility shift assay using the UTR-S probe. Competitions were performed with increased amounts of unlabeled UTR-S (lanes 3 to 5) and polylinker pGEM RNA (lanes 6 to 8). (C) Gel mobility shift assay using the sense or antisense labeled UTR-S probes incubated with BAEC cytosolic extracts. The amount of each RNA probe used was 1 ng. 1× = 10 ng of RNA; C, complex; F, free probe.

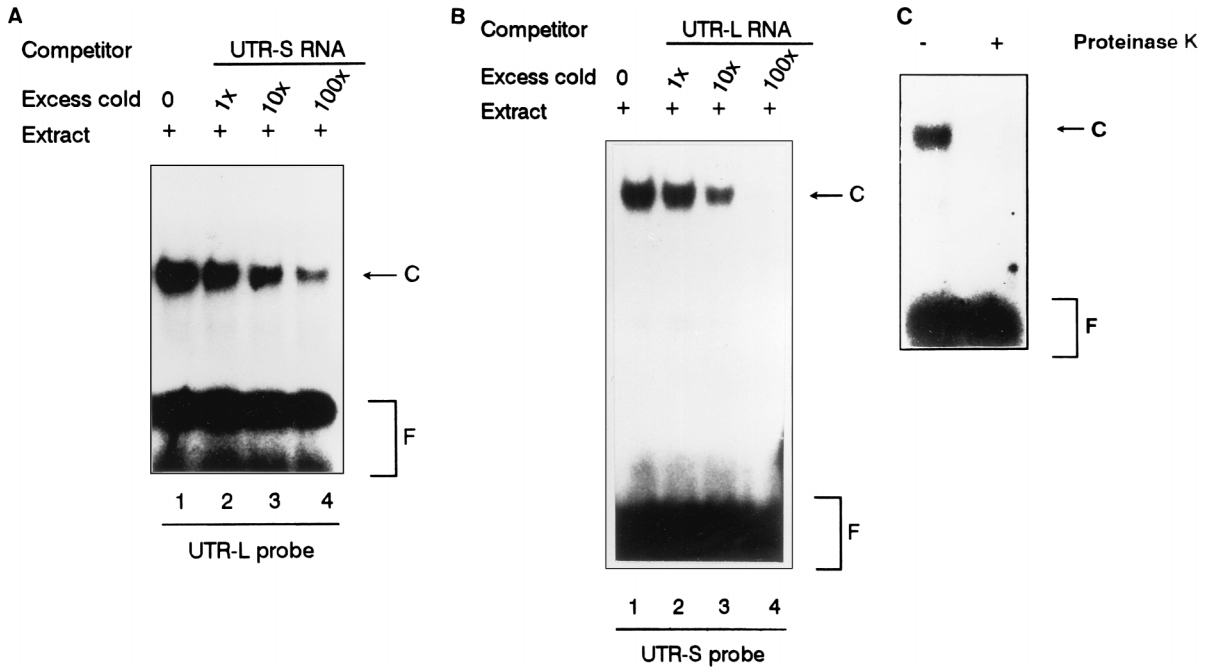

FIG. 3. Competitive experiments between UTR-L and UTR-S probes. (A) Gel mobility shift assay showing competition experiments using labeled UTR-L probe together with increased amounts of unlabeled UTR-S probe. (B) Competition experiments using labeled UTR-S with unlabeled UTR-L. (C) Treatment of endothelial cytosolic extracts with proteinase K prior to incubation with the UTR-UC probe fully abolished the gel-shifted band. The amount of each RNA probe used was 1 ng. 1× = 10 ng of RNA; C, complex; F, free probe.

Western blot analysis. eNOS protein expression was analyzed in confluent BAEC by Western blotting as previously described (18). BAEC were incubated with TNF-α (10 ng/ml) during different periods of time. BAEC were then lysed in Laemmli buffer containing 2-mercaptoethanol. Proteins were separated on denaturing sodium dodecyl sulfate–10% polyacrylamide gels (15 μg/lane) and blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol of Tris-HCl per liter, 137 mmol of NaCl per liter, 0.1% Tween 20). Western blot analysis was performed with a monoclonal antibody against eNOS. Blots were incubated with the first antibody (1:500) for 1 h at room temperature and, after extensive washing, with the second antibody (horse horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody) at a dilution of 1:1,500 for 1 h further. Specific eNOS protein was detected by enhanced chemiluminescence (ECL; Amersham Iberica). Prestained protein markers were used for molecular mass determinations. As previously reported (18), this monoclonal antibody specifically recognizes the eNOS isoform (135 kDa) and does not cross-react with the inducible isoform, because the band of eNOS protein was undetectable in a homogenate of lipopolysaccharide-treated mouse macrophages and it reacted with positive controls obtained from homogenates of human endothelial cells (data not shown).

RESULTS

Binding of cytosolic endothelial proteins to the 3′ UTR of eNOS mRNA. To determine whether endothelial cell proteins bound specifically to the 3′ UTR of eNOS mRNA, we generated a series of plasmids whose in vitro transcription yielded RNAs containing different segments of the 3′ UTR of eNOS mRNA (Fig. 1).

We first examined the ability of endothelial cytoplasmic extracts to bind to a probe corresponding to the entire 3′ UTR (probe UTR-L) (Fig. 1), by using an RNA gel mobility shift assay. The addition of endothelial cytoplasmic extract to the labeled UTR-L probe resulted in a gel-shifted band (Fig. 2A, lane 1). To analyze the specificity of the mRNA-protein complex, we performed competition experiments. Increased concentrations of unlabeled UTR-L prevented labeled UTR-L-protein complex formation (Fig. 2A, lanes 3 to 5), while a nonrelated RNA, pGEM RNA, did not prevent complex formation (Fig. 2A, lanes 6 to 8). The UTR-L probe contained 393 nucleotides of 3′ UTR eNOS mRNA. Therefore, to localize more specifically the binding site for endothelial cell cytosolic extracts in 3′ UTR eNOS mRNA, further experiments were performed using a probe containing 261 nucleotides of the 3′ end of the 3′ UTR, which we named UTR-S (Fig. 1). A shifted band similar to that observed with the UTR-L probe was obtained with the UTR-S probe (Fig. 2B, lane 1). UTR-S-endothelial cytosolic complex formation was also prevented by unlabeled UTR-S (Fig. 2B, lanes 3 to 5) but not by the nonspecific competitor, pGEM RNA (Fig. 2B, lanes 6 to 8). The complex between endothelial cytosolic extracts and the UTR-S probe was not observed when the extracts were incubated with probes consisting of labeled antisense UTR-S (Fig. 2C).

The unlabeled UTR-S probe prevented the endothelial cytosolic protein from binding to the labeled UTR-L probe (Fig. 3A), and the unlabeled UTR-L removed the protein-UTR-S RNA complex (Fig. 3B). These results indicated that no additional binding site was present in 3′ UTR eNOS mRNA outside the UTR-S fragment. Treatment of the cytosolic extracts with proteinase K prior to their incubation with the UTR-UC probe abolished complex formation (Fig. 3C), indicating the involvement of cytosolic proteins in complex formation.

Endothelial cytosolic proteins specifically bind to the U+C-rich region of the eNOS 3′ UTR. Analysis of the nucleotide sequence of UTR-S shows two differentiated regions: (i) an adenine–uridine (A+U)-rich region with two AUUUA pentamers in the 3′ half of the UTR-S probe, and (ii) a uridine–cytidine (U+C)-rich region in the 5′ half of the UTR-S probe. To determine whether the AU-rich region, the UC-rich region, or both were implicated in complex formation, we made probes containing these two particular fragments (Fig. 1) and analyzed complex formation with endothelial cytosolic proteins by RNA gel mobility shift assays.

Gel-shifted bands were observed with the labeled probe containing the UC-rich region (UTR-UC RNA) but not with UTR-AU-rich RNA labeled with either [^{32}P]UTP or [^{32}P]CTP

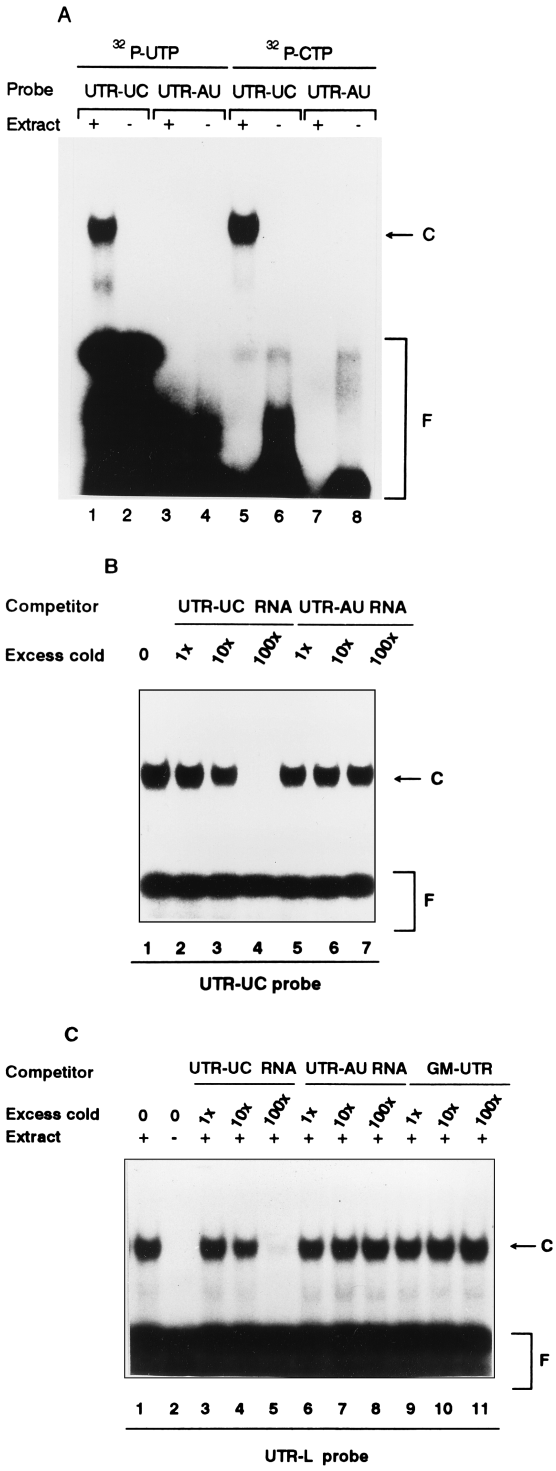

FIG. 4. Specificity of complex formation with UTR-UC. (A) Gel mobility shift assay showing the binding of BAEC cytosolic extracts to labeled probes containing the uridine–cytidine-rich region (UTR-UC, lanes 1 and 5) and the adenine–uridine-rich region (UTR-AU, lanes 3 and 7) of eNOS mRNA. Both probes were labeled with either [^{32}P]UTP (lanes 1 and 3) or [^{32}P]CTP (lanes 5 and 7). (B) Gel mobility shift assay using the UTR-UC probe. Competition experiments were performed with increased amounts of unlabeled UTR-UC (lanes 2 to 4) or UTR-AU (lanes 5 to 7) probes (1× = 10 ng of RNA). (C) Inhibition of complex formation with labeled UTR-L by increased amounts of unlabeled UTR-UC (lanes 3 to 5), but not by unlabeled UTR-AU (lanes 6 to 8) or unlabeled GM-CSF 3′ UTR RNA (GM-UTR, lanes 9 to 11). The GM-CSF 3′ UTR probe was used as a prototype of the AU-rich element. The amount of each RNA probe used was 1 ng. 1× = 10 ng of RNA; C, complex; F, free probe.

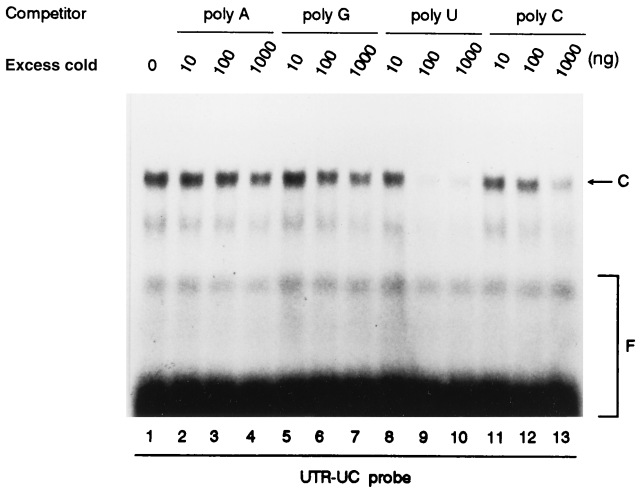

FIG. 5. Competitions with RNA homopolymers. Gel mobility shift assays were carried out with the UTR-UC probe and endothelial cytosolic extracts. As indicated, increased amounts of poly(A) (lanes 2 to 4), poly(G) (lanes 5 to 7), poly(U) (lanes 8 to 10), and poly(C) (lanes 11 to 13) RNA homopolymers were used as competitors (from 10 to 1,000 ng). C, complex; F, free probe. The amount of radiolabeled RNA probe used was 1 ng.

(Fig. 4A). The complex between labeled UTR-UC and the cytosolic proteins was removed by the unlabeled UTR-UC probe, but not by unlabeled UTR-AU RNA (Fig. 4B). Unlabeled UTR-UC RNA prevented complex formation with the labeled UTR-L probe (Fig. 4C, lanes 3 to 5), while competition experiments with probes containing the AU-rich region had no impact on complex formation (Fig. 4C, lanes 6 to 8). Furthermore, unlabeled 3′ UTR RNA of GM-CSF, which is a prototype of AU-dependent mRNA half-life regulation, did not prevent this protein-UTR-L complex formation (Fig. 4C, lanes 9 to 11). Therefore, endothelial cytosolic proteins bind to a U+C-rich region localized in the 3′ UTR of eNOS mRNA.

To further analyze the specificity of nucleotide C in the binding of the endothelial cytosolic proteins to the 3′ UTR, we performed experiments using unlabeled RNA homopolymers [poly(A), poly(C), poly(G), and poly(U)] as competitive probes of the labeled UTR-UC probe. The relative abilities of the homopolymers to remove the cytosolic protein-UTR-UC complex were as follows: poly(U) > poly(C) > poly(G) > poly(A) (Fig. 5).

Identification of the binding sequence in 3′ UTR eNOS mRNA. To identify the sequence in the 129-base fragment of the UTR-UC RNA that binds to the endothelial cytosolic proteins, we performed RNase T_1 mapping assays. Results from these experiments are presented in Fig. 6A.

RNA gel mobility shift assays were performed with [^{32}P]CTP-labeled UTR-UC RNA. Endothelial cytosolic extracts and the RNA protected by the complex were electroeluted from the gel (Fig. 6A, left panel) (see Materials and Methods).

Protected RNA (untreated by RNase T_1) migrated as a 38-base fragment upon denaturing gel electrophoresis (Fig. 6A, right panel, lane 5). To determine the sequence of the protected fragment, it was subsequently digested with RNase T_1 (Fig. 6A, lane 6), which cleaves RNA after G. The RNase T_1 digestion of the protected 38-base RNA showed a subset of fragments with 25, 7, and 4 bases. The entire UTR-UC RNA (not incubated with endothelial cytosolic extracts), after being digested with RNase T_1, was run in parallel (Fig. 6A, lane 2).

In the 129-base UTR-UC RNA, there is only one 25-base fragment (Fig. 6B) between two G's. It is accompanied upstream by a 7-base fragment and downstream by a 4-base

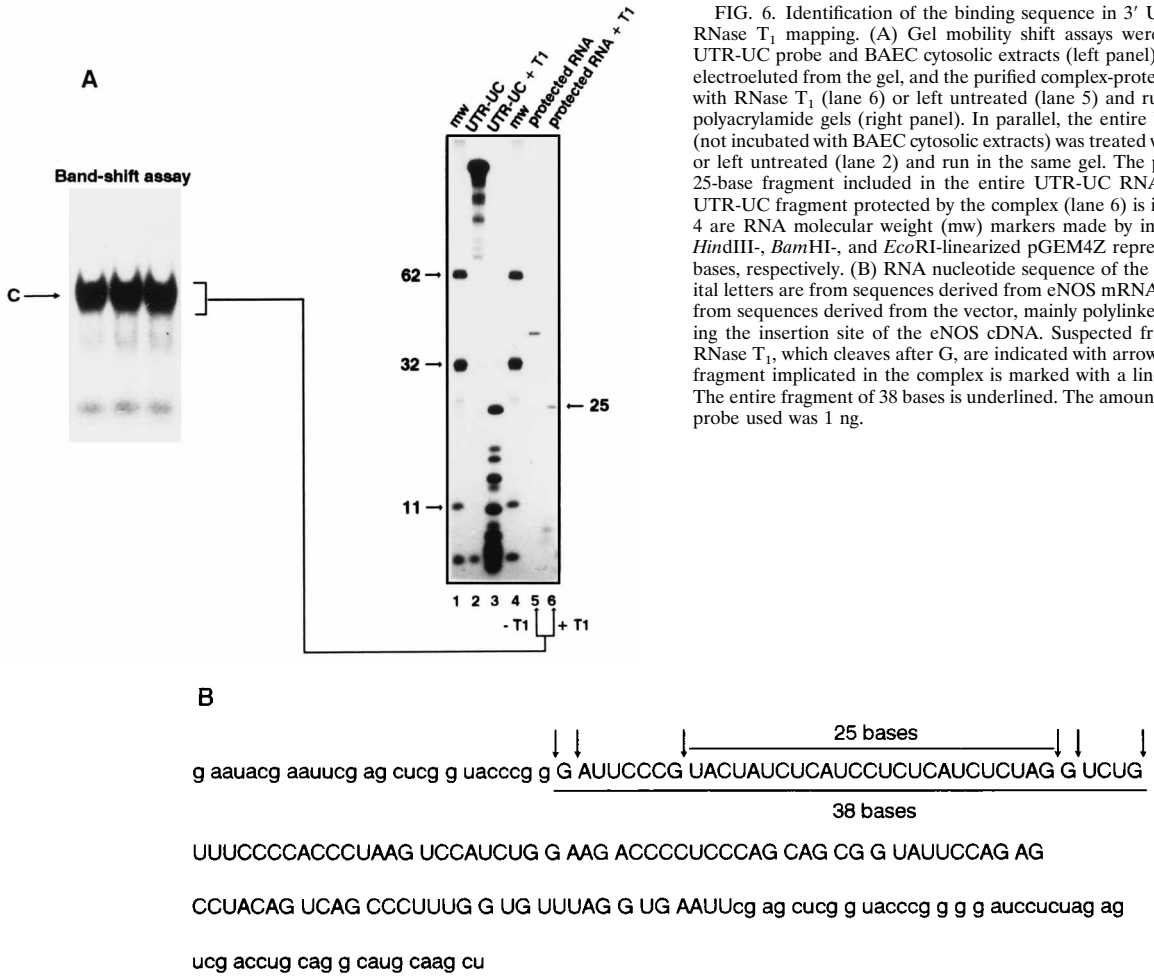

FIG. 6. Identification of the binding sequence in 3′ UTR eNOS mRNA by RNase T_1 mapping. (A) Gel mobility shift assays were performed with the UTR-UC probe and BAEC cytosolic extracts (left panel). Complexes (C) were electroeluted from the gel, and the purified complex-protected RNA was treated with RNase T_1 (lane 6) or left untreated (lane 5) and run on 15% denaturing polyacrylamide gels (right panel). In parallel, the entire UTR-UC RNA probe (not incubated with BAEC cytosolic extracts) was treated with RNase T_1 (lane 3) or left untreated (lane 2) and run in the same gel. The position of the unique 25-base fragment included in the entire UTR-UC RNA (lane 3) and in the UTR-UC fragment protected by the complex (lane 6) is indicated. Lanes 1 and 4 are RNA molecular weight (mw) markers made by in vitro transcription of *Hin*dIII-, *Bam*HI-, and *Eco*RI-linearized pGEM4Z representing 62, 32, and 11 bases, respectively. (B) RNA nucleotide sequence of the UTR-UC probe. Capital letters are from sequences derived from eNOS mRNA; lowercase letters are from sequences derived from the vector, mainly polylinker sequences surrounding the insertion site of the eNOS cDNA. Suspected fragments generated by RNase T_1, which cleaves after G, are indicated with arrows. The unique 25-base fragment implicated in the complex is marked with a line above the sequence. The entire fragment of 38 bases is underlined. The amount of radiolabeled RNA probe used was 1 ng.

fragment. Thus, RNase T_1 mapping of the protected RNA can account for 38 bases (Fig. 6B), consisting of the unique 25-base fragment in addition to 8 bases upstream and 5 bases downstream, including two G residues that are not labeled and, therefore, not shown in Fig. 6A. These results indicate that the 25-base fragment and surrounding regions are the protein binding site in eNOS mRNA. This region is highly rich in U and C residues (37% and 34%, respectively).

Changes in the binding activity of the cytosolic proteins by TNF-α stimulation of endothelial cells. Several proteins that bind to specific sequences in the 3′ UTR of many mRNAs have been implicated in the regulation of their half-lives (5, 8, 10, 32). It has been demonstrated that TNF-α stimulation of BAEC decreases eNOS mRNA half-life from >24 h to >3 h (22, 35). Therefore, we assessed whether TNF-α could change the complex formation between eNOS UTR-UC and the endothelial cytosolic proteins.

For this purpose, cytosolic extracts from BAEC were obtained at different times after TNF-α stimulation. Cytosolic extracts obtained from TNF-α-stimulated BAEC showed increased, time-dependent binding between UTR-UC and cytosolic proteins (Fig. 7A). Complex formation showed the highest activity at 12 h, and the specific binding of endothelial cytosolic proteins obtained from TNF-α-stimulated BAEC to 3′ UTR-S was prevented by the unlabeled UTR-UC probe (Fig. 7B). As already mentioned for the cytosolic extracts obtained from unstimulated endothelial cells, unlabeled UTR-AU failed to remove the complex between labeled UTR-S and the endothelial cytosolic proteins obtained from TNF-α-stimulated endothelial cells (Fig. 7B), suggesting a potential link between both binding activities (those found in TNF-α-treated and untreated BAEC). Furthermore, it is worth noting that the shifted bands detected with the cytosolic extracts obtained from TNF-α-stimulated and unstimulated BAEC run to a very similar level, supporting the hypothesis that they should be the same protein. These results also demonstrated the specificity of TNF-α-induced binding activity. In addition, incubation of cytosolic extracts with proteinase K also abolished complex formation with the UTR-UC probe (data not shown).

Northern blot analysis of BAEC total RNA showed that eNOS mRNA levels decreased in TNF-α-stimulated BAEC (Fig. 8A), and this was accompanied by a marked reduction in eNOS protein expression (Fig. 8B). The time-dependent decrease in the levels of eNOS mRNA induced by TNF-α also occurred in the presence of actinomycin D, indicating that the effect of TNF-α on eNOS mRNA expression was dependent on mRNA destabilization (data not shown). Interestingly, a negative correlation between cytosolic protein activity and eNOS mRNA stability was observed (Fig. 8C).

DISCUSSION

In the present study, we demonstrate that eNOS mRNA contains a previously undescribed *cis* element in the 3′ UTR.

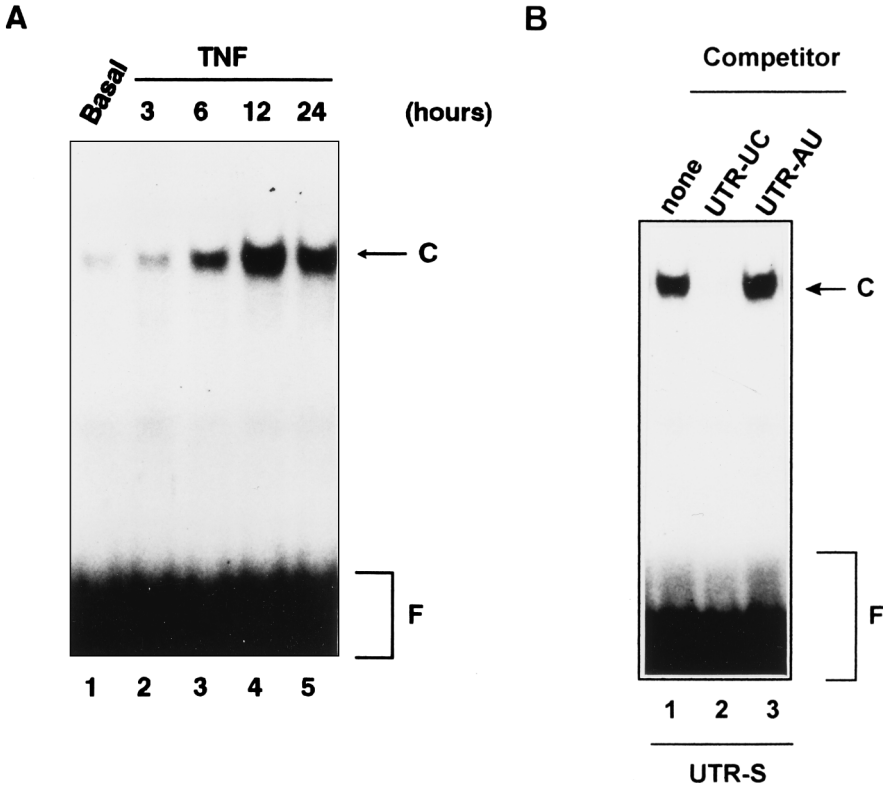

FIG. 7. Regulation of cytosolic protein binding activity by TNF-α. (A) BAEC were stimulated with TNF-α (10 ng/ml) for different periods of time, and their cytosolic extracts were analyzed by gel mobility shift assay using the UTR-UC probe. The highest binding activity was found at 12 h. (B) Inhibition of complex formation between labeled UTR-S and cytosolic extracts obtained from TNF-α-incubated BAEC. UTR-UC (100 ng) and UTR-AU (1,000 ng) were used as competitors. Endothelial cytosolic extracts were obtained 12 h after TNF-α stimulation. C, complex; F, free probe. The amount of radiolabeled RNA probe used was 1 ng.

The specific sequence within this 3′ UTR that interacts with cytosolic BAEC proteins has also been identified.

Endothelial cytosolic extract components bind to 3′ UTR eNOS mRNA. In the first set of experiments we showed that complexes were formed between BAEC cytosolic lysates and complete in vitro-transcribed labeled 3′ UTR eNOS mRNA. The resultant RNA-cytosolic lysate complexes were detected by gel mobility shift assays. The specificity of the interaction was assessed by competition experiments with unlabeled 3′ UTR RNAs.

To further characterize the 3′ UTR fragment that bound the endothelial cytosolic extracts, we used a probe containing 261 nucleotides of the 3′ end of eNOS 3′ UTR. We termed this probe UTR-S. No additional binding site, outside of this UTR-S fragment, appeared in the 3′ UTR eNOS mRNA, since unlabeled UTR-S probes removed the complex formed between the BAEC cytosolic extract and the complete labeled 3′ UTR (probe UTR-L).

Computer analysis of the nucleotide sequence of the 3′ UTR showed two different regions. In the 3′ half of the UTR-S fragment, there are two AUUUA pentamers. Many ephemeral mRNAs, including those coding for lymphokines, cytokines and oncogenes, have an AU-rich sequence in their 3′ UTRs (1, 3, 5, 6). Despite the fact that 3′ UTR eNOS mRNA also possesses an AUUUA-rich region, we did not observe gel-shifted bands by using a labeled UTR-AU probe. However, gel-shifted bands were obtained when we tested a UC-rich fragment contained in the 129 bases from the 5′ end of UTR-S.

The cytosolic component bound to the 3′ UTR eNOS mRNA was proteins. This was determined by the fact that proteinase K preincubation of the BAEC cytosolic extracts fully inhibited complex formation with the UTR-UC probe.

Other reports about proteins that interact with the 3′ UTR of mRNAs, independently of AUUUA motifs, have appeared (2, 9). In this regard, proteins that are capable of binding to the α-globin 3′ UTR in a C-rich fragment have been described, although their relationship to α-globin mRNA stability remains to be clarified (33).

The proteins that interacted with 3′ UTR eNOS mRNA remain to be identified. However, previous unpublished data from our group seem to indicate that a 60-kDa endothelial cytosolic protein interacts with 3′ UTR eNOS mRNA.

In the present study, the sensitivity of complex formation to poly(C) and poly(U) competitions suggested that both C and U were needed for the interaction between the endothelial cytosolic proteins and the 3′ UTR. However, since UTR-AU and GM-CSF-UTR RNA did not modify the complex formation, the aforementioned results were at least unexpected and they could indicate that there are U residues essential for stabilization and/or complex formation between the endothelial cytosolic proteins and 3′ UTR eNOS mRNA.

Studies with RNase T_1 showed that a 25-base fragment located within this UC-rich fragment seems to be the binding site for the endothelial cytosolic compound. However, further and more specific analysis of the binding site, i.e., performing site-directed mutagenesis within these 25 bases, is needed.

Potential role of endothelial cytosolic proteins in eNOS mRNA destabilization. Different reports have demonstrated a potent destabilization of eNOS mRNA in endothelial cells exposed to cytokines, i.e., TNF-α (22, 35). Thus, decreased expression of

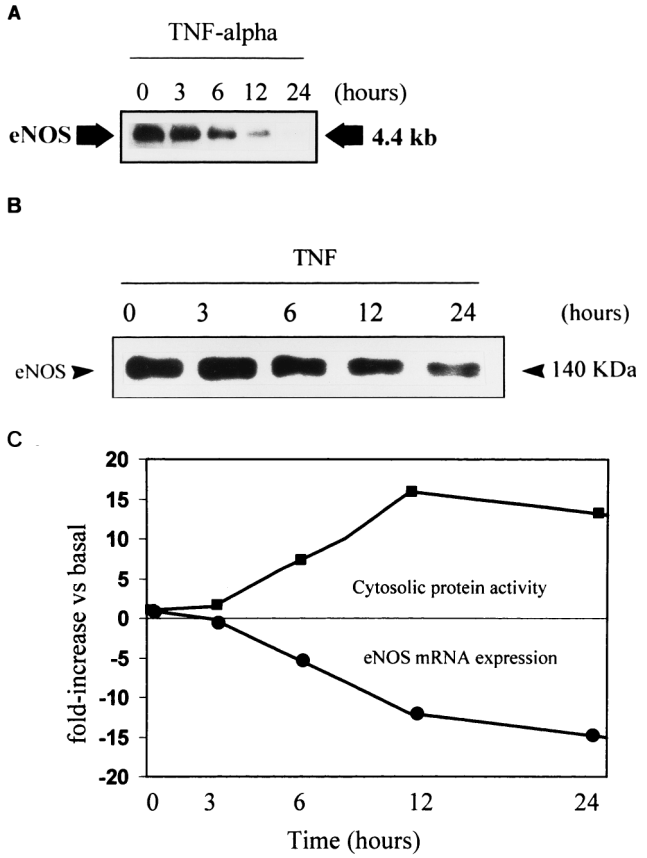

FIG. 8. eNOS mRNA and eNOS protein expression in TNF-α-incubated endothelial cells. (A) Northern blot analysis of eNOS mRNA expression after exposure of BAEC to TNF-α (10 ng/ml) for different periods of time. Total RNA (20 μg) was analyzed by Northern blotting with eNOS probe. (B) Representative Western blot demonstrating the expression of eNOS protein in BAEC after exposure to TNF-α (10 ng/ml) for different periods of time. (C) Relationship between changes in cytosolic protein binding activity and eNOS mRNA expression in TNF-α-stimulated BAEC, corrected by GAPDH expression. A negative correlation between cytosolic protein activity and eNOS mRNA levels was observed.

endothelial eNOS may contribute to the development of vascular disorders such as atherosclerosis or myocardial ischemia, in which increased production of TNF-α and impaired endothelium-dependent vasodilator response have been reported (3, 14, 19, 20).

eNOS mRNA expression was found to be slightly decreased 3 h after TNF-α stimulation of BAEC; the decrease was more marked at 12 h, with nearly no detectable message 24 h after TNF-α incubation. A similar effect was observed in the presence of actinomycin D, strongly suggesting that a change in stability of the eNOS message is the predominant mechanism for the downregulation of its expression. In addition, a correlative decrease in the eNOS protein expression was also obtained. Similar findings were previously reported by Mohamed et al. (22) and Yoshizumi et al. (35).

Therefore, our final aim was to assess whether the binding of endothelial cytosolic extracts could be modified by TNF-α. After TNF-α stimulation, cytosolic lysates obtained from BAEC showed increased complex formation with the UTR-UC probe. In addition, unlabeled UTR-UC, but not UTR-AU, abolished complex formation between UTR-S and the cytosolic proteins obtained from TNF-α-stimulated BAEC, supporting the specificity of these proteins for the UTR UC-rich region. These results also suggested that these cytosolic proteins should be the same ones that recognized the 25-base fragment mentioned in the experiments performed with unstimulated BAEC. The kinetics of TNF-α-dependent complex formation correlated negatively with the decreased levels in eNOS RNA message, suggesting that these cytosolic proteins may have a role in the destabilization of eNOS mRNA. A TNF-α-stimulated cytosolic protein, which binds to the 3′ UTR of glucose transporter mRNA, has been described (31). However, in contrast to the results of our study, the binding of this protein stabilized glucose transporter mRNA (31).

The activity of other reported 3′ UTR binding proteins correlated inversely with the destabilization of mRNAs. For example, a protein that binds to adrenergic receptor mRNA was upregulated when the mRNA was destabilized by adrenergic agonists (25). Furthermore, Bickel et al. have identified three proteins that interact with the 3′ UTR of GM-CSF mRNA and decrease GM-CSF mRNA levels (4). Our present experimental design did not allow us to establish the exact mechanism of TNF-α-stimulated endothelial cytosolic protein activity in the regulation of eNOS mRNA stability. Furthermore, the data presented here do not address whether the TNF-α-dependent increase in endothelial cytosolic protein binding activity was due to a greater affinity of the proteins for the 3′ UTR binding site or to the synthesis of new proteins. Mohamed et al. (22) and Yoshizumi et al. (35) have shown that destabilization of the eNOS message by TNF-α was largely prevented by coincubation with cycloheximide, and they postulated that synthesis of new proteins was required for this effect. Finally, it is noteworthy that the binding site of endothelial cytosolic proteins flanks a potential stem-loop structure in the 3′ UTR of eNOS mRNA. Therefore, the binding of endothelial cytosolic proteins to the 25 bases may modify the tridimensional configuration of this region, showing an RNase active site. A similar mechanism has been described for iron-regulated transferrin receptor mRNA stability (24).

The loss of eNOS expression and the resulting decrease in the capacity to produce NO in response to physiological stimuli could compromise the ability of the endothelium to protect against thrombosis, vasoconstriction, and subintimal proliferation (23). Increased levels of cytokines have been found in a variety of cardiovascular disorders, in which an endothelial dysfunction seems to have a main role in their development (19, 20).

In the present study, we describe an unknown *cis* element contained within the 3′ UTR of eNOS mRNA. In addition, we speculate that cytosolic proteins may be important in the regulation of the stability of eNOS mRNA. A better understanding of the regulatory mechanisms of eNOS expression may lead to new strategies in the prevention and treatment of endothelial dysfunction associated with vascular diseases.

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