

Structure-Activity Relationship of Staurosporine Analogs in Regulating Expression of Endothelial Nitric-Oxide Synthase Gene

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ABSTRACT

In human umbilical vein endothelial cells and in human umbilical vein endothelial cell-derived EA.hy 926 cells, staurosporine (Stsp) and its glycosidic indolocarbazole analogs 7-hydroxystaurosporine (UCN-01) and 4'-*N*-benzoyl staurosporine (CGP 41251) enhanced nitric-oxide synthase (NOS) III mRNA expression (analyzed by RNase protection assay), protein expression (determined by Western blot), and activity [measured by rat fetal lung fibroblast (RFL-6) reporter cell assay] in a concentration- and time-dependent manner. In contrast, the bisindolylmaleimide analogs GF 109203X, Ro 31-8220 and Gö 6983 had no effect on NOS III expression, and Gö 6976, a methyl- and cyanoalkyl-substituted nonglycosidic indolocarbazole derivative of Stsp, even reduced NOS III expression in a concentration-dependent fashion. The up-regulation of NOS III expression by Stsp and analogs appears to be a transcriptional event

because Stsp, 7-hydroxystaurosporine, and CGP 41251 enhanced the activity of a 1.6-kb human NOS III promoter fragment transiently transfected into EA.hy 926 endothelial cells. Stsp and analogs did not affect the stability of the NOS III mRNA. Stsp is known as a potent protein kinase (PK) inhibitor. Data obtained with other kinase inhibitors (and stimulators) indicated, however, that the effect of Stsp and analogs on NOS III expression was unrelated to the activities of PKC, PKA, PKG, or tyrosine kinase(s). Stsp analogs such as CGP 41251 also counteracted the NOS III mRNA-decreasing effect of tumor necrosis factor- α . These findings demonstrate that Stsp analogs represent a new class of compounds positively interacting with the transcription of the endothelial NOS III gene. Such compounds may prove useful in the prophylaxis and therapy of vascular disease.

Nitric oxide (NO) produced by NO synthase (NOS) III in vascular endothelium is an important endogenous vasodilator agent. NO dilates blood vessels by directly stimulating soluble guanylyl cyclase in vascular smooth muscle. Mice deficient of the NOS III gene develop hypertension. Also pulmonary hypertension is associated with diminished NOS III expression (for review, see Förstermann et al., 1998, 1994). In addition, endothelial NO has numerous vasoprotective and probably antiatherosclerotic properties. NOS III-derived NO inhibits platelet aggregation by stimulating platelet soluble guanylyl cyclase, and is a major factor responsible for the prevention of platelet adhesion to the vascular wall. Besides protection from thrombosis, this also pre-

vents the release of platelet-derived growth factors that stimulate smooth muscle proliferation (for review, see Förstermann et al., 1998, 1994).

Endothelial NO decreases the expression of monocyte chemoattractant protein-1 (Zeiher et al., 1995) and of surface adhesion molecules such as CD11/CD18 (Kubes et al., 1991), P-selectin (Davenpeck et al., 1994), vascular cell adhesion molecule-1 (De Caterina et al., 1995), and intercellular adhesion molecule-1 (Gauthier et al., 1995), thereby preventing leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall. This offers protection against an early phase of atherogenesis. Also, the reduced influx of lipoproteins into the vascular wall (Cardona-Sanclemente and Born, 1995) and the inhibition of low-density lipoprotein oxidation (Hogg et al., 1993) may contribute to the antiatherosclerotic properties of NO. Finally, by inhibit-

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; TNF- α , tumor necrosis factor- α ; PKC, protein kinase C; Stsp, staurosporine; UCN-01, 7-hydroxystaurosporine; CGP 41251, 4'-*N*-benzoyl staurosporine; GF 109203X, {3-[1-[(3-dimethylaminopropyl)-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)]maleimide}; Ro 31-8220, {3-[1-[(3-amidinopropyl)-1*H*-indol-3-yl]-4-(1-methyl-1*H*-indol-3-yl)]maleimide}; Gö 6983, {3-[1-[(3-dimethylaminopropyl)-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)]maleimide}; Gö 6976, {12-(2-cyanoethyl)-13-methyl-5-oxo-7*H*-indolo[2,3-*b*]pyrrolo[3,4-*c*]carbazole}; PMA, phorbol-12-myristate-13-acetate; SOD, superoxide dismutase; IBMX, 3-isobutyl-1-methylxanthine; HUVEC, human umbilical vein endothelial cell; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; SV-40, simian virus 40; PET, β -phenyl-1,*N*²-ethenoguanosine; RFL-6, rat fetal lung fibroblast cells.

ing the proliferation and migration of vascular smooth muscle cells (Dubey et al., 1995), NO can protect against a later phase of atherogenesis. In agreement with this hypothesis, pharmacological inhibition of NOS caused accelerated atherosclerosis in experimental models (Cayatte et al., 1994).

Although NOS III is being classified as a constitutively expressed NOS isozyme, its expression can be regulated by a variety of stimuli such as cytokines [tumor necrosis factor- α (TNF- α), transforming growth factor- β 1], bacterial lipopolysaccharide, oxidized lipoproteins, estrogens, shear stress, growth status, hypoxia, and activators of protein kinase C (PKC) (Förstermann et al., 1998). Decreased endothelial NO production has been seen in pathophysiological conditions such as atherosclerosis, diabetes, and hypertension (Förstermann et al., 1998). In advanced human atherosclerosis, a reduced NOS III expression (and NO production) has been reported (Oemar et al., 1998). In view of the protective effects of NO, compounds that increase NOS III activity and/or expression are of significant therapeutic interest.

The indolocarbazole alkaloid staurosporine (Stsp) is a natural product first isolated from *Streptomyces staurosporeus* (Omura et al., 1977). It was initially described as an inhibitor of PKC (Tamaoki et al., 1986). Since its discovery, Stsp has been the lead molecule for the synthesis of a variety of novel PK inhibitors that are widely used in biomedical research. Two of these Stsp derivatives, 7-hydroxystaurosporine (UCN-01) and 4'-N-benzoyl staurosporine (CGP 41251) are in clinical trials as anticancer drugs.

In the current study, we show a novel effect of Stsp and some of its indolocarbazole derivatives, i.e., the regulation of NOS III expression. We describe the structure-activity relationship of different Stsp analogs in regulating NOS III gene expression and demonstrate that the stimulation of NOS III expression is unrelated to the effect of these compounds on PKs A, C, G and tyrosine kinases.

Materials and Methods

Reagents. Stsp, GF 109203X, {3-[1-[(3-dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)]maleimide}, Ro 31-8220 {3-[1-[(3-(amidinothio)propyl)-1H-indol-3-yl]-4-(1-methyl-1H-indol-3-yl)]maleimide}, Gö 6983 {3-[1-[(3-dimethylaminopropyl)-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)]maleimide}, Gö 6976 {12-(2-cyanoethyl)-13-methyl-5-oxo-7H-indolo[2,3-b]pyrrolo[3,4-c]carbazole}, actinomycin D, and Rp-cAMPS were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). UCN-01 (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) was a generous gift from Professor A. Gescher, University of Leicester (Leicester, UK). CGP 41251 was provided by Novartis Pharma AG (Basel, Switzerland). Phorbol-12-myristate-13-acetate (PMA), 8-bromo-cAMP, dibutyryl-cAMP, 8-bromo-cGMP, superoxide dismutase (SOD), 3-isobutyl-1-methylxanthine (IBMX), and TNF- α were from Sigma (Deisenhofen, Germany). β -phenyl-L- N^2 -ethenoguanosine (PET)-cGMP and Rp-8-PET-cGMPs were from Biolog (Bremen, Germany). [α - 32 P]UTP and [γ - 32 P]ATP were obtained from ICN (Asse-Relegem, Belgium). [125 I]-cGMP was from BioTrend (Cologne, Germany). Restriction enzymes, polynucleotide kinase, and oligonucleotides were purchased from Pharmacia (Freiburg, Germany). SuperFect transfection reagent was obtained from Qiagen (Hilden, Germany). Dual-luciferase reporter assay system was obtained from Promega (Mannheim, Germany). Superscript reverse transcriptase was purchased from Life Technologies (Eggenstein, Germany). DNase I, RNase A, T3, and T7 RNA polymerase were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The mouse monoclonal antibody to cPKC (PKC-MC5), the rabbit polyclonal antibody to PKC μ (PKC N20), and Protein

A-Agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody to human NOS III was purchased from Transduction Laboratories (Lexington, KY). A mouse monoclonal antibody to β -tubulin and the horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated by collagen digestion and cultured in endothelial cell growth medium (PromoCell, Heidelberg, Germany). HUVECs from passages 3 to 5 were used in the experiments. HUVEC-derived EA.hy 926 endothelial cells (Edgell et al., 1983) were grown under 10% CO₂ in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (PAA Laboratories, Cölbe, Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 \times hypoxanthine, amethopterin/methotrexate, and thymine (Life Technologies).

Rat fetal lung fibroblasts (RFL-6 cells) (Ishii et al., 1991) (American Type Culture Collection, Manassas, VA) were cultured at 5% CO₂ in Ham's F12 nutrient mixture (Life Technologies) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. RFL-6 cells were used as reporter cells for the determination of NO production by EA.hy 926 cells (Ishii et al., 1991).

RNase Protection Assay for NOS III mRNA Analyses. Total RNA was isolated from EA.hy 926 cells by guanidinium thiocyanate-phenol-chloroform extraction. For the generation of radiolabeled antisense RNA probes, plasmids pCR-NOS III-Hu and pCR- β -actin-Hu were used. pCR-NOS III-Hu was generated by cloning a 426-base pair cDNA fragment corresponding to position 3111–3536 of the human NOS III cDNA into the *EcoRV* site of pCR-Script (Stratagene, La Jolla, CA) (Li et al., 1998). pCR- β -actin-Hu contains a 354-base pair fragment of the human β -actin cDNA (position 270–623) (Kleiner et al., 1996).

pCR-NOS III-Hu and pCR- β -actin-Hu were linearized with *Sma*I or *Bst*EII, respectively, extracted with phenol/chloroform, and concentrated by ethanol precipitation. Then 0.5 μ g of each DNA was in vitro transcribed with T7/T3 RNA polymerase and [α - 32 P]UTP at 37°C for 1 h. After transcription, the template DNA was degraded with DNase I for 45 min. The radiolabeled RNA was purified with NucTrap probe purification columns (Stratagene).

RNase protection assays were performed with a mixture of RNase A and RNase T1 according to the manufacturer's instructions (Roche Molecular Biochemicals). Briefly, following denaturation, 20 μ g of total RNA was hybridized with 200,000-cpm-labeled NOS III antisense RNA probe and 40,000-cpm-labeled β -actin antisense RNA probe at 51°C for 16 h in a volume of 40 μ l of hybridization buffer [40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.7, 1 mM EDTA, 400 mM NaCl, 50% formamide]. Then the mixture was digested by adding 300 μ l of digestion buffer (10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) containing 3.5 μ g of RNase A and 25 U of RNase T1 for 30 min at 30°C. The reaction was stopped by proteinase K digestion (70 μ g/sample in 70 μ l of 7.15 mM Tris-HCl, pH 7.4, 7.15 mM EDTA, 2.85% SDS; 15 min at 37°C) 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 \times 1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA. The gels were electrophoresed for 1 to 2 h, dried, and exposed to X-ray films. Densitometric analyses were performed with a phosphor-imager (BioRad, Richmond, CA). The sizes of 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.

Western Blot for NOS III Protein Analyses. EA.hy 926 cells (untreated or incubated for 20 h with 10 nM Stsp, 1 μ M UCN-01, or 1 μ M CGP 41251) were homogenized in ice-cold homogenization buffer containing 20 mM of the detergent 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate. The composition of the homogenization buffer was 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM

EGTA, 2 mM dithiothreitol (DTT), 7 mM glutathione, 10% glycerol, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, 20 U/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were incubated for 30 min at 4°C with gentle agitation followed by a centrifugation at 100,000g for 1 h. Protein concentrations in the supernatant was determined with the Bradford assay.

For Western blotting, 50 μ g of protein sample was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% gels). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (BioRad). Blots were blocked overnight with 3% (w/v) BSA and 0.05% (w/v) Tween 20 in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) at 4°C. All subsequent steps were performed at room temperature. The membrane was cut into two parts at the position 87 kDa. The upper part was incubated for 45 min with a polyclonal rabbit anti-NOS III antibody, and the lower one with a mouse monoclonal antibody to β -tubulin. The incubation buffer was TBS containing 0.5% (w/v) gelatin and 0.05% (w/v) Tween 20. After washing in TBS/gelatin/Tween, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in TBS/gelatin/Tween for 30 min. The blots were washed stepwise with TBS/gelatin/Tween, TBS/Tween, and TBS alone. Immunocomplexes were developed with an enhanced horseradish peroxidase/luminol chemiluminescence reagent (DuPont-NEN, Boston, MA) according to the manufacturer's instruction.

Detection of PKC Translocation. For the detection of PKC translocation, soluble and particulate protein fractions of EA.hy 926 cells were prepared. EA.hy 926 cells were left untreated or incubated for 2, 6, or 24 h with 10 nM Stsp or 10 nM of the PKC stimulator phorbol-12-myristate-13-acetate (PMA). The cells were homogenized in ice-cold homogenization buffer and centrifuged at 100,000g for 1 h. The supernatant (soluble fraction) was removed and the pellet was washed in homogenization buffer containing 1 M NaCl and recentrifuged at 100,000g for 30 min. The supernatant was discarded and the pellet solubilized by agitation in homogenization buffer containing 20 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (30 min; 4°C). After another centrifugation step at 100,000g for 1 h, the supernatant (containing the solubilized particulate fraction) was obtained. Western blotting was performed as described above, with a monoclonal antibody PKC-MC5, which recognizes all cPKC-members (α , β _I, β _{II}, and γ).

Kinase Assay for Determination of PKC μ Activity. EA.hy 926 cells were plated in 90-mm dishes and grown to confluence. The cells ($\sim 6 \times 10^6$ cell/dish) were left untreated or incubated with 10 nM PMA and 10 or 100 nM Stsp for 15, 30, or 90 min. After washing with PBS, the cells were lysed in 10 mM Tris-HCl, 140 mM NaCl, 1% Triton X-100, 1% desoxycholate, 10 μ g/ml leupeptin, 0.2 mM PMSF, and 1 mM DTT. PKC μ was immunoprecipitated with an anti-PKC μ antibody (PKC μ N-20; 1:100 dilution) and Protein A-Agarose.

PKC μ in vitro autophosphorylation was performed by incubation of the immunoprecipitated PKC μ with the phosphorylation buffer containing (final concentration) 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2 mM sodium orthovanadate, 10 μ g/ml leupeptin, 0.2 mM PMSF, 10 μ M ATP, and 1 μ Ci [γ -³²P]ATP at 30°C for 30 min. The reaction was terminated by adding the SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 4% (w/v) SDS, 20% (w/v) glycerol, 0.02% (w/v) bromophenol blue, and 40 mM DTT) and analyzed by SDS-PAGE. The gel was dried and visualized by autoradiography. The 115-kDa radioactive band corresponded to autophosphorylated PKC μ .

Transient Transfection and Reporter Gene Assay. EA.hy 926 cells were plated in six-well plates 24 h before transfection. Transient transfection was performed with 3 μ l/well SuperFect reagent (Qiagen) according to the manufacturer's instructions. Each well received 1 μ g of plasmid DNA, i.e., 0.9 μ g pGL₃-Basic (Promega, Madison, WI) or 0.9 μ g of pGL₃-NOS III-Hu-1600 and 0.1 μ g pRL-simian virus 40 (SV40) (Promega). pGL₃-NOS III-Hu-1600 contains a

1.6-kb NOS III promoter fragment (−1600 to +23) cloned before the luciferase gene of pGL₃-Basic (analogous to Kleinert et al., 1998). Plasmid pRL-SV40 contains the renilla-luciferase gene driven by an SV40 promoter and was cotransfected for normalization. On the next day, the cells transfected with pGL₃-NOS III-Hu-1600 were left untreated or incubated with 10 nM Stsp, 1 μ M UCN-01, or 1 μ M CGP 41251 for 12 h. Then the cells were lysed with passive lysis buffer (Promega) and luciferase/renilla-luciferase activities were measured with the Dual-Luciferase System (Promega). The luciferase activity was normalized by the renilla-luciferase activity after subtraction of background.

Reporter Cell Assay for Determination of NO Production. NO production by EA.hy 926 cells was bioassayed with RFL-6 rat lung fibroblasts as reporter cells (Ishii et al., 1991). EA.hy 926 cells were grown to confluence and then either left untreated, or incubated with 10 nM Stsp, 1 μ M UCN-01, and 1 μ M CGP 41251 for 20 h. For determination of NO production, EA.hy cells and confluent RFL-6 cells were washed twice with Locke's solution (154.0 mM NaCl, 5.6 mM KCl, 2.0 mM CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5.6 mM glucose, 10.0 mM HEPES, pH 7.4). Then EA.hy cells were incubated with Locke's solution containing 20 U/ml SOD and 100 μ M L-arginine, and RFL-6 cells with Locke's solution containing 0.6 mM IBMX for 20 min. After the preincubation, EA.hy cells were incubated for 2 min at 37°C in 1 ml of Locke's solution containing 20 U/ml SOD, 0.3 mM IBMX, 100 μ M L-arginine, and 10 μ M A23187. Then the conditioned media containing the NO released from the EA.hy cells were transferred to the RFL-6 cells, and another incubation of 2 min at 37°C was performed. The reaction was stopped by aspiration of the solution, adding 1 ml of ice-cold 50 mM sodium acetate, pH 4.0, and rapidly freezing the cells with liquid nitrogen. The cGMP content of the RFL-6 samples was determined by radioimmunoassay as described (Ishii et al., 1991). The basal cGMP content of the RFL-6 cells (receiving no conditioned media) was subtracted from all samples.

Statistics. Statistical differences between mean values were determined by ANOVA followed by Fisher's protected least-significant difference test for comparison of multiple means.

Results

Effect of Stsp Analogs on NOS III mRNA Expression in Human Endothelial Cells. Human EA.hy 926 endothelial cells were incubated with 1 to 10 nM Stsp; 1 to 1000 nM UCN-01, CGP 41251, Gö 6983, Gö 6976, or 10 to 1000 nM GF 109203X; and Ro 31-8220 for 18 h. As shown in Fig. 1, Stsp, UCN-01, and CGP 41251 enhanced NOS III mRNA expression in a concentration-dependent manner. Stsp proved to be 100-fold more potent than UCN-01 and CGP 41251, but all three compounds had similar efficacies (Fig. 1). Compounds GF 109203X, Ro 31-8220, and Gö 6983 did not affect NOS III mRNA expression. Compound Gö 6976 even decreased NOS III mRNA expression significantly (Fig. 1). A significant up-regulation of the NOS III mRNA (>2-fold) was already observed after a 6-h incubation with 10 nM Stsp or 1 μ M UCN-01 or 1 μ M CGP 41251.

The effect of Stsp and analogs on NOS III mRNA expression was confirmed in native HUVECs. Incubation of the HUVECs for 18 h with 10 nM Stsp, 1 μ M UCN-01, or 1 μ M CGP 41251 resulted in up-regulation of NOS III mRNA to $251.7 \pm 16.1\%$ of control, $177.4 \pm 12.3\%$ of control, and $213.5 \pm 11.9\%$ of control, respectively (means \pm S.E. of three experiments each).

Stsp, UCN-01, and CGP 41251 Enhanced NOS III Protein Expression in Human Endothelial Cells. As shown in Fig. 2, a 20-h incubation with 10 nM Stsp, 1 μ M CGP

41251, or 1 μ M UCN-01 also increased NOS III protein content in EA.hy 926 cells, by Western blot with 50 μ g of total cell protein. Densitometric analyses of the NOS III protein bands, normalized for β -tubulin bands, demonstrated increases to $306.9 \pm 44.4\%$ for Stsp, $247.8 \pm 30.4\%$ for CGP 41251, and $305.0 \pm 43.2\%$ for UCN-01 (means \pm S.E. of three experiments).

Stsp, UCN-01, and CGP 41251 Increased Endothelial NO Production. RFL-6 fibroblasts showed basal cGMP lev-

els of 2.02 ± 0.05 pmol/ 10^6 cells (six experiments). Incubation of the RFL-6 cells for 2 min with conditioned medium from control EA.hy 926 cells stimulated with 10 μ M the calcium ionophore A23187 increased the cGMP content to 9.81 ± 0.17 pmol/ 10^6 cells. The NO-stimulated net increase in cGMP (after subtraction of basal cGMP) was set 100%. As shown in Fig. 3, preincubation of EA.hy 926 with 10 nM Stsp, 1 μ M UCN-01, or 1 μ M CGP 41251 for 20 h increased the NO-stimulated cGMP levels \sim 2-fold.

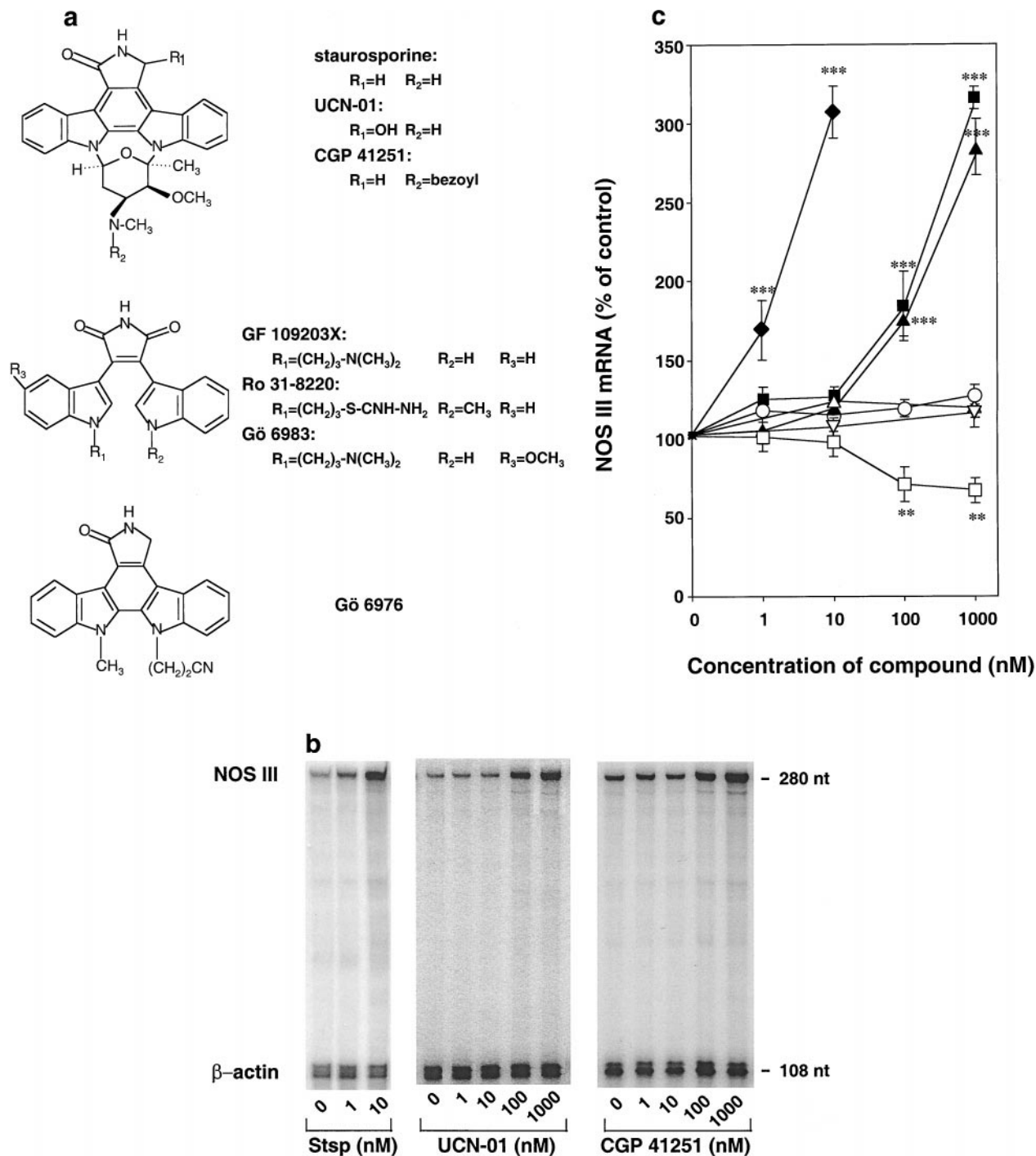


Fig. 1. Structure-activity relationship of Stsp analogs in regulating NOS III mRNA expression. The Stsp analogs used were divided into three groups based on their structure (a). Human EA.hy 926 endothelial cells were incubated with these compounds for 18 h and NOS III mRNA was analyzed with RNase protection assay with antisense RNA probes to human NOS III and β -actin (for standardization). b, representative RNase protection gels. c, the result of densitometric analyses of several gels (\blacklozenge , Stsp; \blacksquare , UCN-01; \blacktriangle , CGP 41251; \triangle , GF 109203X; ∇ , Ro 31-8220; \circ , G6 6983; \square , G6 6976.). Symbols represent means \pm S.E. of four or five experiments ($^{**}P < .01$; $^{***}P < .001$ compared with untreated cells).

Stsp, UCN-01, and CGP 41251 Enhanced NOS III Promoter Activity. When EA.hy 926 cells transfected with pGL₃-NOS III-Hu-1600 (containing a 1.6-kb human NOS III promoter fragment before the luciferase reporter gene) were analyzed for luciferase activity, the promoter fragment showed a significant basal activity compared with the vector pGL₃-Basic alone (Fig. 4). Incubations with 10 nM Stsp, 1 μ M UCN-01, or 1 μ M CGP 41251 for 12 h markedly increased the activity of the human NOS III promoter (Fig. 4).

Stsp Did Not Change Stability of NOS III mRNA. Confluent EA.hy 926 cells were preincubated for 18 h with or without 10 nM Stsp and then 10 μ g/ml actinomycin D was added to inhibit gene transcription. NOS III mRNA level was analyzed at different time points thereafter. The NOS III mRNA levels before actinomycin D (time zero) were set 100% for both groups. As shown in Fig. 5, incubation with Stsp (10

nM) did not change the stability of the NOS III mRNA.

Effect of PKC Inhibitors on Stsp-Induced NOS III mRNA Expression. Incubation of EA.hy 926 cells for 18 h with 1 μ M of GF 109203X, Ro 31-8220, or Gö 6983 did not affect the Stsp-induced NOS III mRNA expression. However, Gö 6976, an inhibitor of cPKC and PKC μ , which reduced basal NOS III expression (see above) also reduced the Stsp-induced NOS III expression to the same extent (six experiments; Fig. 6).

Stsp Did Not Activate cPKC or PKC μ When Stimulating NOS III Expression. We have shown previously that the phorbol ester PMA, which causes the same degree of NOS III up-regulation as Stsp, caused a translocation of PKC α from the cytosol to the membrane at 2 and 6 h and a down-regulation of the kinase at 24 h (Li et al., 1998) (Fig. 7). This demonstrated an activation of the kinase followed by a down-

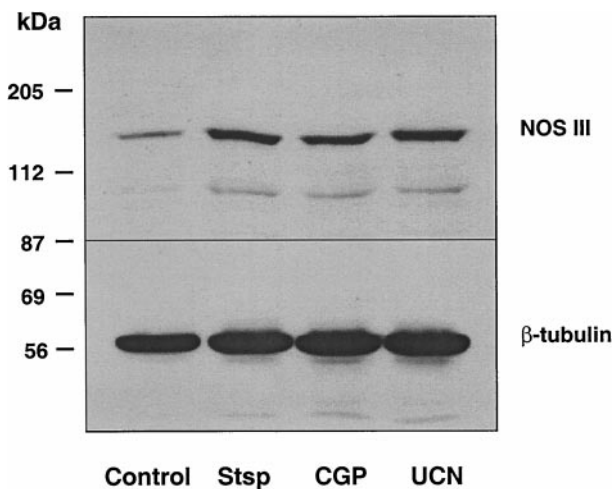


Fig. 2. Stsp, CGP 41251, and UCN-01 increase NOS III protein expression in EA.hy 926 cells. Total protein was isolated from cells either receiving no treatment (control), or incubated with 10 nM Stsp, 1 μ M CGP 41251, or 1 μ M UCN-01 for 20 h. Western blots were performed with a polyclonal anti-NOS III antibody (top) and a monoclonal antibody to β -tubulin (bottom, for normalization). The blot shown is representative of three independent experiments with similar results.

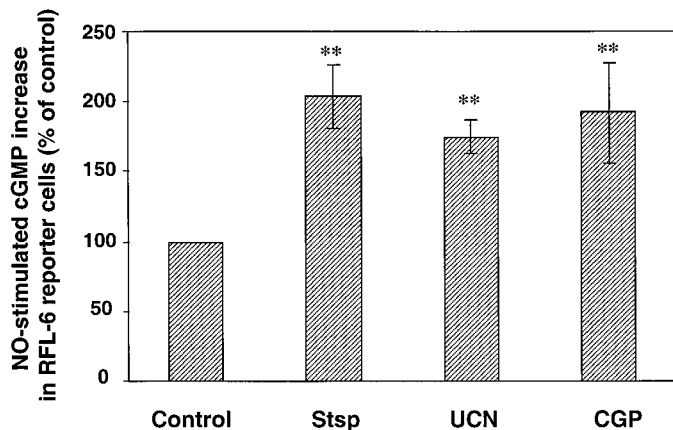


Fig. 3. Stsp, UCN-01, and CGP 41251 increase NO production. EA.hy 926 cells were either left untreated (control) or were treated with 10 nM Stsp, 1 μ M UCN, or 1 μ M CGP for 20 h. Then, cells were stimulated with 10 μ M calcium ionophore A23187 for 2 min before the transfer of conditioned media to RFL-6 reporter cells. The cGMP content in the RFL-6 cells was measured by radioimmunoassay. The cGMP production stimulated by NO from A23187-stimulated control EA.hy 926 cells (7.79 ± 0.12 pmol/ 10^6 RFL-6 cells) was set 100%. Data represent mean \pm S.E. of six experiments (** $P < .01$, compared with control).

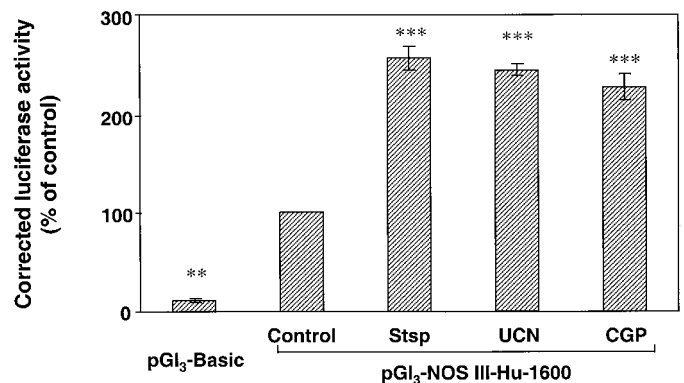


Fig. 4. Stsp, UCN-01, and CGP 41251 enhance NOS III promoter activity. EA.hy 926 cells were transfected with plasmid pGL₃-Basic (containing a promoterless luciferase gene) or pGL₃-NOS III-Hu-1600 (containing a 1.6-kb NOS III promoter fragment cloned before the luciferase gene). Plasmid pRL-SV40 (containing the renilla-luciferase gene driven by an SV40 promoter) was cotransfected for normalization. The pGL₃-NOS III-Hu-1600-transfected cells were either left untreated (control) or exposed to 10 nM Stsp, 1 μ M UCN, or 1 μ M CGP for 12 h. Then, the cells were lysed and light units determined. The relative luciferase activity (corrected with renilla-luciferase activity) was taken as a measure of NOS III promoter activity. Columns represent means \pm S.E. of six independent experiments (** $P < .01$; *** $P < .001$ compared with control).

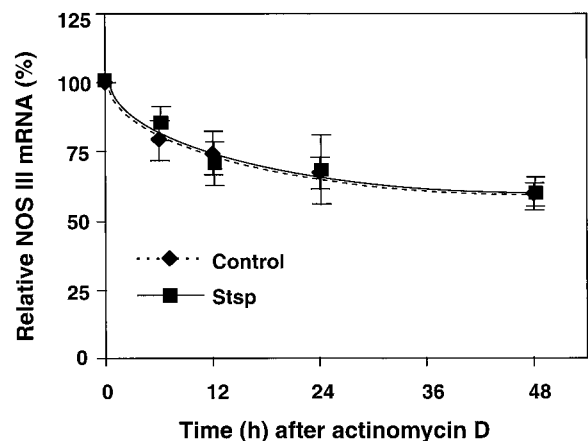


Fig. 5. Stsp does not affect the stability of NOS III mRNA in EA.hy 926 cells. Cells were either left untreated (control) or incubated with 10 nM Stsp for 18 h. Then, the inhibitor of transcription, actinomycin D (10 μ g/ml), was added to the culture medium. RNA was prepared 6, 12, 24, or 48 h thereafter and NOS III mRNA determined by RNase protection assays. NOS III mRNA levels at the time of addition of actinomycin D (time zero) were set 100%. Symbols represent means \pm S.E. of six independent experiments.

regulation at later times. There have been reports that Stsp also can activate PKC under certain conditions (Stanwell et al., 1996). However, in our current experiments, Stsp did not produce any translocation or down-regulation of cPKC (Fig. 7). Also the activity of PKC μ remained unchanged after Stsp. As shown in Fig. 8, PMA significantly increased the extent of PKC μ autophosphorylation (indicating activation also of this PKC isoform), whereas Stsp did not cause any significant change in PKC μ activity (Fig. 8).

Stsp Does Not Increase NOS III mRNA Expression by Interfering with PKA, PKG, or Tyrosine Kinase(s). Stsp is a potent, but nonspecific inhibitor of PKs (Ruegg and Burgess, 1989). Therefore, we tested if inhibition (or activation) of kinases other than PKC could be involved in the stimulatory effect of Stsp on NOS III expression. However, neither the PKA inhibitor Rp-cAMPS (30 μ M) nor the cell permeable cAMP analogs (and PKA activators) 8-bromo-

cAMP (1 mM) and dibutyl-cAMP (1 mM), nor the PKG inhibitor Rp-8-bromo-PET-cGMPS (100 μ M), or the PKG stimulators 8-bromo-cGMP (100 μ M) and PET-cGMP (100 μ M) had any effect on NOS III expression in EA.hy 926 cells (Fig. 9). Finally, the protein tyrosine kinase inhibitors genistein (100 μ M) and erbstatin A (10 μ M) had no significant effect on basal or Stsp-induced NOS III mRNA expression (Fig. 10). Interestingly, the same protein tyrosine kinase inhibitors markedly reduced (by ~50%) the NOS III mRNA expression stimulated by PMA (four experiments; data not shown).

Effect of CGP 41251 on NOS III mRNA Expression in Human Endothelial Cells Treated with TNF- α . Human EA.hy 926 endothelial cells were incubated with 0.1 to 1 ng/ml TNF- α alone or in combination with 1 μ M CGP 41251 for 6 h. As previously reported (Yoshizumi et al., 1993), TNF- α down-regulated NOS III mRNA levels. As shown in

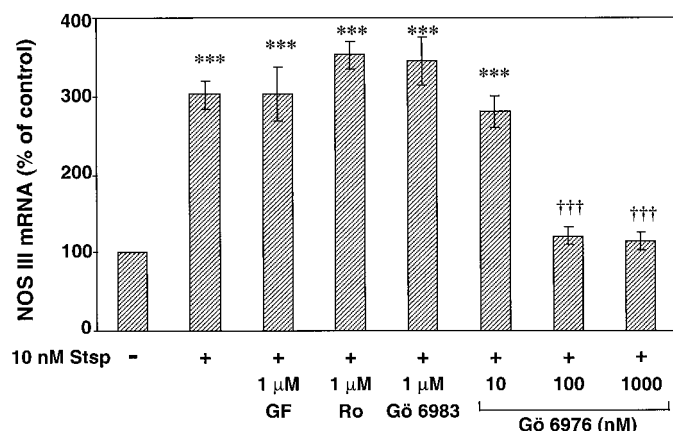


Fig. 6. Effect protein kinase C inhibitors on Stsp-stimulated NOS III mRNA expression. EA.hy 926 cells were treated with Stsp alone or with Stsp combined with GF 109203X, Ro 31-8220, Gö 6983, or Gö 6976 for 18 h. NOS III mRNA levels were determined with RNase protection assay. Columns represent means \pm S.E. of six independent experiments (***) $P < .001$ versus untreated cells; (†††) $P < .001$ versus Stsp treatment alone).

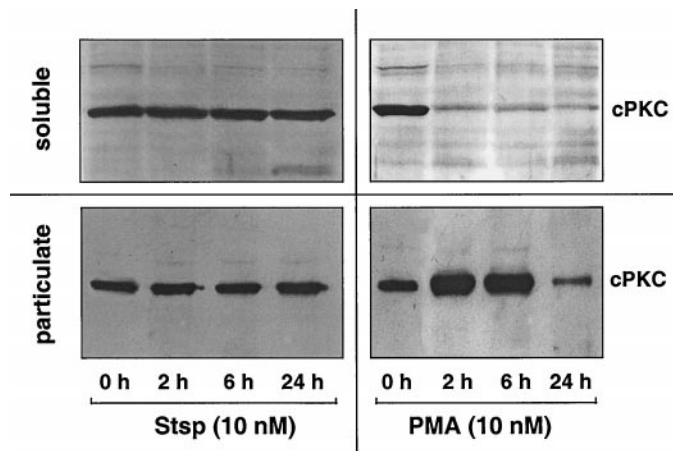


Fig. 7. Stsp does not induce translocation of cPKC. EA.hy 926 cells were either left untreated (0 h) or were incubated with 10 nM PMA or 10 nM Stsp for 2, 6, or 24 h. Subcellular fractions (soluble and particulate) were isolated and Western blots performed with an antibody to cPKC that recognizes all cPKC members α , β , β , and γ . The blots shown are representative of three independent experiments with similar results. The effect of the PKC-stimulating phorbol ester PMA is shown for comparison.

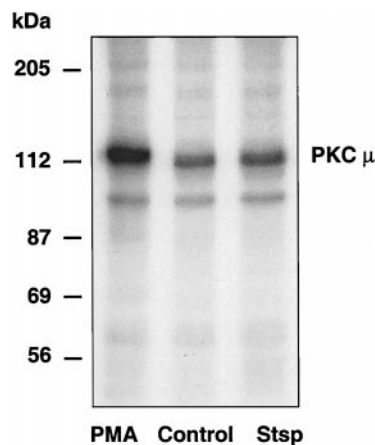


Fig. 8. Stsp does not activate PKC μ . EA.hy 926 cells were incubated with 10 nM PMA or Stsp for 30 min. Then PKC μ was immunoprecipitated and an *in vitro* autophosphorylation was performed in the presence of [γ - 32 P]ATP. The samples were then analyzed with SDS-PAGE. PMA increases PKC μ autophosphorylation, whereas Stsp (10 nM) does not. Also at 100 nM, Stsp did not change PKC μ activity (data not shown). The gel shown is representative of six independent experiments with similar results.

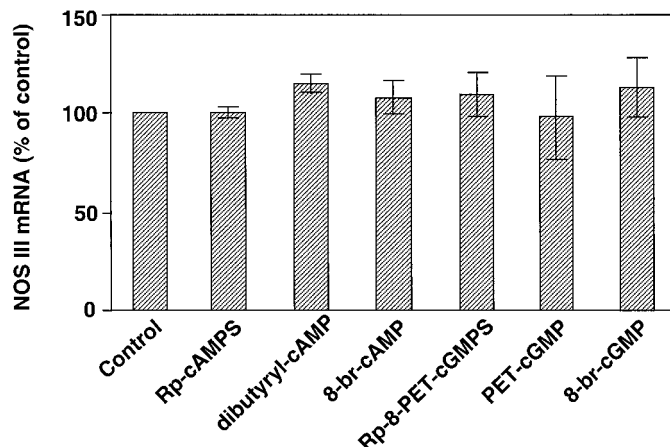


Fig. 9. Protein kinase A (PKA) and PKG are not involved in NOS III mRNA expression in EA.hy 926 cells. NOS III mRNA was analyzed by RNase protection assay using RNA samples from cells incubated for 18 h with the PKA inhibitor Rp-cAMPS (30 μ M), the PKA stimulators dibutyl-cAMP (1 mM) or 8-bromo-cAMP (1 mM), the PKG inhibitor Rp-8-PET-cGMPS (100 μ M), and the PKG stimulators PET-cGMP (100 μ M) or 8-bromo-cGMP (100 μ M). Bars represent means \pm standard error of 6 independent experiments.

Fig. 11, CGP 41251 was able to functionally antagonize the effect of TNF- α on NOS III mRNA.

Discussion

The current study identified Stsp and some of its analogs as efficacious stimulators of expression of the vasoprotective and antiatherosclerotic enzyme NOS III. Other structurally related compounds (the bisindolylmaleimides) had no stimulating effect on NOS III expression and one compound (Gö 6976) was even inhibitory. When correlating the chemical structures of Stsp and its derivatives with their effect on NOS III mRNA expression, an interesting structure-activity relationship emerged. The indolocarbazoles with an intact aromatic ring structure (Stsp, UCN-01, CGP 41251, and Gö 6976; Fig. 1a) were effective in regulating NOS III gene

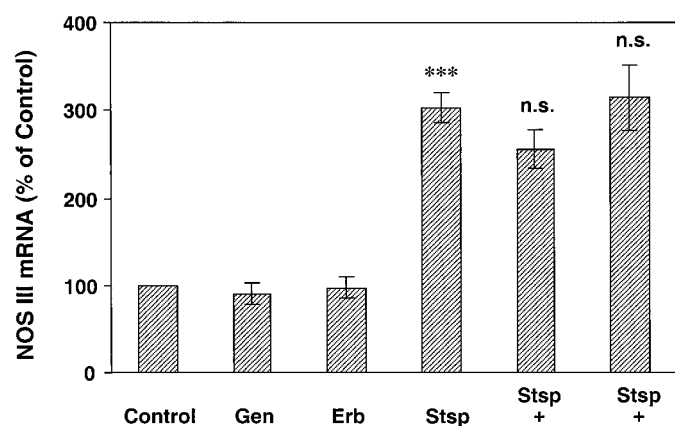


Fig. 10. Tyrosine kinases do not play a role in the basal and Stsp-induced NOS III expression in EA.hy 926 cells. Cells were incubated for 18 h with 100 μ M genistein (Gen), 10 μ M erbstatin A (Erb), or 10 nM Stsp, alone or in combination. Then NOS III mRNA levels were determined by RNase protection assay. Columns represent means \pm S.E. of four independent experiments (** P < .001 compared with control; n.s., not significant versus Stsp alone).

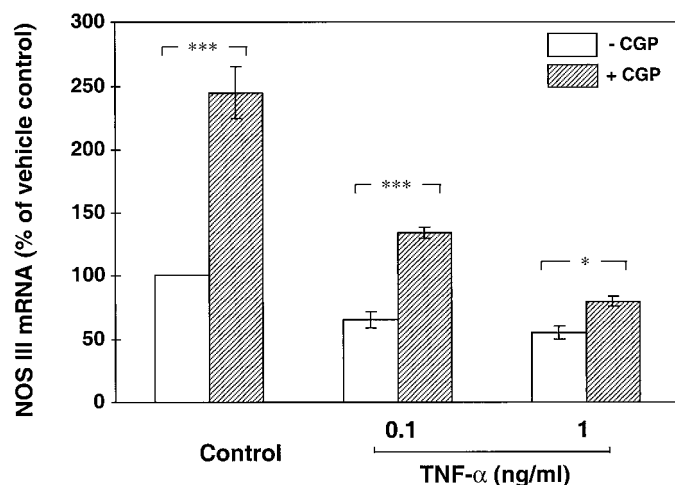


Fig. 11. Effect of CGP 41251 on the NOS III mRNA expression of untreated (control) EA.hy 926 endothelial cells and cells whose NOS III expression had been down-regulated with TNF- α . EA.hy 926 cells were incubated for 6 h with 1 μ M CGP 41251 (or its vehicle 0.1% dimethyl sulfoxide) and/or TNF- α , alone or in combination. NOS III mRNA was analyzed with RNase protection assay followed by densitometry. Columns represent means \pm S.E. of three experiments (* P < .05; *** P < .001).

expression. Stsp, UCN-01, and CGP 41251 enhance NOS III expression, whereas Gö 6976, a methyl- and cyanoalkyl-substituted nonglycosidic indolocarbazole, had the opposite effect. In contrast, the bisindolylmaleimides with an opened central aromatic ring (GF 109203X, Ro 31-8220, and Gö 6983, Fig. 1a) had neither stimulatory nor inhibitory effects on NOS III expression in concentrations up to 1 μ M.

Consistent with the mRNA expression, Stsp, UCN-01, and CGP 41251 also increased NOS III protein expression and NOS activity in EA.hy 926 cells (Figs. 2 and 3). The regulation of NOS III gene expression by glycosidic indolocarbazole analogs of Stsp is likely to be a transcriptional event because Stsp, UCN-01, and CGP 41251 enhance human NOS III promoter activity (Fig. 4) without affecting NOS III mRNA stability (Fig. 5). The molecular basis of the increased NOS III transcription remains to be elucidated.

A feature shared by all the Stsp analogs used herein is their inhibitory effect on PKC, although they differ in their isoform selectivity. PKC defines a family of serine/threonine-specific protein kinases, which consists of at least 12 isoforms (Mellor and Parker, 1998; Toker, 1998). They are grouped into three categories. The conventional PKC members (cPKCs α , β_1 , β_2 , and γ) are calcium-dependent and activated by diacylglycerol/phorbol esters. The novel PKC members (nPKCs δ , ϵ , η , and θ) are unresponsive to calcium but activated by diacylglycerol/phorbol esters. The atypical PKCs (aPKCs ζ and ι/λ) are unresponsive to both calcium and diacylglycerol/phorbol esters. PKC μ is likely to represent a novel subtype that does not fit into any of the known PKC subgroups (Gschwendt et al., 1996; Nishikawa et al., 1997). The Stsp analogs show different specificity for the different isoforms. Stsp inhibits cPKCs, nPKCs, and PKC μ at nanomolar concentrations, but is less potent for aPKCs (Martiny-Baron et al., 1993; Gschwendt et al., 1996). UCN-01 inhibits cPKC and nPKC members (Mizuno, 1993, 1995) and CGP 41251 is a selective inhibitor of the cPKCs (Geiges et al., 1997). GF 109203X is somewhat selective for cPKCs and nPKCs (Toullec et al., 1991; Martiny-Baron et al., 1993). Ro 31-8220 shows selectivity for cPKCs and nPKCs (Yeo and Exton, 1995), and Gö 6983 inhibits cPKCs, nPKCs, and aPKCs, but not PKC μ (Gschwendt et al., 1996). Gö 6976 is considered a selective inhibitor of cPKCs and PKC μ (Martiny-Baron et al., 1993; Gschwendt et al., 1996). Some years ago, Ohara et al. (1995) described a stimulatory effect of Stsp on NOS III expression in bovine endothelial cells and concluded that PKC inhibition was responsible for the effect. The current findings render this hypothesis unlikely because a number of established PKC inhibitors such as GF 109203X, Ro 31-8220, Gö 6983, and Gö 6976 did not enhance NOS III expression. The same compounds reversed the stimulatory effect of PKC activators on NOS III expression (Li et al., 1998).

The glycosidic indolocarbazoles Stsp, UCN-01, and CGP 41251 had a similar efficacy in up-regulating NOS III gene expression, but Stsp was 100-fold more potent than its derivatives UCN-01 and CGP 41251 (Fig. 1, b and c). This does not correlate with the potency of these compounds as cPKC inhibitors. The EC₅₀ values of Stsp and UCN-01 as cPKC inhibitors are in the range of 1 to 2 nM; the EC₅₀ of CGP 41251 is 20 to 30 nM (Marte et al., 1994; Mizuno et al., 1995; Geiges et al., 1997).

A recent observation with mouse keratinocytes demon-

strated that Stsp could paradoxically activate some PKC isoforms and some of the Stsp effects could be prevented with GF 109203X (Stanwell et al., 1996). This led to the idea that Stsp might increase NOS III expression by activating PKC. We tested this hypothesis in our EA.hy 926 cell model. In assays of PKC activity (Figs. 7 and 8) there was no evidence for PKC activation by Stsp. Also, as shown in Fig. 6, the PKC inhibitors GF 109203X, Ro 31-8220, and Gö 6983 (up to 1 μ M) did not block Stsp-induced NOS III up-regulation. Therefore, we feel that the stimulatory effect of Stsp and analogs on NOS III expression is unrelated to PKC inhibition or activation.

It has been shown that Stsp and related analogs inhibit PKC by competing with the ATP binding in the catalytic domain of PKC (Herbert et al., 1990; Martiny-Baron et al., 1993). Probably due to this mechanism of action, Stsp is a nonspecific PK inhibitor that also can inhibit PKA, PKG, and protein tyrosine kinases (Ruegg and Burgess, 1989; Herbert et al., 1990). However, the experiments presented in Figs. 9 and 10 provided no evidence for the interaction of Stsp and analogs with any of these kinases when stimulating NOS III expression.

To investigate the potential benefit of NOS III regulators with a Stsp-related structure under pathophysiological conditions of reduced NOS III expression, we have tested the effect of CGP 41251 on endothelial cells whose NOS III had been down-regulated with TNF- α . As shown in Fig. 11, CGP 41251 was also active under these conditions.

Collectively, Stsp analogs modulate NOS III gene expression when they have an intact indolocarbazole structure. The glycosidic indolocarbazoles Stsp, UCN-01, and CGP 41251 enhance NOS III expression and NO production by increasing transcription, whereas Gö 6976, a nonglycosidic indolocarbazole, reduces NOS III expression. The molecular mechanism of action remains unclear at this time, but the regulation of NOS III expression seems to be unrelated to the effect of the compounds on PKA, PKC, PKG, or tyrosine kinases.

One limiting factor for the therapeutic use of Stsp-like compounds as "vasculoprotective" drugs is cytotoxicity. However, minor structural modifications of the Stsp molecule can markedly reduce toxicity. For example, a simple hydroxyl group substitution at the C-7 position of Stsp resulted in compound UCN-01 that exhibits a significantly reduced toxicity. Based on the release of lactate dehydrogenase from cells (Courage et al., 1996), the following rank order of cytotoxicity has been established: Stsp \gg UCN-01 $>$ Ro 31-8220 $>$ CGP 41251 $>$ GF 109203X. In rats and dogs, the maximum tolerated dose of UCN-01 produced peak plasma concentrations of 0.2 to 0.3 μ M, i.e., concentrations higher than those effective in the current study. Human beings tolerated plasma concentrations of UCN-01 as high as 6 μ M without signs of toxicity, however plasma protein binding was $>95\%$ (Fuse et al., 1998; Sausville et al., 1998). UCN-01 and CGP 41251 are currently in preparation for clinical evaluation as anticancer drugs. These or related compounds could represent an interesting pharmacological approach to a new class of compounds that prevent or attenuate vascular disease by stimulating the expression of the vasoprotective and antiatherosclerotic enzyme NOS III.

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