Tumor Necrosis Factor Downregulates an Endothelial Nitric Oxide Synthase mRNA by Shortening Its Half-life

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Nitric oxide (NO), which accounts for the biological properties of endothelium-derived relaxing factor, is generated by NO synthase (NOS). The vascular endothelium contains two types of NOS: one is constitutively expressed (cNOS), and the other is inducible. Endothelium-mediated vasorelaxation is impaired in atherosclerotic vessels. To determine whether tumor necrosis factor (TNF)-α, which is commonly found in atherosclerotic lesions, has an effect on NOS message, we measured cNOS mRNA levels in TNF-treated human umbilical vein endothelial cells (HUVECs) by RNA blot analysis with a cNOS cDNA probe. TNF-α markedly reduced cNOS mRNA levels in HUVECs in a dose- and time-dependent manner. In response to 3 ng/mL TNF-α, cNOS mRNA levels began to decrease at 4 hours and diminished to only 5% of control levels at 24 hours. As little as 0.1 ng/mL TNF-α reduced cNOS mRNA levels by 50%. This reduction in cNOS message in response to TNF-α depended on protein synthesis as it was blocked by cycloheximide. In nuclear runoff experiments, TNF-α did not change the rate of cNOS gene transcription. cNOS mRNA is very stable under basal conditions, with a half-life of 48 hours; however, treatment with TNF-α shortened this half-life to 3 hours. TNF-α thus appears to decrease cNOS mRNA levels by increasing the rate of mRNA degradation. TNF-induced reductions in cNOS mRNA levels may have an important effect on impaired endothelium-mediated vasorelaxation in atherosclerosis. (Circulation Research 1993;73:205-209)

KEY WORDS • nitric oxide synthase • tumor necrosis factor • atherosclerosis • endothelium • transcriptional regulation

The endothelium regulates vascular tone by producing vasodilating as well as vasoconstricting substances. Nitric oxide (NO), which accounts for the biological activity of endothelium-derived relaxing factor, increases cellular cGMP levels by stimulating soluble guanylate cyclase.1-4 NO is derived from the guanidino nitrogen atom(s) of L-arginine through a reaction catalyzed by NO synthase (NOS). There are two forms of NOS. One is constitutively expressed (cNOS), is calcium and calmodulin dependent, and is present in vascular endothelial cells and the brain.5-9 The other is inducibly expressed (iNOS), is calcium and calmodulin independent, and is identified in endotoxin- or cytokine-treated macrophages, endothelial cells, and vascular smooth muscle cells.10-14 The cDNA encoding endothelial cNOS has been cloned,5-7,9 although the cDNA for endothelial iNOS has not.

In normal vessels, acetylcholine causes vasodilation by increasing the calcium concentration in endothelial cells. The increase in intracellular calcium activates cNOS and thereby increases NO synthesis. By contrast, acetylcholine induces constriction in atherosclerotic vessels.13,16 One possible explanation for this paradox is that cNOS activity is impaired in the vascular endothelial cells of atherosclerotic arteries.16

Cytokines such as tumor necrosis factor (TNF)–α and interleukin-1β are present in atheromatous tissue.17,18 We19 and others20 have shown that cytokines induce expression of growth factors in human umbilical vein endothelial cells (HUVECs). It is likely that cytokines such as TNF-α inhibit cNOS gene expression in vascular endothelial cells and that inhibition of cNOS gene expression contributes to the impaired endotheliument dependent relaxation associated with atherosclerosis.

To show the noninducible character of their cNOS clones, Nishida et al8 and Marsden et al6 treated endothelial cells with TNF-α and found that cNOS mRNA was downregulated by the cytokine. However, in these studies, the endothelial cells were treated with a single high dose of TNF-α (100 U/mL, Nishida et al; 100 ng/mL, Marsden et al). No dose-response or comprehensive time-course studies of TNF-α treatment were reported. Also, the molecular mechanisms causing downregulation of cNOS mRNA by TNF-α were not investigated.

In the present study, we measured cNOS mRNA levels in HUVECs treated with or without TNF-α.
TNF-α caused a marked reduction in cNOS message in a time- and dose-dependent fashion, and this TNF-induced decrease was mediated by shortening of the half-life of cNOS mRNA. Because this reduction in cNOS mRNA levels by TNF-α was blocked by cycloheximide, it must have been mediated by the synthesis of new protein.

Materials and Methods

Cell Culture
HUVECs obtained from Clonetech Corp, San Diego, Calif, were grown in medium 199 (Sigma Chemical Co, St Louis, Mo) supplemented with 20% fetal calf serum, 100 μg/mL heparin (Sigma), and 50 μg/mL endothelial cell growth substance (Collaborative Research, Inc, Bedford, Mass). HUVECs were passaged every 4 to 6 days; cells from passages 3 to 5 were used in the experiments. HUVECs were grown to confluence for 24 hours before stimulation with recombinant human TNF-α, a gift from Knoll Pharmaceuticals, Whipsany, N.J. Twelve hours before TNF-α treatment, fetal calf serum in the culture medium was reduced to 5%.

Amplification of cNOS cDNA by Polymerase Chain Reaction
The reverse transcription polymerase chain reaction was used to amplify an 883-bp cNOS cDNA fragment from HUVEC RNA.19,21 The sequences of the forward (5′GGTCTGGTTCGATCGTCT3′) and reverse (5′TC-CCCATTCCCAATGTGC3′) primers were based on the published human cNOS cDNA sequence.69 Authenticity of the polymerase chain reaction product and isolated cDNA clones was confirmed by dideoxy chain termination sequencing.22

RNA Blot Hybridization
Total RNA was extracted from cultured cells with RNAzol (Biotex Laboratories, Houston, Tex) according to the methods of Chomczynski and Sacchi.10 Ten to twenty micrograms of total RNA per lane was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were hybridized with a random-primed 32P-labeled cNOS cDNA according to standard techniques.22 The hybridized filters were then washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate (at 60°C) and autoradiographed with Kodak XAR film for 24 to 72 hours at −70°C or stored on phosphor screens for 6 hours. Because TNF-α increases the steady-state mRNA level of actin,23 we used an oligonucleotide (5′ACCGTATCGATGTCTTCCAGC3′) complementary to 18S ribosomal RNA to quantitate the amount of RNA loaded onto the agarose gels.19 Filters were washed at 80°C in 50% formamide to remove the cNOS probe and rehybridized to the radiolabeled 18S oligonucleotide.19 The image was displayed, and radioactivity was quantitated on a PhosphorImager running the IMAGEQUANT software (Molecular Dynamics, Sunnyvale, Calif).24

Nuclear Runoff Analysis
Confluent HUVECs treated with and without 10 ng/mL TNF-α for 1 hour were lysed, and nuclei were isolated as described.19 Nuclear suspension (100 μL) was incubated with 0.5 mM each of CTP, ATP, and GTP and with 250 μCi 32P-labeled UTP (3 000 Ci/mmol, New England Nuclear, Boston, Mass). The samples were extracted with phenol/chloroform, precipitated, and resuspended at equal counts per minute per milliliter in hybridization buffer (10×10⁶ cpm/mL). Denatured probes (1 μg) dot-blotted on nitrocellulose filters were hybridized at 40°C for 2 days in the presence of formamide. cDNAs for the cNOS, heparin-binding epidermal growth factor–like growth factor (HB-EGF),19 and von Willebrand factor (vWF)25 genes were used as probes. pUC-18 plasmid DNA (Pharmacia LKB Nuclear, Gaithersburg, Md) was used as a control.

Results

TNF-α Markedly Decreases cNOS mRNA Levels in HUVECs
We detected a single 4.3-kb cNOS message in RNA prepared from HUVECs (Fig 1). Although treatment with TNF-α (3 ng/mL) did not change cNOS message in the first 2 hours, cNOS mRNA levels quickly decreased to 50% of control at 4 hours, and only 5% of the control could be detected at 24 hours (Fig 1). TNF-α decreased cNOS mRNA levels in a dose-dependent fashion (Fig 2). As little as 0.1 ng/mL TNF-α decreased cNOS message to 50% at 14 hours. The inhibitory effect of TNF-α on cNOS mRNA levels peaked at 3 ng/mL (Fig 2). To test whether another cytokine reduces cNOS message levels, we also treated HUVECs with interleu-
kin-1β. Interleukin-1β caused a reduction in cNOS mRNA levels similar to that caused by TNF-α (data not shown).

To determine whether downregulation of cNOS message by TNF-α depends on new protein synthesis, we treated HUVECs with the protein synthesis inhibitor cycloheximide before exposing the HUVECs to TNF-α. This 1.5-hour pretreatment did not change the basal level of cNOS mRNA (data not shown). Cycloheximide blocked the inhibitory effect of TNF-α on cNOS mRNA at both 4 and 8 hours (Fig. 3), indicating that the downregulation process depends on synthesis of new protein.

**TNF-α Has No Effect on the Transcriptional Rate of the cNOS Gene**

To determine the precise mechanism by which TNF-α decreased steady-state cNOS mRNA levels, we first performed nuclear runoff experiments to assess the rate of cNOS gene transcription in the presence or absence of TNF-α and compared it with the rate for HB-EGF, another gene transcribed and regulated by TNF-α in endothelial cells. We performed these experiments twice and obtained identical results. Fig. 4 shows the data from one experiment. TNF-α increased the transcriptional rate of HB-EGF (Fig 4), as our group reported previously.19 In contrast, TNF-α had no effect on the transcriptional rate of cNOS (Fig 4). Thus, the reduction of cNOS message by TNF-α was not mediated by a reduction in the gene’s transcriptional rate.

![Fig 2](image2.png)

**Fig 2.** Dose-response relation of constitutively expressed nitric oxide synthase (cNOS) mRNA downregulation by tumor necrosis factor-α (TNF). Panel A: Autoradiographs show human umbilical vein endothelial cells incubated for 14 hours with the indicated concentrations of TNF. Total RNA was extracted at the end of incubation, and 20 μg total RNA was analyzed by Northern blotting with cNOS and 18S oligonucleotide probes. Panel B: Relative percentage of cNOS mRNA in response to each dose of TNF is plotted against dose. The x axis is in log scale.

![Fig 3](image3.png)

**Fig 3.** Bar graph showing reversal by cycloheximide of the inhibitory effect of tumor necrosis factor (TNF) on constitutively expressed nitric oxide synthase (cNOS) mRNA. Human umbilical vein endothelial cells were treated with (+, dotted bars) or without (−, open bars) cycloheximide (CHX, 20 μg/mL) for 1.5 hours before addition of 3 ng/mL TNF. Human umbilical vein endothelial cells were harvested for RNA extraction 4 and 8 hours after exposure to TNF. No TNF was added to the control lane. RNA blot analysis was performed with 20 μg total RNA probed with cNOS and 18S. The relative percentage of cNOS mRNA is plotted against time. Pretreatment the human umbilical vein endothelial cells for 1.5 hours with cycloheximide had no effect on the basal level of cNOS mRNA (data not shown).

**TNF-α Destabilizes cNOS mRNA**

To determine whether TNF-α decreases the levels of cNOS mRNA by increasing its degradation, we measured the cNOS mRNA half-life in the presence of actinomycin-D (an inhibitor of transcription). cNOS mRNA is very stable in the absence of TNF-α and has an estimated half-life of 48 hours. However, the presence of TNF-α shortened this half-life to 3 hours (Fig 5). Therefore, the reduction in cNOS message by TNF-α appears to be mediated by an increase in the mRNA’s degradation rate, through a process that requires new protein synthesis. We rehybridized the filters with a probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure that our results were not simply due to an overall decrease in mRNA stability caused by a combination of actinomycin D and TNF-α. TNF-α did not change the degradation rate of GAPDH mRNA (data not shown).

**Discussion**

Our study demonstrates that downregulation of cNOS mRNA by TNF-α is both time and dose dependent (Figs 1 and 2). As little as 0.1 ng/mL TNF-α reduced cNOS mRNA levels by 50%, and in response to 3 ng/mL TNF-α, cNOS mRNA levels decreased to 5% of control at 24 hours. In light of a previous study in which we showed that 3 ng/mL TNF-α markedly increased the mRNA levels and transcriptional rate of HB-EGF in HUVECs,19 we infer that this downregulation cannot have been due to an overall suppression of gene expression by TNF-α.

Our study also indicates that TNF-induced downregulation of cNOS message is mediated by a shortening of the mRNA’s half-life (Figs 4 and 5), through a process that requires new protein synthesis (Fig 3). Although TNF-α induces the expression of a variety of genes,20 it is...
FIG 4. Graph showing effect of tumor necrosis factor-α (TNF-α) on transcription of constitutively expressed nitric oxide synthase (cNOS) mRNA. Human umbilical vein endothelial cells at confluence were treated with or without 10 ng/mL TNF-α for 4 and 24 hours. Nuclei were isolated, and in vitro transcription was allowed to resume in the presence of [α-32P]UTP. Equal amounts of 32P-labeled in vitro–transcribed RNA prepared from human umbilical vein endothelial cells treated with or without TNF-α were hybridized to 1 μg denatured cDNA for the cNOS, heparin-binding epidermal growth factor–like growth factor (HB-EGF), and von Willebrand factor (vWF) genes, which had been immobilized previously on nitrocellulose filters. One microgram of pUC-18 plasmid DNA was used as a negative control. Since TNF-α did not change the transcription rate of vWF, the signal density of HB-EGF (○) and cNOS (●) was divided by that of vWF for each time point (to correct for differences among RNA probes used in individual hybridizations). The corrected density for each time point was then divided by that of the individual control and is plotted as a percentage of the control transcriptional rate against time. The background signal density was 1021 PhosphorImaging units. After subtraction of background density, the signal densities (in PhosphorImaging units) at 0, 4, and 24 hours were 2991, 1808, and 4092, respectively, for cNOS; 662, 1300, and 1627, respectively, for HB-EGF; and 1012, 838, and 1216, respectively, for vWF.

known to inhibit only a few of them: collagen, in human fibroblasts;26 thrombomodulin, in human vascular endothelial cells;27 α-actin, in human myoblasts;28 pulmonary surfactant protein, in human pulmonary adenocarcinoma cells;29 and acetyl-CoA carboxylase, in adipocytes.30 TNF-α inhibits the message of these genes by decreasing their rate of transcription. Our study shows that the inhibition of cNOS gene expression by TNF-α is unique in that it is mediated by an increase in mRNA degradation rather than a decrease in transcription.

It has been reported that cytokines induce expression of iNOS in endothelial cells,13,31 although very high concentrations of TNF-α (more than 10 ng/mL) are required to increase NO production in bovine aortic endothelial cells.31 This requirement of a high TNF-α concentration for induction of iNOS is in sharp contrast with the marked reduction in cNOS message caused by as little as 0.1 ng/mL TNF-α (Fig 2). The availability of an endothelial iNOS cDNA clone should allow a more detailed molecular analysis of the differential regulation of these two forms of the NOS gene.

In addition to vasodilation, NO inhibits leucocyte adhesion, platelet aggregation, and proliferation of smooth muscle cells.1,3,4 This fact implies that the basal production of NO by endothelium may protect against atherosclerosis in addition to regulating vascular tone. Cytokines such as TNF-α17 and interleukin-1β18 are found in atheromatous tissue. It will be important to test whether downregulation of cNOS message by these cytokines has an effect on impaired endothelium-mediated vasorelaxation and the progression of atherosclerotic lesions.

Acknowledgments

This study was supported by a grant from the Bristol-Myers Squibb Pharmaceutical Research Institute. This work was done during the tenure of a Clinician Scientist Award to Dr Lee from the American Heart Association. This work was also supported by a Grant-in-Aid to Dr Lee from the American Heart Association and with funds contributed in part by the American Heart Association, Massachusetts Affiliate, Inc. We also extend our gratitude to Dr Edgar Haber for his continued enthusiasm and support of this work. We thank Dr H.S. Sui for her helpful suggestions as well as critical review of the manuscript, Ms Denise Hubble for her technical assistance, and Mr Thomas J. McVarish for his editorial assistance.

References

7. Lamas S, Marsden PA, Li GK, Tempst P, Michel T. Endothelial nitric oxide synthase: molecular cloning and characterization of a


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doi: 10.1161/01.RES.73.1.205

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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