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Activation of Protein Kinase Cα and/or ϵ Enhances Transcription of the Human Endothelial Nitric Oxide Synthase Gene

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ABSTRACT

In primary human umbilical vein endothelial cells (HUVECs), incubation with phorbol-12-myristate-13-acetate (PMA) enhanced basal and bradykinin-stimulated nitric oxide production. In the HUVEC-derived cell line EA.hy 926, PMA and phorbol-12,13-dibutyrate stimulated endothelial nitric oxide synthase (NOS III) mRNA expression in a concentration- and time-dependent manner. Maximal mRNA expression (3.3-fold increase) was observed after 18 hr. NOS III protein and activity were increased to a similar extent. The specific protein kinase C (PKC) inhibitors bisindolylmaleimide I (1 μM), Gö 6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo-[2,3-*a*]pyrrolo-[3,4-*c*]carbazole] (1 μM), Ro-31–8220 [3-[1-[3-(amidinothio)propyl-1*H*-inoyl-3-yl]3-(1-methyl-1*H*-indoyl-3-yl) maleimide methane sulfonate] (1 μM), and chelerythrine (3 μM) did not change NOS III expression when applied alone, but they all prevented the up-regulation of NOS III mRNA produced by PMA. Of the PKC isoforms expressed in EA.hy 926 cells (α, βI, δ, ϵ, η, ζ, λ, and μ), only PKCα and PKCϵ showed changes in protein expression after PMA treatment. Incubation of EA.hy 926 cells with PMA for 2–6 hr resulted in a translocation of PKCα and PKCϵ from the cytosol to the cell membrane, indicating activation of these isoforms. After 24 hr of PMA incubation, both isoforms were down-regulated. The time course of activation and down-regulation of these two PKC isoforms correlated well with the PMA-stimulated increase in NOS III expression. When human endothelial cells (ECV 304 or EA.hy 926) were transiently or stably transfected with a 3.5-kb fragment of the human NOS III promoter driving a luciferase reporter gene, PMA stimulated promoter activity up to 2.5-fold. On the other hand, PMA did not change the stability of the NOS III mRNA. These data indicate that stimulation of PKCα, PKCϵ, or both by active phorbol esters represents an efficacious pathway activating the human NOS III promoter in human endothelium.

Isoform III of NOS (NOS III, ecNOS, eNOS) was identified first in endothelial cells (Förstermann *et al.*, 1991; Pollock *et al.*, 1991) but also is expressed in some other cell types, such as epithelial cells (Shaul *et al.*, 1994; Tracey *et al.*, 1994; Förstermann and Kleinert, 1995; Sakai *et al.*, 1996). Although NOS III is classified as a constitutively expressed NOS isozyme, its expression can be regulated by a variety of stimuli, such as cytokines (e.g., tumor necrosis factor-α, transforming growth factor-β1), bacterial lipopolysaccharide, oxidized lipoproteins, estrogens, shear stress, growth status, and hypoxia (Busse and Fleming, 1995; Förstermann and Kleinert, 1995; Harrison *et al.*, 1996).

NO generated by endothelial NOS III is involved in blood pressure regulation (Huang *et al.*, 1995; Rees *et al.*, 1989) 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 on vascular smooth muscle proliferation (for reviews, see Förstermann *et al.*, 1994; Moncada and Higgs, 1995; Gibbons and Dzau, 1996). Decreased endothelial NO production has been seen in pathophysiological conditions such as atherosclerosis, diabetes, and hypertension (for reviews, see Förstermann *et al.*, 1994; Moncada and Higgs, 1995; Gibbons

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ABBREVIATIONS: NOS, nitric oxide synthase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; Chel, chelerythrine; Gö, Gö 6976; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide; NOS III, endothelial-type nitric oxide synthase; LU, light unit(s); PBS, phosphate-buffered saline; PDBu, phorbol-12,13-dibutyrate; 4αPDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; Ro, Ro-31–8220; SDS, sodium dodecyl sulfate, DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; RT, reverse transcription; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

and Dzau, 1996). In view of the protective effects of NO, stimuli and mechanisms that increase NOS III activity, expression, or both are of significant interest.

PKC represents a family of closely related serine/threonine kinases (Nishizuka, 1992; Hug and Sarre, 1993) that plays a key role in different cellular signal transduction pathways (Nishizuka, 1988). Reports on the regulation of NOS activity by PKC are controversial. PKC inhibitors have been shown to reduce purinoceptor-stimulated (Brown *et al*., 1996) and angiotensin II-stimulated (Saito *et al*., 1996) NO synthesis in bovine endothelial cells. Phorbol esters that activate PKC have been shown to induce NO synthesis in isolated rat aorta (Sakata and Karaki, 1990). On the other hand, the application of phorbol esters inhibited endothelium-dependent vasodilator responses evoked by acetylcholine (Rubanyi *et al*., 1989). In porcine endothelial cells, PKC activation reduced the bradykinin-stimulated release of NO, and calphostin C, a PKC inhibitor, augmented the NO release (Hecker *et al*., 1993). A study performed with bovine aortic endothelial cells suggested that down-regulation or inhibition of PKC could increase endothelial NOS III expression (Ohara *et al*., 1995). This prompted us to investigate the importance of the different PKC isoforms in NOS III expression in human endothelial cells. Our study provides evidence that activation rather than inhibition of PKC up-regulates the activity of the human NOS III promoter.

Materials and Methods

Reagents. BIM, Chel, Gö [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo-[2,3-*a*]pyrrolo-[3,4-*c*]carbazole], PMA, PDBu, 4αPDD, and staurosporine were purchased from Calbiochem (San Diego, CA). Ro [3-[1-[3-(amidinothio)propyl-1*H*-inoyl-3-yl]-3-(1-methyl-1*H*-indoyl-3-yl)maleimide methane sulfonate] was a generous gift of Dr. D. Bradshaw (Roche Research Center, London, UK). Isotopes were obtained from Amersham (Braunschweig, Germany). Restrictions enzymes, polynucleotide kinase, *Taq* polymerase, dNTPs, Ficoll (type 400), oligonucleotides, and oligo(dT) primer were purchased from Pharmacia (Vienna, Austria). Luciferase and β-galactosidase assay systems were obtained from Promega (Madison, WI) and Tropix (Bedford, MA), respectively. Superscript reverse transcriptase was obtained from GIBCO BRL (Gaithersburg, MD). DNase I, DOTAP, RNase A, RNase T1, T3, and T7 RNA polymerase were purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). An antibody specific for PKCα was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies specific for PKCβ_I and PKCζ were generous gifts of Peter Parker (ICRF, London, UK). Antibodies specific for PKCβ_{II}, PKCδ, PKCϵ, PKCη, PKCθ, and PKCμ were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). The antibody to PKCλ was from Transduction Laboratories (Lexington, KY). 3-Isobutyl-1-methylxanthine and bradykinin were purchased from Sigma Chemie (Deisenhofen, Germany).

Cell culture. HUVECs were isolated as described previously (Wohlfart *et al*., 1997) and grown in Iscove's minimal essential medium containing glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and Biotect protection medium. Human endothelial cells EA.hy 926 (Edgell *et al*., 1983) and ECV304 (Takahashi *et al*., 1990) cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1× HAT (hypoxanthine, amethopterin/methotrexate, thymine) (Edgell *et al*., 1983). For NOS III mRNA analyses, confluent EA.hy 926 cells were incubated for 18 hr with PMA (0.1–1000 nM), PDBu (100 nM), 4αPDD (100 nM), BIM (1 μM), Gö (1 μM), Ro (1 μM), Chel (3 μM), or staurosporine (1–100 nM), alone or in combination. To determine the time course of NOS III mRNA increases in response to PMA, confluent EA.hy 926 cells were incubated with 100 nM PMA for 2, 6, 10, 18, or 24 hr. For determination of the stability of the NOS III mRNA in the presence of PMA, cells were incubated with or without PMA for 18 hr; then 10 μg/ml actinomycin D was added to the medium, and the cells were incubated for additional 6, 12, 24, or 48 hr.

Determination of cellular NOS activity through measurement of intracellular cGMP accumulation. Measurements of cGMP levels in HUVECs were made as described previously (Wohlfart *et al*., 1997). Briefly, HUVECs were incubated for 20 hr with or without 10 nM PMA. Then, the cells were washed twice with HEPES/Tyrode's solution (prewarmed at 37°) and preincubated for 15 min with 3-isobutyl-1-methylxanthine (0.1 mM) and superoxide dismutase (20 units/ml). Then, cells were stimulated with bradykinin (10 nM; Sigma) for 3 min. The reaction were stopped by rapid removal of the incubation medium and extraction of the cells with an ice-cold mixture of 1 N formic acid/acetone (15:85, v/v). After removal of the solvent, cGMP was determined using a specific radioimmunoassay (DuPont-New England Nuclear, Boston, MA).

Cloning of a human NOS III cDNA fragment. Two micrograms of total RNA from EA.hy 926 cells were annealed with 0.5 μg of an oligo(dT) primer (Pharmacia) and reverse-transcribed with Superscript 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 GACATTGAGAGCAAAGGGCTGC (sense) and CGGCTTGTCACCTCCTGG (antisense) corresponding to positions 3111–3133 and 3518–3536 of the human NOS III cDNA (Marsden *et al*., 1992). PCR was performed in a 100-μl volume containing 1× *Taq* polymerase buffer (Pharmacia), 0.2 mM concentration of dNTPs, 1.5 mM $MgCl_2$, 2 units of *Taq*-polymerase, 50 pmol of oligonucleotide primers, and RT products (10% of the RT reaction). After an initial denaturation step at 95° for 5 min, 30 cycles were performed (1 min at 95°, 1 min at 60°, and 1 min at 72°), followed by a final 10-min extension step at 72°. The PCR products (30 μl) were analyzed on a 1.5% agarose gel containing 0.1 mg/ml ethidium bromide. The amplified cDNA fragments (426 bp) were cloned into the *Eco*RV 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 using the dideoxy chain termination method (^{T7}Sequencing Kit; Pharmacia).

Preparation of antisense RNA probes. To generate radiolabeled antisense RNA probes for RNase protection assays, pCR-NOS III-Hu and pCR-β-actin-Hu (Kleinert *et al*., 1996) were linearized with *Sma*I or *Bst*EII, extracted with phenol-chloroform, and concentrated by ethanol precipitation. Then, 0.5 μg of each DNA was *in vitro* transcribed using T7/T3 RNA polymerase (Pharmacia) and [α-^{32}P]UTP. After a 1-hr incubation, the template DNA was degraded with DNase I for 45 min. The radiolabeled RNA was purified using NucTrap probe purification columns (Stratagene).

RNA extraction and RNase protection analyses. Total RNA was isolated from EA.hy 926 cells by guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). RNase protection assays were performed with a mixture of RNase A and RNase T1 according to the manufacturer's instructions (Boehringer-Mannheim). Briefly, after denaturation, 20 μg of total RNA (prepared as described above) was hybridized with 200,000 cpm labeled NOS III antisense RNA probe and 40,000 cpm labeled β-actin antisense RNA probe at 51° for 16 hr in a volume of 40 μl of hybridization buffer [40 mM PIPES, pH 6.7, 1 mM EDTA, 400 mM NaCl, 50% formamide]. The mixture was digested by the addition of 300 μl of digestion buffer (10 mM Tris·HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) containing 3.5 μg of RNase A and 37.5 units of RNase T1 for 30 min at 30°. The reaction was stopped by proteinase K digestion (70 μg/sample in 70 μl of 7.15 mM Tris·HCl, pH 7.4, 7.15 mM EDTA, and 2.85% SDS for 15 min at 37°) and phenol extraction. The reaction products were concentrated by ethanol precipitation and analyzed by

electrophoresis on denaturing urea-polyacrylamide gels (8 M urea/6% polyacrylamide). The electrophoresis buffer was 1× TBE (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA). The gels were electrophoresed for 1–2 hr, dried, and exposed to X-ray films. Densitometric analyses were performed using a PhosphorImager (Bio Rad, Hercules, CA). The protected RNA fragments of NOS III and β-actin were 280 and 108 nucleotides, respectively. The density of each NOS III band was normalized with the corresponding β-actin band.

Determination of NOS activity by the conversion of L-arginine to L-citrulline. Approximately 10^6 EA.hy 926 cells (untreated or incubated for 18 hr with 100 nM PMA, 100 nM PDBu, or 100 nM 4αPDD) were washed with 5 ml of PBS and scraped into 1 ml of PBS. After centrifugation (100 × *g*, 5 min, 4°), the cell pellet was resuspended in 50 μl of lysis buffer (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 100 μM phenylmethylsulfonyl fluoride). Cells were lysed by three cycles of freeze-thawing using liquid nitrogen. The homogenates were centrifuged (1500 × *g*, 15 min, 4°). The protein content of the supernatant was determined using the Bradford assay (BioRad, Hercules, CA). For the conversion assay, 5 μg of the cellular protein was incubated in 100 μl of assay volume containing 1 mM NADPH, 3 μM tetrahydrobiopterin, 5 μM FAD, 5 μM FMN, and 1000 Bq of [^{14}C]L-arginine. The reaction was stopped after 15 min by the addition of two volumes of ice-cold methanol. The dried reaction products were redissolved in 20 μl of water and spotted onto Polgram SIL N-HR thin layer chromatography plates (Macherey-Nagel, Düren, Germany). [^{14}C]L-Arginine was separated from [^{14}C]L-citrulline using chloroform/methanol/ammonium hydroxide/water (0.5:4.5:2.0:1.0, v/v/v/v) as the solvent system. Thin layer chromatography plates were dried and autoradiographed.

Protein preparation and Western blotting. For the determination of NOS III and PKC protein expression, total protein was isolated from EA.hy 926 cells as described previously (Wallerath *et al.*, 1997). In brief, EA.hy 926 cells (untreated or incubated for 20 hr with 100 nM PMA or 100 nM PDBu) were homogenized in ice-cold homogenization buffer (50 mM Tris·HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreithol, 7 mM glutathione, 10% glycerol, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 20 units/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride) containing 20 mM concentration of the detergent CHAPS. Homogenates were incubated for 30 min at 4° followed by a centrifugation at 100,000 × *g* for 1 hr. Supernatants were used for protein determination (Bradford assay). Western blotting was performed as described previously (Kleinert *et al.*, 1996). Briefly, 50 μg of each protein sample were separated by SDS-7.5% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (BioRad). All subsequent steps were performed at room temperature. Blots were blocked for 30 min with 3% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 in TBS (10 mM Tris·HCl, pH 7.4, 150 mM NaCl) and then incubated for NOS III protein detection with a polyclonal rabbit anti-NOS III antibody (Transduction Laboratories) in TBS containing 0.5% (w/v) gelatin and 0.05% (w/v) Tween 20 for 45 min. After washing in TBS/gelatin/Tween, the blots were incubated with horseradish peroxidase-conjugated second antibodies diluted 1:1500 in TBS/gelatin/Tween for 30 min. The blots were washed stepwise with TBS/gelatin/Tween, TBS/Tween, and TBS alone. Immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (DuPont-New England Nuclear) according to the manufacturer's instruction.

Analysis of PKC expression and translocation. PKC proteins were detected in Western blots with antibodies specific for the different PKC isoforms (α, βI, βII, δ, ϵ, η, θ, ζ, λ, and μ). Here, the antibody incubation buffer was PBS containing 0.1% (v/v) Triton X100 and 1% (w/v) low-fat dry milk powder. Blots were incubated with primary antibody overnight and then washed with PBS/Triton/milk powder and incubated with secondary antibody for 1 hr.

For the detection of PKC translocation, soluble (cytosolic) and particulate (membrane) protein fractions were separated. EA.hy 926 cells were kept untreated or incubated with 100 nM PMA for 2, 6, or 24 hr. Then, cells were homogenized in ice-cold homogenization buffer and centrifuged at 100,000 × *g* for 1 hr. The supernatant (cytosolic fraction) was removed, and the pellet was washed in homogenization buffer containing 1 M NaCl and centrifuged at 100,000 × *g* for 30 min. The supernatant was discarded, and the pellet solubilized by agitation in homogenization buffer containing 20 mM CHAPS (30 min, 4°). After another centrifugation step at 100,000 × *g* for 1 hr, the supernatant (containing the solubilized particulate fraction) was obtained. Western blotting was performed as described.

Cloning of the 5′-flanking region from the human NOS III gene. Genomic DNA was isolated from human EA.hy 926 cells by RNase/proteinase K digestion and phenol-chloroform extraction as described previously (Sambrook *et al.*, 1989). This DNA was used for amplification of the 5′-flanking DNA of the human NOS III gene. PCR was performed as described using as primers the oligonucleotides TGATGCTGCCTGTCACCTTG (5′) and TACTGTGCGTCCACTCTGCTGC (3′). The sequences were based on published 5′-flanking sequences of the human NOS III gene (Marsden *et al.*, 1993). 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-5′. The DNA sequence of the cloned PCR products were determined using the ^{T7}Sequencing Kit (Pharmacia). This human NOS III promoter fragment was used to screen a cosmid library containing human chromosomal DNA cloned in pWE15 (Stratagene). Eight individual cosmid clones were isolated and characterized. An *Apa*I fragment containing the human NOS III promoter sequence (positions −3470 to +115) was cloned and sequenced (GenBank accession no. AF032908). To clone the human NOS III promoter in front of a luciferase reporter gene, this fragment was used as template in a PCR with the oligonucleotides gtgagaagcttGAGAGAAAGAGCTGTCCCCGGGGCCTTGGGG (5′) (P-ES1a) and gtgagtcatgaGTTACTGTGCGTCCACTCGCTGCTGCCTGC (3′) (P-ES1b) as primers (extra nucleotides to generate *Hin*dIII and *Bsp*HIII restriction sites are displayed in lowercase letters). The resulting PCR fragment was restricted with *Hin*dIII and *Bsp*HIII and cloned into pGL$_3$-Basic (Promega; containing a promoterless luciferase reporter gene) restricted with *Hin*dIII and *Nco*I to generate pNOS III-Hu-3500-Luc.

To generate a human NOS III promoter luciferase reporter gene construct that also contains a neomycin resistance gene, the *Apa*I fragment was used in a PCR with P-ES1a and the oligonucleotide gtgagtcatgaGGCCCTGCTTGCCGCACAGCCCAAGGCCC (3′) (P-ES2) as primers. The resulting PCR fragment (containing an additional 90 base pairs of the human NOS III transcript) was restricted with *Hin*dIII and *Bsp*HIII and cloned in pGL$_3$-neo (pGL$_3$-Basic containing a neomycin resistance gene under the control of the thymidine kinase promoter) previously restricted with *Hin*dIII and *Nco*I to generate pNOS III-Hu-3500-Luc-neo.

Transient transfection and reporter gene assay. Because the transfection efficiency of EA.hy 926 cells was very low, the human endothelial cell line ECV304 was used transient transfection experiments. Cells were plated onto 30-mm cell culture dishes 24 hr before transfection. The cells (at ≈80% confluence) were transfected by lipofection with DOTAP according to the manufacturer's recommendations (Boehringer-Mannheim); 2.5 μg pGL$_3$-Basic (Promega) or pNOS III-Hu-3500-Luc was used; 2.5 μg of pCH110 (Pharmacia; containing the β-galactosidase gene driven by an SV40 promoter) was cotransfected for normalization. The cells were washed with culture medium 6 hr after transfection and incubated with 100 nM PMA for 18 hr. Extracts (200 μl) were prepared 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 previously (Kleinert *et al.*, 1996). The LU of the luciferase assay were

normalized by the LU of the β-galactosidase assay after subtraction of extract background.

Stable transfection of EA.hy 926 cells and reporter gene assay. EA.hy 926 cells were plated onto 30-mm cell culture dishes 24 hr before transfection. The cells (at ≈80% confluence) were transfected with DOTAP according to the manufacturer's recommendations (Boehringer-Mannheim); 10 μg pGL_3-Basic pNOS III-Hu-3500-Luc-neo or 10 μg salmon sperm DNA was used. The cells were washed with culture medium 6 hr after transfection and incubated with medium for 18 hr. Then, the cells were split to dilute them and incubated with medium containing 1 mg/ml G418. Single clones were selected from the pNOS III-Hu-3500-Luc-neo-transfected cells and propagated in medium containing 1 mg/ml G418. For analysis of phorbol ester enhancement of NOS III promoter activity, the stably transfected cells were incubated with PMA (10 nM) for 18 hr. Extracts (200 μl) were prepared using the reporter lysis buffer (Promega), and luciferase activities were determined as described.

Statistical analysis. Statistical differences between mean values were determined by analysis of variance followed by Fisher's protected least significant difference test for comparison of mean values.

Results

The phorbol ester PMA enhances cGMP production in HUVECs incubated with bradykinin. Preincubation with PMA (10 nM) for 20 hr enhanced both basal and bradykinin (10 nM)-stimulated NO production of HUVECs by ≈2-fold (Fig. 1). To investigate the molecular mechanism of this stimulation, subsequent experiments were performed in the HUVEC-derived stable cell line EA.hy 926 (Edgell *et al.*, 1983).

Phorbol esters enhance NOS III mRNA expression in human endothelial EA.hy 926 cells. Human endothelial EA.hy 926 cells were incubated for 18 hr with PMA (0.1–1000 nM), and total RNA was prepared. As shown in Fig. 2, PMA enhanced NOS III mRNA expression in a concentration-dependent manner. A concentration as low as 0.1 nM PMA produced a doubling of NOS III mRNA. NOS III mRNA concentrations reached plateau between 1 and 100 nM PMA and decreased at higher concentrations of PMA. Fig. 3 demonstrates the time dependence of the PMA effect. A significant up-regulation of the NOS III mRNA was observed after a 6-hr incubation with 100 nM PMA (271.6 ± 38.6% of control, mean ± standard error). Maximum stimulation was reached at 18 hr (326.1 ± 24.2% of control). Another active phorbol ester, PDBu (100 nM, 18 hr), simulated NOS III mRNA expression, whereas 4αPDD (100 nM, 18 hr), which does not activate PKC, had no effect on NOS III mRNA expression (Fig. 4).

Phorbol esters enhance NOS III protein and activity in human endothelial EA.hy 926 cells. As shown in Fig. 5, both PMA and PDBu (100 nM, 20 hr each) increased NOS III protein content in the EA.hy 926 cells (as determined with Western blotting using 50 μg of total protein from each sample). Densitometric analyses of the NOS III protein bands indicated an increase to 225.5 ± 8.2% after 100 nM PMA and an increase to 258.2 ± 22.8 after PDBu (mean ± standard error, four experiments). 4αPDD had no effect (three experiments).

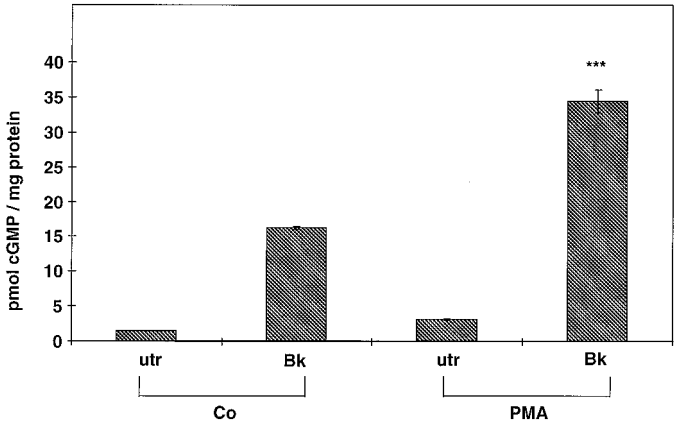

Fig. 1. Effect of PMA on NOS activity in HUVEC. Cells were either left untreated (control; *Co*) or preincubated for 20 hr with the phorbol ester PMA (10 nM). NOS III activity was determined by measuring the cGMP content in untreated (*utr*) cells and cells stimulated for 3 min with bradykinin (*Bk*, 10 nM). *Bars*, mean ± standard error of eight independent experiments. ***, $p < 0.001$ compared with either control/bradykinin or untreated/PMA.

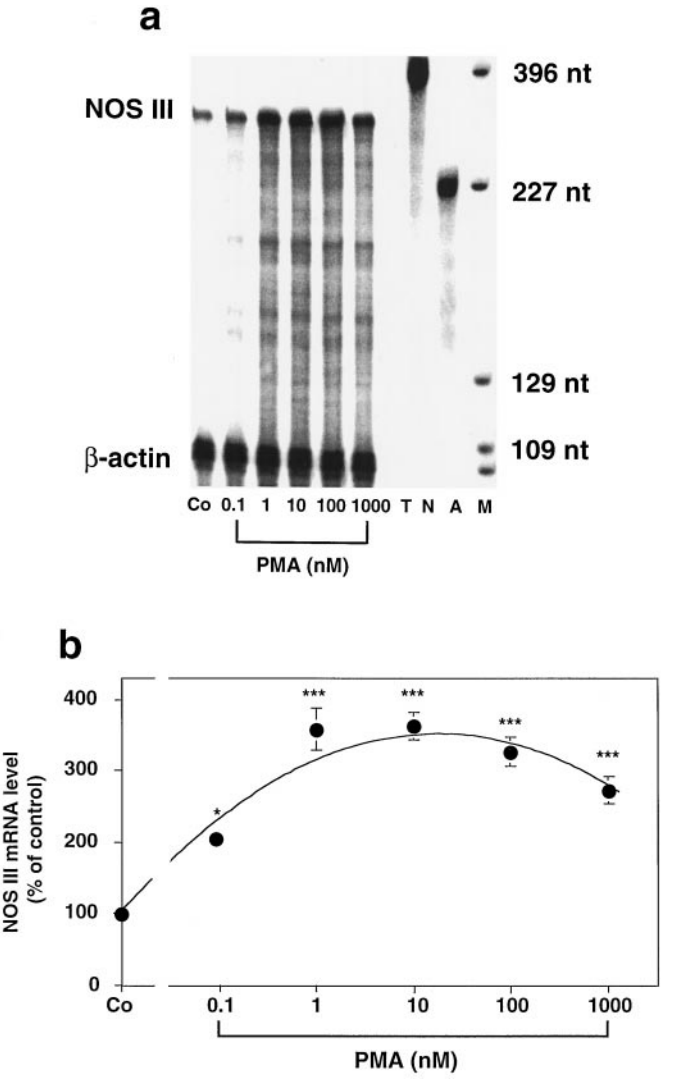

Fig. 2. PMA enhances NOS III mRNA expression in EA.hy 926 cells. RNase protection analyses were performed using antisense RNA probes to human NOS III and β-actin (for standardization). RNAs were prepared from untreated EA.hy 926 cells (control cells, *Co*) and EA.hy 926 cells incubated with PMA (0.1–1000 nM) for 18 hr. a, Autoradiograph of a representative gel. *T*, tRNA control. *N*, NOS III antisense probe alone. *A*, β-actin antisense probe alone. *M*: molecular weight markers (pGl_2-Basic; Promega; restricted with *Hin*fI). b, Densitometric analyses of five different gels. Values are mean ± standard error. *, $p < 0.05$; ***, $p < 0.001$ compared with control.

In additional experiments, EA.hy 926 cells were incubated with 100 nM PMA, 100 nM PDBu, or 100 nM 4αPDD for 20 hr, and NOS III activity was determined by the [^{14}C]L-arginine to [^{14}C]L-citrulline conversion assay. As demonstrated in Fig. 6, both PMA and PDBu, but not 4αPDD, increased NOS III activity in the EA.hy 926 cells.

PKC inhibitors had no effect on NOS III mRNA level but reduced the PMA-induced up-regulation. As shown in Fig. 7a, an 18-hr incubation with the specific PKC inhibitors BIM, Gö, Ro (1 μM each), and Chel (3 μM) did not significantly affect the NOS III mRNA expression. However, the PMA (100 nM)-induced up-regulation of NOS III mRNA was significantly reduced by all four PKC inhibitors (Fig. 7b).

EA.hy 926 cells express different isoforms of PKC. Western blotting using isoform-specific anti-PKC antibodies demonstrated the expression of PKCα, PKCβI, PKCδ, PKCε, PKCη, PKCζ, PKCλ, and PKCμ in EA.hy 926 cells (Fig. 8).

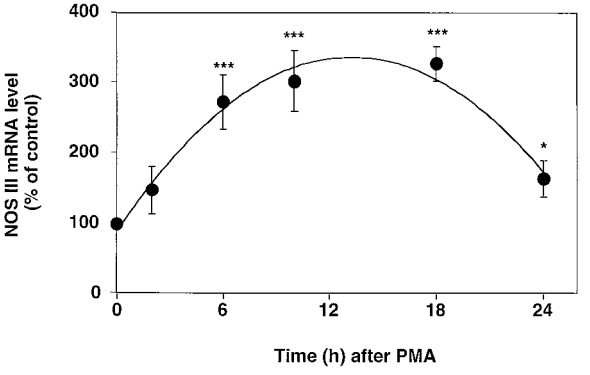

Fig. 3. Time course of PMA-induced NOS III mRNA expression in EA.hy 926 cells. Cells were exposed to PMA (100 nM) for different periods of time. Total RNA was isolated, and NOS III mRNA was determined with RNase protection analyses (as shown in Fig. 2). NOS III mRNA levels were quantified by densitometric scanning of the autoradiographs. Values are mean ± standard error of four experiments. *, $p < 0.05$; ***, $p < 0.001$ compared with time 0.

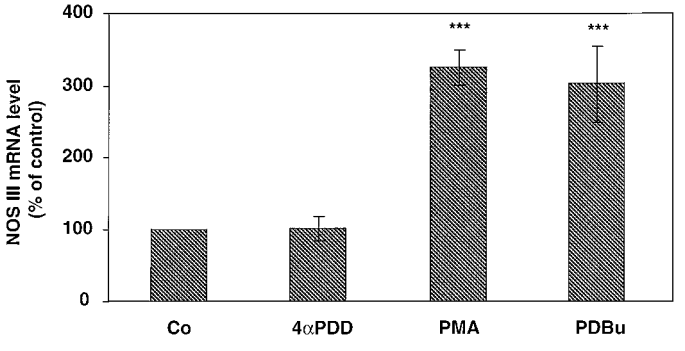

Fig. 4. Effect of different phorbol esters on NOS III mRNA expression in EA.hy 926 cells. Cells were either left untreated (*Co*) or incubated for 18 hr with the phorbol esters 4αPDD, PMA, or PDBu (100 nM each). NOS III mRNA was quantified with RNase protection analyses. *Bars*, mean ± standard error of three independent experiments. ***, $p < 0.001$ compared with control.

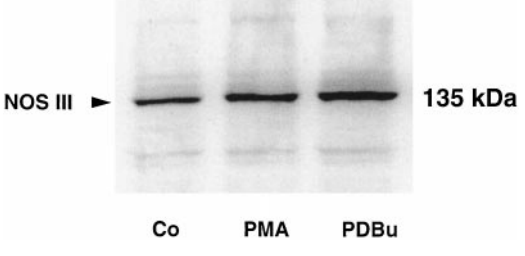

Fig. 5. Effect of phorbol esters on NOS III protein expression in EA.hy 926 cells. Total protein was isolated from cells either receiving no treatment (*Co*) or incubated with PMA or PDBu (100 nM each) for 20 hr. Western blots were performed using a polyclonal anti-NOS III antibody. The blot shown is representative of three independent experiments with similar results.

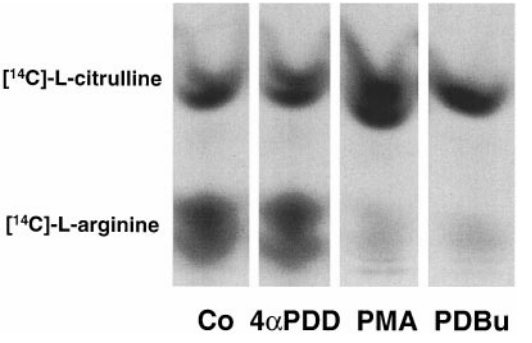

Fig. 6. Effect of different phorbol ester on NOS activity in EA.hy 926 cells. Cells were either kept untreated (*Co*) or incubated for 20 hr with the phorbol esters 4αPDD, PMA, or PDBu (100 nM each). Cell extracts were prepared, and NOS III activity was determined in terms of conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline. Amino acids were separated by thin layer chromatography, and chromatograms were autoradiographed. The autoradiograph shown is representative of three independent experiments with similar results.

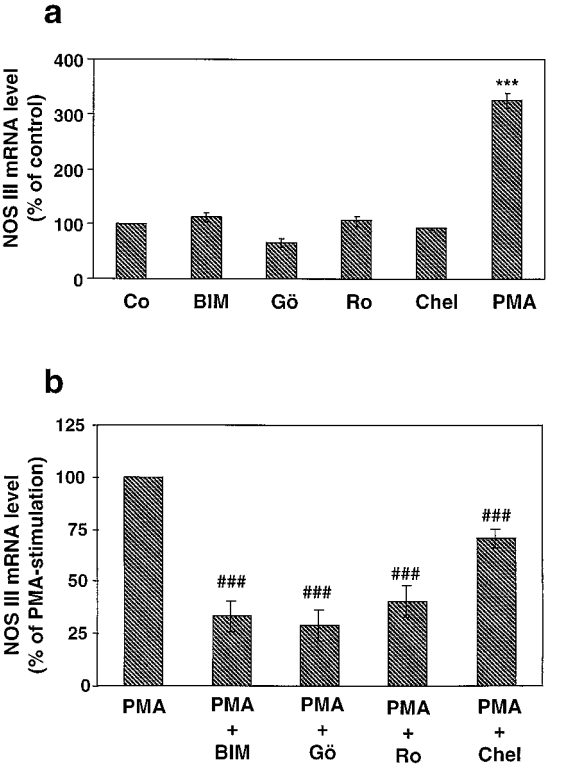

Fig. 7. Effect of PKC inhibitors on basal and PMA-stimulated NOS III mRNA expression in EA.hy 926 cells. Densitometric analyses of RNase protection analyses using RNAs isolated from EA.hy 926 cells. a, Effect of the specific PKC inhibitors BIM, Gö, Ro (1 μM each), and Chel (3 μM) for 18 hr on basal NOS III mRNA expression. *Co*, untreated control cells; treatment with PMA (100 nM) is shown for comparison. ***, $p < 0.001$ compared with control cells. b, Effect of specific PKC inhibitors on the PMA (100 nM)-stimulated NOS III mRNA expression. ###, $p < 0.001$ compared with PMA-treated cells. *Bars*, mean ± standard error of four to eight independent experiments.

PKC isoforms βII and θ were not expressed (two experiments, data not shown). Strictly neuronal expression had been described for PKCγ (Hug and Sarre, 1993); therefore, expression of this isoform was not analyzed in endothelial cells. Incubation of endothelial cells with PMA (100 nM) for 30 hr significantly down-regulated PKCα and PKCε but left protein levels of the other isoforms unchanged (Fig. 8).

Time course of the PMA-induced translocation and down-regulation of PKCα and PKCε in EA.hy 926 cells. PKCα and PKCε were found to be down-regulated by long term treatment with PMA (Fig. 8); therefore, we determined the time course of their translocation and down-regulation. Fig. 9 shows a representative Western blot analysis for PKCα. Before PMA treatment, more than half of the PKCα protein was located in the soluble fraction. Incubation with PMA (100 nM, 2 and 6 hr) induced a translocation of PKC protein from the soluble to the particulate fraction. After 24 hr, PKCα was markedly down-regulated. Only small amounts of protein were found in the particulate fraction (Fig. 9). PKCε showed a closely similar pattern of regulation (three experiments, data not shown). The time course of PKC translocation (activation) and down-regulation paralleled the time course of the PMA-induced NOS III mRNA increase shown in Fig. 3.

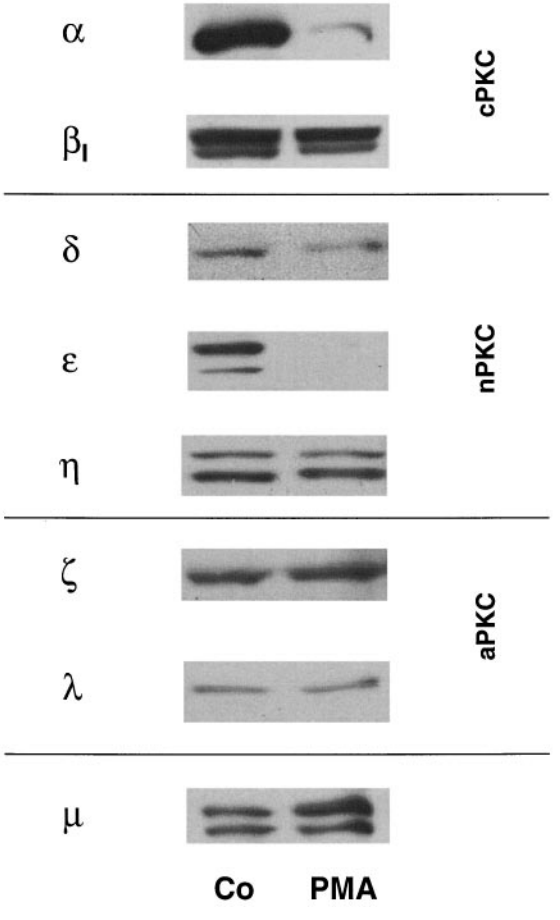

Fig. 8. Effect of PMA on the protein expression of different PKC isoforms in EA.hy 926 cells. Western blot analyses using antibodies specific for the conventional PKC isoforms α and βI; the novel PKC isoforms δ, ε, and η; the atypical PKC isoforms ζ and λ, and the PKCμ. EA.hy 926 cells were kept untreated (*Co*) or incubated with PMA (100 nM) for 30 hr. Total protein was isolated, and Western blots were performed. The blots shown are representative of two independent experiments with similar results.

PMA enhances the activity of a NOS III gene promoter fragment transiently transfected into human endothelial ECV304 cells and stably transfected into EA.hy 926 cells. Because EA.hy 926 cells exhibited a very low transfection efficiency on transient transfection, ECV304 cells were used in these experiments. They were transfected with pGL_3-Basic (containing a promoterless luciferase reporter gene) or pNOS III-Hu-3500-Luc (containing a 3470-bp fragment of the 5′-flanking sequence of the human NOS III gene cloned before the luciferase reporter gene). Transfected cells were incubated with or without PMA (100 nM) for 18 hr, and cytoplasmic cell extracts were prepared. Fig. 10, *top*, shows the relative luciferase activity (corrected for β-galactosidase activity; see Materials and Methods). The 3470-bp NOS III promoter fragment showed significant basal activity compared with pGl_3-Basic. PMA (100 nM) increased the activity of the human NOS III promoter fragment by 2.3-fold, demonstrating that the PMA stimulation of endothelial cells enhances the activity of the NOS III gene promoter (Fig. 10, *top*).

Similarly, in EA.hy 926 cells stably transfected with pNOS III-Hu-3500-Luc-neo, an incubation with PMA (0.1–100 nM) for 18 hr enhanced NOS III promoter activity up to 2.6-fold (Fig. 10, *bottom*). A comparable stimulation of the NOS III promoter activity (up to 2.4-fold) was seen after an 18-hr incubation of the stably transfected EA.hy 926 cells with PDBu (0.1–100 nM, three experiments, data not shown).

PMA does not change the stability of the NOS III mRNA. EA.hy 926 cells were incubated for 18 hr with or without PMA (100 nM). Then, 10 μg/ml actinomycin D was added to inhibit gene transcription, and RNA was prepared at different times thereafter. As shown in Fig. 11, the half-life of human NOS III mRNA in untreated EA.hy 926 cells was ≈44 hr. Incubation with PMA (100 nM) did not change the stability of the NOS III mRNA.

Discussion

Because of the vascular protective function of endothelial NOS III (Förstermann *et al*., 1994; Moncada and Higgs, 1995; Gibbons and Dzau, 1996), an up-regulation of this gene is of significant scientific (and possibly clinical) interest. The current study demonstrates that activation of PKC isoforms α, ε, or both represents an efficacious mechanism of increasing transcription of this gene (at least *in vitro*.). Given the potency of NO in the vascular system, a ≤3-fold increase in

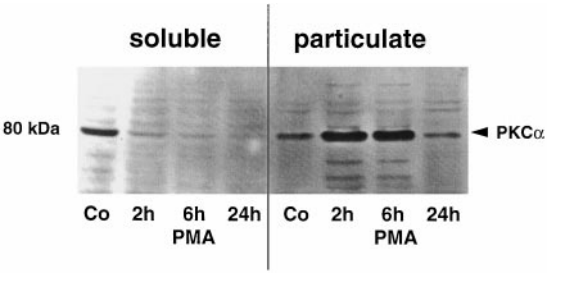

Fig. 9. PMA induces a translocation and down-regulation of PKCα. Western blot analysis using an antibody to PKCα. EA.hy 926 cells were either kept untreated (*Co*) or incubated with PMA (100 nM) for 2, 6, or 24 hr. Subcellular fractions (soluble and particulate) were isolated, and Western blots were performed. The blots shown are representative of three independent experiments with similar results.

NOS III mRNA, protein, and activity could have major functional consequences.

Evidence accumulated in the current study indicates that activation (not down-regulation or inhibition) of PKC triggers the pathway or pathways leading to NOS III induction. First, NOS III expressional stimulation was only seen with the PKC-stimulating phorbol esters PMA and PDBu (Figs. 2–7). The phorbol ester 4αPDD, which does not activate PKC, had no effect on NOS III expression (Figs. 4 and 6). Second, specific PKC inhibitors such as BIM, Gö, Ro, and Chel did not change NOS III mRNA expression by themselves (Fig. 7). They did, however, prevent the stimulatory effect of active phorbol esters (Fig. 7). Third, the time course of the PMA-induced NOS III mRNA expression correlated well with the time course of PKC activation, not with its down-regulation and inactivation (compare Figs. 3 and 9). When PKC was down-regulated (after 24 hr), NOS III mRNA levels returned toward basal levels.

The issue of NOS III expressional regulation by PKC is somewhat contentious because a previous study performed with bovine aortic endothelial cells concluded that down-regulation or inhibition of PKC elevated endothelial NOS III expression in that cell type (Ohara *et al.*, 1995). These authors found that staurosporine, a nonspecific kinase inhibitor, increased NOS III expression (Ohara *et al.*, 1995). We verified in our human endothelial cell system that staurosporine produced an increase in NOS III mRNA that was similar in magnitude to that of the active phorbol esters. NOS III mRNA levels reached a maximum of 333 ± 7% of control (five experiments) after 24 hr of incubation with 10 nM staurosporine. However, as shown in Fig. 7a, this effect was not mimicked by any other PKC inhibitor tested. There are some reports that staurosporine can activate, rather than inhibit, PKC (Stanwell *et al.*, 1996a, 1996b). However, in EA.hy 926 cells, no translocation or down-regulation of PKC was detected with staurosporine using an anti-PKCα antibody (two experiments, data not shown). In addition, the staurosporine effect on NOS III expression was not inhibited by the specific PKC inhibitors BIM, Ro, or Chel (two experiments, data not shown). Therefore, staurosporine seems to up-regulate NOS III expression by an unknown mechanism unrelated to PKC. This mechanism awaits further clarification.

To date, 12 isozymes of PKC have been identified in mammalian tissues. They have been subdivided into conventional PKC isoforms (α, βI, βII, γ), novel PKC isoforms (δ, ϵ, η, θ), atypical PKC isoforms (ζ, ι/λ), and yet another subgroup (PKCμ) (Nishikawa *et al.*, 1997). The conventional PKC members can be activated by calcium, phospholipids, diacylglycerol, and phorbol esters; the novel PKC members are activated by the same compounds but are calcium independent. In resting endothelial cells, PKC is located predominantly in the cytosol (Hecker *et al.*, 1993). After activation, the PKCs translocate from the cytosol to the particulate fraction (Mochly-Rosen, 1995). This seems essential for the function of many, if not all, PKC isoforms. Phosphorylation of endogenous PKC substrates has been shown to increase on PKC translocation (Graff *et al.*, 1989), and an inhibition of PKC translocation diminished PKC-mediated biological responses (Yedovitzky *et al.*, 1997).

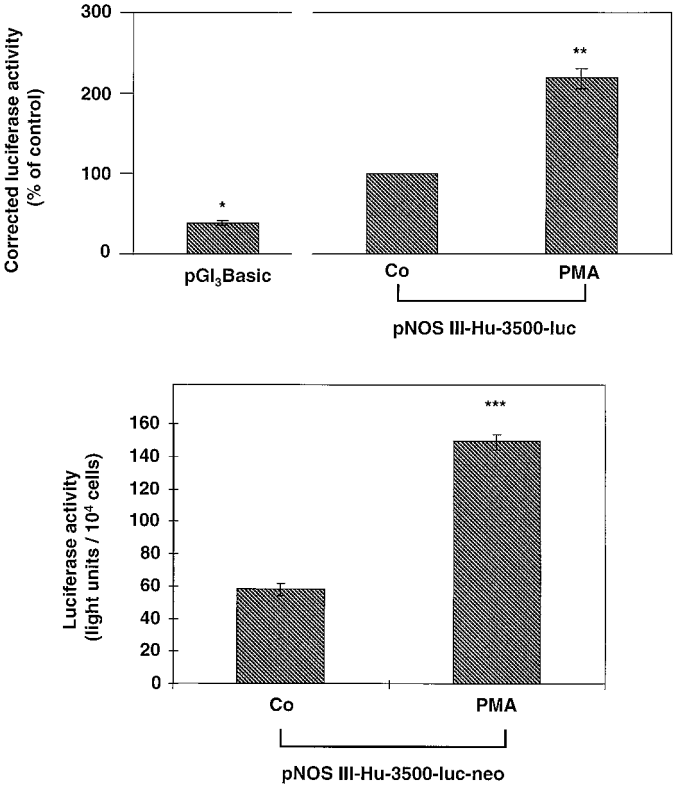

Fig. 10. PMA increases NOS III promoter activity in human endothelial ECV 304 and EA.hy 926 cells. *Top*, human endothelial ECV304 cells were transiently transfected with pGl$_3$-Basic (containing a promoterless luciferase gene) or pNOS III-3500-Hu-Luc (containing a 3.5-kb NOS III promoter fragment cloned before the luciferase gene). pCH110 (containing the β-galactosidase gene) was cotransfected. The pNOS III-3500-Hu-Luc-transfected cells were either kept untreated (*Co*) or exposed to PMA (100 nM) for 18 hr. Then, the cells were lysed, and LU were determined. *Bottom*, human endothelial EA.hy 926 cells were stably transfected with pNOS III-3500-Hu-Luc-neo (containing a 3.5-kb NOS III promoter fragment cloned before the luciferase gene and a neomycin resistance gene). Stable cells were either kept untreated (*Co*) or incubated with PMA (10 nM) for 18 hr. Then, the cells were lysed, and luciferase activity was determined. The relative luciferase activity (corrected for β-galactosidase activity for the transiently transfected cells) was taken as a measure of NOS III promoter activity. *Bars*, mean ± standard error of three to five independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.01$ compared with the respective control.

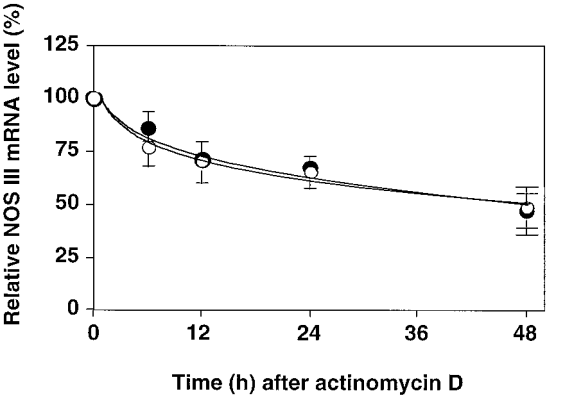

Fig. 11. PMA does not affect the stability of NOS III mRNA in EA.hy 926 cells. Cells were either kept untreated (control, ○) or incubated with PMA (100 nM, ●) for 18 hr. Then, the inhibitor of transcription, actinomycin D (10 μg/ml), was added to the culture medium. RNA was prepared 6, 12, 24, or 48 hr thereafter, and NOS III mRNA was determined by quantitative RNase protection assays. In both groups, NOS III mRNA levels at the time of addition of actinomycin D (0 hr) were set 100%.Values are mean ± standard error of four independent experiments.

Phorbol esters like PMA and PDBu have a diacylglycerol-like structure and activate conventional and novel PKCs, followed by down-regulation after prolonged exposure (Nishizuka, 1984). EA.hy 926 endothelial cells express a number of PKC isoforms (Fig. 8), but only PKCα and PKCϵ showed translocation and down-regulation after PMA treatment. This suggests that one of these isoforms (or both) trigger the signaling process leading to enhanced NOS III expression. This hypothesis is consistent with the finding that the PKC inhibitor Gö was the most efficacious blocker of the PMA-induced stimulation of NOS III mRNA expression. Gö has been described as a preferential inhibitor of the conventional PKCs (α, β, and γ) (Martiny-Baron *et al.*, 1993; Gschwendt *et al.*, 1996).

The signaling mechanism triggered by the activated PKC or PKCs and leading to the up-regulation of NOS III mRNA is still largely unclear. Our data indicate, however, that the up-regulation is a result of an increased gene transcription and does not involve changes in NOS III mRNA stability (Figs. 10 and 11). In conclusion, we demonstrated that activation of protein kinase C isoforms α, ϵ, or both represents an efficacious means of increasing the transcription of the human NOS III gene.

Acknowledgments

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