Estrogens Increase Transcription of the Human Endothelial NO Synthase Gene

Analysis of the Transcription Factors Involved

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Abstract—Estrogens have been found to reduce the incidence of cardiovascular disease that has been ascribed in part to an increased expression and/or activity of the vasoprotective endothelial NO synthase (NOS III). Some reports have shown that the level of expression of this constitutive enzyme can be upregulated by estrogens. The current study investigates the molecular mechanism of the NOS III upregulation in human endothelial EA.hy 926 cells. Incubation of EA.hy 926 cells with 17β-estradiol or the more stable 17α-ethinyl estradiol enhanced NOS III mRNA and protein expression up to 1.8-fold, without changing the stability of the NOS III mRNA. There was no enhancement of NOS III mRNA after incubation of EA.hy 926 cells with testosterone, progesterone, or dihydrocortisol or when 17α-ethinyl estradiol was added together with the estrogen antagonist RU58668, indicating a specific estrogenic response. Nuclear run-on assays indicated that the increase in NOS III mRNA is the result of an estrogen-induced enhancement of NOS III gene transcription. In transient transfection experiments using a 1.6 kb human NOS III promoter fragment (which contains no bona fide estrogen-responsive element, ERE), basal promoter activity was enhanced 1.7-fold by 17α-ethinyl estradiol. In electrophoretic mobility shift assays, nuclear extracts from estrogen-incubated EA.hy 926 cells showed no enhanced binding activity either for the ERE-like motif in the human NOS III promoter or for transcription factor GATA. However, binding of transcription factor Sp1 (which is essential for the activity of the human NOS III promoter) was significantly enhanced by estrogens. These data suggest that the estrogen stimulation of the NOS III promoter could be mediated in part by an increased activity of transcription factor Sp1. (Hypertension. 1998;31:582-588.)

Key Words: 17α-ethinyl estradiol ■ 17β-estradiol ■ nitric oxide synthase ■ transcription factor Sp1

Sex differences in the incidence of coronary heart disease are well established. The incidence of coronary heart disease is relatively low among premenopausal women and increases sharply with the occurrence of menopause.1,2 The beneficial effect of estrogens in replacement therapy of postmenopausal women3,4 and the increased risk of coronary heart disease in young bilateral oophorectomized women5 support a fundamental role for estrogens as cardioprotective agents (for review see Reference 6). Part of this effect may result from an estrogen-mediated enhancement of the activity and/or expression of endothelial nitric oxide synthase (NOS III or eNOS). NO generated by this endothelial enzyme is involved in blood pressure regulation7,8 and exerts protective effects in the cardiovascular system such as inhibition of platelet aggregation and adhesion, prevention of leukocyte adhesion to the vascular wall, and reduction of vascular smooth muscle proliferation (for review see References 9 through 11). Decreased endothelial NO production has been seen in pathophysiological conditions such as atherosclerosis, diabetes, and hypertension (for review see Reference 12).

In recent years, in vivo evidence has been presented for acute vascular effects of estrogens leading to improved endothelium-mediated vasodilatation and/or NO release.13–15 Other studies in which long-term treatment with estrogens was used either indicate improved vascular NOS activity or increased expression of endothelial NOS.16,17 In addition, there is more direct evidence indicating that estrogens can upregulate the expression of NOS III mRNA and protein. In guinea pigs, near-term pregnancy and treatment with estradiol (but not progesterone) increased calcium-dependent NOS activity in various tissues. Pregnancy and estradiol both also enhanced NOS III mRNA in skeletal muscle, suggesting an induction of the enzyme.18,19 An increase in NOS III mRNA has also been seen in the aortas of pregnant or estrogen-treated, but not progesterone- or testosterone-treated, rats.20 It has been technically difficult to reproduce these in vivo or ex vivo findings in cell culture models, which is a prerequisite for studying the molecular mechanism or mechanisms. Hayashi et al21 and Hishikawa et al22 demonstrated an increase in NOS III protein in human umbilical vein and human aortic endothelial cells.
respectively, but the mechanism of this upregulation remained unclear. A recent study on bovine endothelial cells claimed that 17α-ethinyl estradiol did not enhance the expression of NOS III but that it increased the release of bioactive NO by inhibiting superoxide anion production.21

In the current study we demonstrate that 17α-ethinyl estradiol and 17β-estradiol enhance NOS III mRNA and protein expression, whereas other steroid hormones do not. The increased NOS III expression results from an increased NOS III promoter activity with unchanged mRNA stability. Nuclear extracts from estrogen-treated EA.hy 926 cells display increased transcription factor Sp1 activity which is essential for NOS III transcription.

Methods

Reagents

17α-ethinyl estradiol, 17β-estradiol, bovine serum albumin (fraction V), dihydrocortisol, polyvinylpyrrolidone, progesterone, testosterone, and actinomycin D were purchased from Sigma. The estrogen antagonist 11β-[4-[5-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-triien-3,17-β-diol (RU58668) was a gift from Roussel-Uclaf, Paris, France. Isotopes were from New England Nuclear/DuPont. Restriction enzymes, polynucleotide kinase, T7 DNA polymerase, dNTPs, Ficoll (type 400), oligonucleotides, and oligo-dT primers were obtained from Pharmacia. Luciferase and β-galactosidase assay systems were from Promega and Tropix/PE Applied Biosystems, respectively. Superscript reverse transcriptase was purchased from Gibco/BRL. DNase I, DOTAP, RNase A, RNase T1, R.Nase T3, and T7 RNA polymerase were from Boehringer Mannheim.

Cloning of a Human NOS III cDNA Fragment

Two micrograms total RNA from EA.hy 926 cells was annealed with 0.5 μg of an oligo-dT primer (Pharmacia) and reverse-transcribed with Superscript reverse transcriptase (RT, Gibco-BRL) according to the manufacturer’s instructions. RT-generated cDNAs encoding for human NOS III were amplified using PCR. Oligonucleotide primers for NOS III were GACATTTGAGACAAAAAGCTCG (sense) and CCGCGTTGCACACTCTCGG (antisense), corresponding to positions 3111 to 3133 and 3518 to 3536 of the human NOS III cDNA.22 PCR was performed in a 100 μL volume containing 1X Taq polymerase buffer (Pharmacia), 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl2, 2 μL Taq polymerase, 50 pmol oligonucleotide primers and RT products (1/10 of the RT reaction). After an initial denaturation step at 95°C for 5 minutes, 30 cycles were performed (1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C) followed by a final 10-minute extension step at 72°C. The PCR products (30 μL) were analyzed on a 1.5% agarose gel containing 0.1 μg/mL ethidium bromide. The amplified cDNA fragments (426 bp) were cloned into the EcoRV site of pCR-Script (Stratagene) using the Sure Clone Ligation Kit (Pharmacia), generating the cDNA clone pCR-NOS III-Hu. DNA sequences of the cloned PCR product were determined from plasmid templates with the T7 Sequencing Kit (Pharmacia) using the dyeoxy chain termination method.

Cloning of the 5′-Flanking Region From the Human NOS III Gene

Chromosomal DNA was isolated from human EA.hy 926 cells by RNase/proteinase digestion and phenol/chloroform extraction as described previously.22 This DNA was used for amplification of the 5′-flanking DNA of the human NOS III gene. The PCR was performed as described above using the following oligonucleotides as primers: TGTAGTGCCTGTCACCTTG (5′) and TACTGTGCCTCCACTCTGCG (3′). The sequences were based on published 5′-flanking sequences of the human NOS III gene.23 The amplified DNA fragment (1616 bp, positions −1596 to +20) was cloned into the Sma I site of pUC 18, generating pUC-NOS III-Hu-S. The DNA sequence of the cloned PCR products was determined using the T7 Sequencing Kit (Pharmacia). The human NOS III 5′-flanking sequence was then inserted into the luciferase gene–containing plasmid pG52-Basic (Promega) generating pNOS III-Hu-5′Luc.

Preparation of Antisense RNA Probes

To generate radiolabeled antisense RNA probes for RNase protection assays, pCR-NOS III-Hu and pCR-β-actin-Hu were linearized with Smal I or BstEII, extracted with phenol/chloroform, and concentrated by ethanol precipitation. One half of a microgram of this DNA was in vitro transcribed using T3 RNA polymerase (Pharmacia) and α-32P-UTP. After a 1-hour incubation, the template DNA was degraded with DNase I for 45 minutes. The radiolabeled RNA was purified using NuTrap probe purification columns (Stratagene).

RNase Protection Analyses

RNase protection assays were performed with a mixture of RNase A and RNase T1 according to the manufacturer’s instructions (Boehringer Mannheim). Briefly, following denaturation, 20 μg of total RNA (prepared as described above) was hybridized with 200 000 cpm–labeled NOS III antisense RNA probe and 20 000 cpm–labeled β-actin antisense RNA probe at 51°C for 16 hours in a volume of 40 μL hybridization buffer (40 mmol/L PIPES, pH 6.7, 1 mmol/L EDTA, 400 mmol/L NaCl, 50% formamide). Then the mixture was digested by adding 300 μL digestion buffer (10 mmol/L Tris/HCl, pH 7.4, 300 mmol/L NaCl, and 5 mmol/L EDTA) containing 3.5 μg RNase A and 37.5 μg RNase T1, for 30 minutes at 30°C. The reaction was stopped by proteinase K digestion (70 μg/sample in 70 μL of 7.15 mmol/L Tris/HCl, pH 7.4, 7.15 mmol/L EDTA, 2.85% SDS; 15 minutes at 37°C) and phenol extraction. The reaction products were concentrated by ethanol precipitation and analyzed by electrophoresis on denaturing urea-polyacrylamide gels (8 mol/L urea, 6% polyacrylamide). The electrophoresis buffer was 1X TBE (89 mmol/L Tris, pH 8.3, 89 mmol/L boric acid, and 20 mmol/L EDTA). The gels were electrophoresed for 1 to 2 hours, dried, and exposed to x-ray.
NOS III Induction by Estrogens

The protected RNA fragments of NOS III and β-actin were 280 and 108 nt, respectively. Densitometric analyses were performed using a Phospho-Imager (Bio-Rad). The protected NOS III bands were normalized using the protected β-actin bands (NOS III minus RNA background)/(β-actin minus tRNA background)×100.

Transient Transfection of ECV304 Cells and Luciferase/β-Galactosidase Assays

ECV304 endothelial cells were plated in 60-mm cell culture dishes at least 24 hours before transfection. The cells (approximately 80% confluent) were transfected by lipofection with DOTAP according to the manufacturer’s recommendations (Boehringer Mannheim) using 5 μg of pNOS III-Hu-Luc or pGI2-Basic (Promega), and 0.1 μg of pCH110 (Pharmacia; containing the β-galactosidase gene under the control of the SV40 promoter/enhancer) for normalization. ECV304 cells were used instead of EA.hy 926 cells because transfection efficiency was poor with EA.hy 926 cells. The cells were washed with culture medium 9 hours after transfection and incubated with 17α-estradiol (10 or 100 nmol/L) for 24 hours after transfection. Extracts (400 μL) were prepared 18 hours later using the reporter lysis buffer (Promega). The luciferase- and β-galactosidase activities of the extracts were determined using the Luciferase Assay System (Promega) and the Galacto-Light System (Tropix) as described. The light units (LU) of the luciferase assay were normalized by the LU of the β-galactosidase assay after subtraction of extract background, (LUmin, minus background)/(LUp, minus background)×100.

Nuclear Run-On Assay and Hybridization of De Novo Radiolabeled RNA

Nuclear run-on assays were performed as described. Briefly, EA.hy 926 cells incubated with or without estrogen were scraped from the cell culture plates with a rubber “policeman,” collected by centrifugation, and washed twice with ice-cold phosphate-buffered saline. The cell pellet (1×10^9 to 3×10^9 cells) was resuspended in 1 mL NP40 lysis buffer (10 mmol/L Tris/HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.5% [vol/vol] NP40), incubated on ice for 5 minutes, and centrifuged at 500 g for 5 minutes. The supernatant was removed, and the nuclear pellet was washed twice with 2 mL NP40 lysis buffer. Then the nuclei were resuspended in 100 μL nuclei freezing buffer (50 mmol/L Tris/HCl, pH 8.3, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 40% [vol/vol] glycerol) and stored frozen until used. For run-on transcription, the nuclei were mixed with 100 μL transcription buffer (10 mmol/L Tris/HCl, pH 8.0, 5.5 mmol/L MgCl₂, 300 mmol/L KCl, 0.5 mmol/L each of ATP, CTP, and GTP, and 80 μCi of α-32P-UTP (800 Ci per mmol, New England Nuclear). The transcription reaction was carried out for 45 minutes at 30°C. Then, 400 U DNase I (Boehringer Mannheim) was added, and the incubation continued for another 15 minutes at 30°C. After the addition of 80 μg proteinase K and 1% SDS (final concentration), the samples were incubated at 37°C for an additional 30 minutes. After a phenol/chloroform extraction, nucleic acids were collected by ethanol precipitation. The radiolabeled RNA was hybridized at 65°C for 48 hours to DNA immobilized on nitrocellulose filters as described previously. The DNA consisted of linearized plasmids containing either the whole bovine NOS III cDNA (kindly provided by Dr William C. Sessa) or the whole human β-actin cDNA. Bacterial DNA (pC-R-Script, Stratagene, alone) was used as a negative control. The reaction was carried out in 6× SSC (0.9 mol/L NaCl; 0.09 mol/L Na citrate, pH 7.0), 5× Denhardt’s reagent (0.1 g Ficoll, type 500, Pharmacia; 0.1 g of polyvinylpyrrolidone, Sigma; 0.1 g bovine serum albumin, Fraction V, Sigma in 100 mL H₂O), and 0.1% (wt/vol) SDS. After hybridization, the filters were washed twice with 2× SSC and 0.1% (wt/vol) SDS at room temperature for 30 minutes followed by two washes with 0.5× SSC and 0.1% (wt/vol) SDS at 65°C for 1 hour. Filters were air-dried and exposed to x-ray film. Densitometric analyses were performed using a Phospho-Imager (Bio-Rad).

Electrophoretic Mobility Shift Assay

Binding activities of the estrogen receptor and the transcription factors Sp1 and GATA in the nuclei of estrogen-treated or untreated cells were determined by electrophoretic mobility shift assays using the Promega gel shift assay system. Nuclear proteins were extracted from EA.hy 926 cells by detergent lysis. Ten micrograms of nuclear protein was incubated with 17.5 fmol [32P]-labeled double-stranded oligonucleotide containing either a bona fide estrogen receptor binding motif from the promoter of the Xenopus laevis vitellogenin A2 gene (5'-GTCAGGTCACAGTGACCTGATC-3'; positions −329 to −308), an ERE-like sequence from the human NOS III promoter (5'-CTGCCTGTACCTGGCTACTGGC-3'; positions −1591 to −1569), or a Sp1-binding motif (5'-ATTCTAGTCGGGCGGGGCGACG-3'), or a GATA-binding motif (5'-CTTGGATTACAGAAATGTTATC-3'). Specificity of binding was determined by adding excess (1.75 pmol) unlabeled oligonucleotide. DNA-protein complexes were analyzed on 5% polyacrylamide gels (buffer 6.7 mmol/L Tris/HCl, pH 7.5; 3.3 mmol/L Na acetate; 1 mmol/L EDTA). The gels were dried and autoradiographed on x-ray film or a Phospho-Imager screen (Bio-Rad).

Statistical Analysis

Statistical differences between mean values were determined by ANOVA followed by the Fisher’s protected least significant difference test for comparison of mean values.

Results

Estrogens Enhance NOS III mRNA and Protein in EA.hy 926 Endothelial Cells

In human endothelial EA.hy 926 cells, 17α-ethinyl estradiol enhanced NOS III mRNA in a concentration-dependent fashion (Fig 1a and 1b). Similar increases in NOS III mRNA levels were obtained when EA.hy 926 cells were incubated with 17β-estradiol (Fig 1c). Incubation of EA.hy 926 cells with 17α-ethinyl estradiol also enhanced NOS III protein expression (Fig 2).
In contrast to 17α-ethinyl estradiol, incubation of EA.hy 926 cells with the steroids testosterone, progesterone, and dihydrocortisol did not result in any significant enhancement of NOS III mRNA expression (Fig 3). When 17α-ethinyl estradiol (100 nmol/L) was added to EA.hy 926 cells preincubated with the estrogen antagonist RU58668 (1 μmol/L), there was no stimulation of NOS III mRNA expression (n=3).

17α-Ethinyl Estradiol Does Not Modify the Stability of NOS III mRNA in EA.hy 926 Endothelial Cells

When transcription was blocked with 10 μg/mL actinomycin D added to the incubation medium of EA.hy 926 cells and RNA was prepared from these cells 6 to 72 hours later, NOS III mRNA levels were found to decline over time (Fig 4). The approximate half-life of the mRNA was 48 hours. Pretreatment of EA.hy 926 cells with 17α-ethinyl estradiol (10 or 100 nmol/L) enhanced the NOS III mRNA levels as shown in Fig 1, but did not change the stability of the mRNA (Fig 4).

17α-Ethinyl Estradiol Enhances NOS III Gene Transcription and Increases the Activity of a NOS III Promoter Fragment Transfected into Human ECV304 Endothelial Cells

To analyze the effect of 17α-ethinyl estradiol on the activity of the endogenous human NOS III promoter, EA.hy 926 cells were incubated with or without 10 or 100 nmol/L 17α-ethinyl estradiol, and nuclei were prepared. Nuclear run-on analyses performed with these nuclei demonstrated that incubation of the cells with 17α-ethinyl estradiol enhanced NOS III gene transcription 1.8-fold (Fig 5).
In an alternative approach, human endothelial ECV304 cells were transfected with pNOS III-Hu-Luc that contained a 1.6-kb fragment of the 5'-flanking sequence of the human NOS III gene cloned before a luciferase reporter gene. When the transfected cells were incubated for 18 hours with 10 or 100 nmol/L 17α-ethinyl estradiol, promoter activity was increased up to 1.7-fold (Fig 6).

Effect of 17α-Ethyl Estradiol on the Binding of Nuclear Extracts to Transcription Factor-Binding DNA Motifs

The known sequence of the human NOS III promoter contains no bona fide ERE but several ERE half sites. At positions 21585 to 21573, the human NOS III promoter contains the sequence 5'-CTGTCACCTTGACCC-3'. This sequence has the highest homology to the published ERE consensus sequence 5'-AGGTCANNNTGACCT-3'. Therefore, we performed electrophoretic mobility shift assays using oligonucleotides that contained either a bona fide ERE-containing oligonucleotide (complex 1e, n=3) or the ERE-like sequence of the human NOS III promoter (n=3, data not shown).

Figure 6. NOS III promoter activity is enhanced by 17α-ethinyl estradiol. Human endothelial ECV304 cells were transfected with pGI2-Basic (containing a promoterless luciferase gene) or pNOS III-Hu-Luc (containing a 1.6-kb NOS III promoter fragment cloned before the luciferase gene). Twenty-four hours after transfection, endothelial cells were incubated without or with 17α-ethinyl estradiol (10 or 100 nmol/L) for an additional 18 hours. Then cell extracts were prepared and luciferase activity was determined. Luciferase activity was normalized with β-galactosidase activity as described in “Methods.” Bars represent mean luminescence activity (± SEM) from four experiments. The asterisks indicate significant differences from pNOS III-Hu-Luc transfected but untreated cells (*P<.05, **P<.001).

Because the enhancement of NOS III promoter activity by 17α-ethinyl estradiol was unlikely to represent a direct ERE-estrogen receptor interaction, we tested the effect of 17α-ethinyl estradiol on the binding activity of other transcription factors known to be important for the activity of the human NOS III promoter. Recent data by three independent groups have demonstrated an essential role of transcription factor Sp1.37–39 As shown in Fig 7a, 17α-ethinyl estradiol enhanced the binding activity of the estrogen receptor to the full-length bovine NOS III cDNA (upper lane, NOS III), the full-length human β-actin cDNA (middle lane, β-Actin), or plasmid DNA alone (lower lane, pCR-Script). To this filter, radiolabeled RNA was hybridized that was obtained by in vitro transcription with nuclei from untreated EA.hy 926 cells (Untr) or EA.hy 926 cells incubated with 17α-ethinyl estradiol (10 or 100 nmol/L) for 18 hours. Panel b shows densitometric analyses of three different filters. Bars represent mean density (± SEM). The asterisk indicates a significant difference from untreated cells (P<.05).

Figure 5. Nuclear run-on analyses are shown. Panel a shows an autoradiography of a representative filter with immobilized linearized plasmids containing either the full-length bovine NOS III cDNA (upper lane, NOS III), the full-length human β-actin cDNA (middle lane, β-Actin), or plasmid DNA alone (lower lane, pCR-Script). To this filter, radiolabeled RNA was hybridized that was obtained by in vitro transcription with nuclei from untreated EA.hy 926 cells (Untr) or EA.hy 926 cells incubated with 17α-ethinyl estradiol (10 or 100 nmol/L) for 18 hours. Panel b shows densitometric analyses of three different filters. Bars represent mean ± SEM. The asterisk indicates a significant difference from untreated cells (P<.05).

Figure 4. Determination of the stability of the NOS III mRNA is shown. EA.hy 926 cells were preincubated without (Untr) or with 100 nmol/L 17α-ethinyl estradiol for 18 hours (17α-Eest). Then, actinomycin D (10 μg/mL) was added to the incubation mixture and the incubation was continued for 6, 12, 24, 48, or 72 hours. At those points in time, RNA was prepared and subjected to RNase protection analysis with antisense RNA probes to human NOS III and β-actin (for normalization). Circles represent mean ± SEM of the densitometric analyses of four different gels. The NOS III mRNA levels at the time of addition of actinomycin D (0 h) were set 100%.
AP-1, NF-1, and NF-κB; as well as heavy metal-, acute-phase response–, shear stress–, and sterol-regulatory cis elements. The human NOS III promoter contains consensus sequences for the binding of transcription factors Sp1, GATA, AP-1, NF-1, and NF-κB; as well as heavy metal–, acute-phase response–, shear stress–, and sterol-regulatory cis elements. The known promoter sequence contains no TATA box and no bona fide ERE. Functional relevance has only been demonstrated for a few of these binding sites. Deletion and mutation analyses revealed an essential role of the Sp1 binding site at position −103 for promoter activity. Also deletion of the consensus GATA site at position −230 reduced human NOS III promoter activity by about 30%. The modest upregulation of NOS III mRNA by incubation of bovine aortic endothelial cells with transforming growth factor-β1 has been reported to be a result of enhanced promoter activity through increased binding of the transcription factor NF-1 to its responsive element in the bovine NOS III promoter.

Reporter gene assays with constructs containing 1.6 kb of the 5′-flanking sequence of the human NOS III promoter showed a significant basal activity of this human promoter in endothelial cells (Fig 6). In our transfection experiments, the activity of this NOS III promoter fragment was enhanced 1.7-fold by 17α-ethinyl estradiol (Figs 6), thus explaining the total increase in NOS III mRNA seen in endothelial cells (Fig 1).

Incubation of EA.hy 926 cells with 17α-ethinyl estradiol enhanced the binding activity of nuclear extracts to a bona fide ERE. However, when the sequence from the human NOS III promoter that is closest to the consensus ERE was used, no binding of nuclear proteins occurred. Therefore, it seems that the estrogen-induced enhancement of NOS III promoter activity is not a direct result of an interaction of the activated estrogen receptor with the NOS III promoter. Increased NOS III transcription in EA.hy 926 cells was paralleled by an increased binding activity transcription factor Sp1 (Fig 7b), whereas binding activity of transcription factor GATA remained unchanged. Therefore, it is tempting to speculate that in the absence of a functional ERE, the estrogen-induced increase in promoter activity is the result of an enhanced binding activity of the essential transcription factor Sp1.

Recently, Arnal et al have reported that estrogen did not change NOS III expression in human umbilical vein endothelial cells, but rather that it reduced the superoxide production of the endothelial cells, thus allowing for more active NO to be released. The enzyme or enzymes responsible for superoxide production and potentially regulated by estrogens have not been identified. Our cell model does show an increase in NOS III mRNA and protein in response to estrogens and therefore mimics the in vivo situation described by other groups. The increase in NOS III expression demonstrated in the current study may seem relatively small, but it is likely to be of significant physiological relevance. In fact, a greater effect could be physiologically critical given the regulatory effects of NOS III on blood pressure and platelet function (for review see References 9 through 12). In addition, recent studies have indicated a variety of cardiovascular protective functions of endothelial-derived NO that goes beyond vasodilatation and inhibition of platelet aggregation. These include the reduced expression of chemokines and adhesion molecules as well as the inhibition of smooth muscle proliferation in the vascular wall (for review see References 9 through 11). In this context, even moderate increases in endothelial NOS expression may bear significant protective effects against cardiovascular disease, and this mechanism could account for at least part of the cardiovascular protective effects of estrogens.

Sp1 binding activities (n=4). A mutation of the putative binding site for transcription factor GATA on the human NO III promoter has been shown to reduce transcription by about 30%. However, in our electrophoretic mobility shift assays, nuclear extracts from estrogen-treated endothelial cells showed unchanged binding activity to oligonucleotides that contained the GATA consensus sequence (n=4, data not shown).

Discussion

The current work used a cell culture model to elucidate the molecular mechanism or mechanisms of the estrogen-stimulated increases in NOS III mRNA and/or protein expression previously seen under in vivo and in vitro conditions. The upregulation of the NOS III mRNA demonstrated in EA.hy 926 endothelial cells was an estrogen-specific effect; other steroid hormones such as testosterone, progesterone, and dihydrotestosterone did not change NOS III expression (Fig 3). The stimulatory effect of estrogens seems to be purely transcriptional; mRNA stability remained unchanged after incubation of EA.hy 926 endothelial cells with 17α-ethinyl estradiol (Fig 4). The estrogen concentrations required for this effect were within the physiological range. They were slightly higher than those found in the plasma of nonpregnant females but lower than those found in human plasma during pregnancy.

The human NOS III promoter contains consensus sequences for the binding of transcription factors Sp1, GATA, AP-1, NF-1, and NF-κB; as well as heavy metal−, acute-phase response−, shear stress−, and sterol-regulatory cis elements. The known promoter sequence contains no TATA box and no bona fide ERE.
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References

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