

Materials and Methods

Cell Culture

HUVECs were purchased from Clonetics and cultured in medium 199 (M-199; BioWhittaker) containing endothelial cell growth supplement, 10% fetal bovine serum, and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, and 20 µg/mL neomycin). Cos-7 and Bovine aortic endothelial cells (BAECs) were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin).

Purification and Identification of eNOS mRNA 3'-UTR Binding Protein(s)

Cytosolic protein was extracted from confluent unstimulated HUVECs and HUVECs stimulated with TNF- α for 24 hr as described¹. Protein extracts were concentrated using a centrifugation filter device (100,000 molecular weight cutoff; Millipore). Streptavidin-agarose beads suspension (600µl, Gibco BRL Life Science) was equilibrated four times with washing buffer (150mM KCl, 1.5mM MgCl₂, 0.5mM DDT, 10mM Tris-HCl, pH 7.5). Packed beads were suspended in 50µL of washing buffer. Biotinylated human eNOS 3'-UTR (15-20µg) was added, and the mixture was incubated on a rotating wheel for 1 hr at 4°C. 10 mg cytosolic protein of HUVECs was then added and incubated for 45 min at 4°C and 15min at RT. After five times washing with the washing buffer, the bound proteins were then eluted with SDS-PAGE sample buffer, concentrated by Ultrafree-MC (Millipore), and analyzed by 12% SDS-PAGE. The gel was stained with coomassie blue and the target protein bands were digested with trypsin, and

followed by subsequent Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) as described previously ².

Expression and purification of glutathione S-transferase-tagged proteins. Purified GST and GST-eEF1A1 fusion proteins were produced as described previously ². DNA fragments encoding full length eEF1A1 and its domains amplified by PCR were inserted into a bacterial expression vector pGEX-2T (Amersham Pharmacia). The resulting constructs were confirmed by DNA sequencing. The glutathione S-transferase fusion proteins were expressed in *E. coli* strain BL21(DE3) by induction with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) after growth to an A_{600} of 1.0 and harvested after 3 h. Lysis and purification of the fusion proteins with glutathione-Sepharose beads were performed in accordance with the manufacturer's guidelines (Pharmacia Biotech).

RNA Electrophoretic Mobility Shift Assay (R-EMSA)

The 3'-UTR RNA of human eNOS were transcribed in vitro from 1 μ g of linearized plasmid using T7 RNA polymerase and labeled internally with 50 μ Ci [³²P]-UTP according to the manufacture's instructions(Ambion). R-EMSA assays were performed essentially as described previously ³. Briefly, radiolabeled riboprobes (10⁵ cpm) were incubated with 1-5 μ g protein or 30 μ g cytosolic extracts in the binding buffer containing (in mmol/L): HEPES 10 (pH 7.4), MgCl₂ 5, KCl 100, and DTT 1; 5% glycerol; 20U RNasin (Ambion), and 50ng/ μ L yeast tRNA (Ambion) for 30 minutes at room temperature. RNase T1 (25 U) was then added and the reaction mixtures were incubated for 10 min at 30 °C. For competition assay, 50-fold excess of cold nonradiolabeled probes were incubated with GST-eEF1A1 for 10 minutes before the addition of

radiolabeled probes. For supershift assay, goat polyclonal antibody to eEF1A (Santa Cruz) was used. The experimental protocol was identical to EMSA except 4 μ g anti-eEF1A antibody was incubated with cytosolic extracts on ice for one hour before the addition of radiolabeled probes. The RNA–protein complexes were separated by electrophoresis in a 4.5% (W/V) non-denaturing polyacrylamide gel containing 1 \times TBE. The gel was running in 1 \times TBE for 2h at 30mA before exposure to x-ray film at -80°C with an intensifying screen.

UV Cross-linking Assay

The 3'-UTR and its deletion mutants RNA probe of human eNOS were prepared as described for the R-EMSA assays. Radiolabeled riboprobes (1X10⁶ cpm) were incubated with 4 μ g GST or GST-eEF1A1 fusion proteins for 20 min at 37°C in 30 μ l binding buffer. Subsequently, the reaction mixture was irradiated for 20 min in a UV-cross-linker model 2400 (Stratagene). RNase T1 was then added to the reaction mixture for 30min at 30°C. The samples were boiled for 3 minutes in Laemmli buffer and separated by electrophoresis on a 10% SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film at -80°C with an intensifying screen.

Immunoprecipitation/Reverse Transcription-PCR Assay

TNF- α unstimulated and stimulated HUVECs were washed twice with cold phosphate-buffered saline, and then lysed for 30 min on ice in 1.5ml of lysis buffer (20mM Tris pH7.4, 0.1% Triton X-100, 2mM EDTA, 25mM NaCl, 10% Glycerol, protein inhibitors, and 100 units of RNase

OUT (Invitrogen). The pre-cleared lysates were incubated 2h at 4°C with 3 µg anti-eEF1A monoclonal antibody (Santa Cruz) and a control mouse IgG, followed by incubation with protein G-Sepharose saturated first with yeast tRNA (100µg/ml). The immunocomplexes were washed four times with 1ml lysis buffer, and the total RNA was purified with Trizol Reagent (Invitrogen) as described. RT-PCR was carried out with SuperScript III one-step RT-PCR kit (Invitrogen) with the following primers (primer 1: 5' TTT AAA GAA GTG GCC AAC GCC GTG 3'; primer 2: 5' AGC GGA TTG TAG CCT GGA ACA TCT 3'). PCR products were analyzed on a 1.5 % agarose gel and visualized by ethidium bromide staining. Each experimental reaction was performed in triplicate.

Construction of adenovirus

Adenovirus harboring eEF1A1 was made using AdMax (Microbix). Briefly, pBHGlox Δ E1,3Cre, including the Δ E1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into Ad293 cells using Fugene 6 (Roche). Adenovirus bearing LacZ was obtained from Clontech. The viruses were propagated on Ad293 cells and purified using CsCl₂ banding followed by dialysis against 20 mmol/l Tris-buffered saline with 2% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the instructions of the manufacturer.

Western Blotting

Proteins were prepared and separated on SDS-PAGE as described². Immunoblotting was performed using antibodies to eNOS (1:1000 dilution; Abgent), to Flag-tag (1:1000 dilution; Sigma), to Myc-tag (1:1000 dilution; Invitrogen), to eEF1A (1:500 dilution; Santa Cruz), and to

α -tubulin (1:5000 dilution; Sigma). Immunodetection was accomplished using a sheep anti-mouse secondary antibody (1:2000 dilution) or donkey anti-rabbit secondary antibody (1:2000 dilution) and the enhanced chemiluminescence kit (Amersham Corp).

Luciferase Reporter Assay

Fire luciferase cDNA fused with eNOS mRNA 3'-UTR (420nt, 3906 to 4325) was cloned in pcDNA3.0 vector. Bovine endothelial cells (70% confluent) and COS7 cells were transfected with the indicated cDNA constructs using FuGENE 6 transfection reagent. 48 h after transfection, cell lysates were assayed for luciferase activities using the dual Luciferase Assay System (Promega). We used *Renilla* luciferase as a control for transfection efficiency. Values for firefly luciferase were normalized to *Renilla* luciferase under the control of the thymidine kinase promoter in the pRLTK vector.

Northern Blotting

Recombinant adenoviruses harboring LacZ (AdLacZ) and human eEF1A1 (AdeEF1A1) were generated by using the AdEasy XL Adenoviral Vector system (Stratagene) according to the manufacturer's instructions. To analyze the effect of eEF1A1 on eNOS mRNA stability, HUVECs (70% confluent) were infected with AdLacZ and AdeEF1A1 at MOI of 100 for 48 hours, then 20 μ g/mL 5,6 -dichloro-1- β -D-ribofuranosylbenzimidazole (DRB, Sigma) was added. Total RNAs were prepared 0-24 hours thereafter. Equal amounts of total RNA (15 μ g) were separated by 1% formaldehyde–agarose gel electrophoresis and the human full-length eNOS cDNA was labeled with the DECAprime II Random Priming DNA Labeling Kit (Ambion). Conditions for hybridization and washing were performed as described^{4, 5}. RNA loading was

determined by ethidium bromide staining of 18 S and 28 S ribosomal RNA on the nylon membranes. The relative amount of eNOS mRNA at 0h DRB was set at 100%.

Gene silencing with small interference RNA

Two pairs of siRNA oligonucleotides for human eEF1A1 (sense strand, 5'- GCA CCA UGA AGC UUU GAG UGA AGC T -3' (eEFsiRNA-A); 5'- GUG CUA ACA UGC CUU GGU UCA AGG G -3' (eEFsiRNA-B); and a pair of control siRNA oligonucleotide: 5'-CAG AGA GGA GGA AAG GAG ACG CAG G-3') were synthesized by Integrated DNA Technologies (Coralville, IA). HUVECs grown to ~50% confluence were transfected with Gene Silencer® (Gene Therapy System, San Diego, CA) transfection agent with target specific siRNA (20 nM) and control siRNA (20 nM) in serum-free M199 medium according to the manufacturer's recommendation. After 3 h post-transfection, fresh complete M199 medium was added, and the cells were cultured for an additional 72 h for protein analyses of eEF1A by Western blotting analysis. The half-life of eNOS mRNA was determined by Northern blotting analysis in the presence and absence of TNF- α treatment.

Statistical Analysis

All values are expressed as the means \pm S.E. Differences between means were analyzed using a two-tailed Student *t* test. Significant differences were taken at $P < 0.05$.

References:

1. Lai PF, Mohamed F, Monge JC, Stewart DJ. Downregulation of eNOS mRNA expression by TNFalpha: identification and functional characterization of RNA-protein interactions in the 3'UTR. *Cardiovasc Res.* 2003;59:160-168.
2. Sun J, Liao JK. Functional interaction of endothelial nitric oxide synthase with a voltage-dependent anion channel. *Proc Natl Acad Sci U S A.* 2002;99:13108-13113.
3. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc.* 2007;2:1849-1861.
4. Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J Biol Chem.* 1995;270:319-324.
5. Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation.* 2002;106:57-62.