Transcriptional Regulation of Endothelial Nitric-oxide Synthase by Lysophosphatidylcholine*

(Received for publication, December 10, 1997, and in revised form, March 11, 1998)

Katarzyna Ciesliki, Artur Zembowicz, Jih-Lu Tang, and Kenneth K. Wu

Vol. 273, No. 24, Issue of June 12, pp. 14885–14890, 1998

THE JOURNAL OF BIOLOGICAL CHEMISTRY

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Published online at http://www.jbc.org

We have shown that lysophosphatidylcholine (lyso-PC) increases endothelial nitric-oxide synthase (eNOS) expression at the transcriptional level (Zembowicz, A., Tang, J.-L., and Wu, K. K. (1995) J. Biol. Chem. 270, 17006–17010). To elucidate the mechanism by which lyso-PC increases the eNOS transcription, we identified Sp1 sites at −104 to −90 and PE3A sites at −40 to −24 as being involved in lyso-PC-induced promoter activity. Site-directed mutagenesis of Sp1 sites resulted in a marked reduction of basal and lyso-PC-induced activity whereas PE3A site mutation abrogated response to lyso-PC. Band shift assays revealed that lyso-PC augmented Sp1 binding activity. Pretreatment of cells or nuclear extracts with okadaic acid reduced the Sp1 binding activity. Furthermore, okadaic acid treatment abrogated the lyso-PC induced promoter augmentation. Lyso-PC increased the nuclear extract protein phosphatase 2A (PP2A) activity, which was suppressed by okadaic acid treatment. These results suggest that lyso-PC up-regulates eNOS transcription by a PP2A-dependent increase in Sp1 binding activity.

Nitric oxide (NO)1 is an important mediator of diverse physiological and pathological processes, including vasodilation, cytotoxicity, and neurotransmission (1–4). Its biosynthesis is catalyzed by nitric-oxide synthase (NOS). Three isoforms of NOS, i.e. neuronal NOS (nNOS or NOS-I), inducible-NOS (iNOS or NOS-II), and endothelial-NOS (eNOS or NOS-III) have been identified and characterized (5–7). NOS-I and -III are constitutively expressed. The inducible form is expressed by stimulation with several inflammatory and mitogenic mediators (8, 9). Altered endothelial NO productions have been implicated in several important cardiovascular disorders: hypertension, atherosclerotic heart disease, and diabetes. The mechanism by which NO synthesis is deranged in these disorders remained unclear. Although NOS-III is considered to be a housekeeping gene, recent studies have provided evidence to suggest that NOS-III is induced by shear stress (10), physical exercise (11), hypoxia (12), estrogen treatment (13), lysophosphatidylcholine (lyso-PC or LPC), and low levels of oxidized low density lipoprotein (14, 15). These findings imply that induction of NOS-III plays an important role in protecting vascular integrity under stress. Work from our laboratory has shown that lyso-PC increases NOS-III expression at the transcriptional level (14). However, the mechanism by which lyso-PC and other inducing agents increase NOS-III gene transcription remains to be elucidated.

The 5′-flanking region of human eNOS gene has been cloned and sequenced (16). The region adjacent to the transcription initiation sites is TATA-less and GC-rich. Functional analysis of the promoter activity conferred by the 5′-flanking region has shown that a canonical Sp1 site situated between −104 to −90 is required for the basal promoter activity (17). However, it is unclear whether this site is involved in lyso-PC-induced transcriptional activation. The purpose of this study is to identify cis-acting elements and nuclear transcriptional activators that are important in augmentation of eNOS transcription in response to lyso-PC stimulation. Our results indicate that Sp1 site at −104 to −90 and PE3A site at −40 to −24 are required for lyso-PC-induced transcriptional activation. Lyso-PC increases the promoter function by enhancing the Sp1 binding activity mediated by the action of protein phosphatase 2A.

EXPERIMENTAL PROCEDURES

Reagents—The following reagents were obtained from the indicated sources: QIA filter Midi kit was from Qiagen; Wizard PCR Prep DNA purification system, restriction endonucleases, T4 polyuridylate kinase, Sp1 consensus oligonucleotide, luciferase assay kit, serine/threonine phosphatase assay system, and pGL3 plasmid were from Promega; Lipofectin reagent, Opti-MEM medium, Klenow enzyme, and T4 DNA ligase were from Life Technologies, Inc.; Tau DNA polymerase was from Fisher; synthetic oligonucleotides were from Genosys; 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC) was from Avanti Polar Lipids; radionucleotides were from Amersham Pharmacia Biotech; Bio-Spin chromatography column was from Bio-Rad; Coomassie protein assay reagent was from Pierce; tissue culture reagents were from Sigma, except endothelial cell mitogen was from Biomedical Technologies. Other reagents were from Sigma.

Preparation of 5′-Promoter Region and Its 5′-Deletion Mutants—A 5′-flanking fragment at nucleotide positions from −1322 to +22 designated GH was obtained by polymerase chain reaction (PCR), using genomic DNA as template and synthetic oligonucleotides as primers: EN1322G (5′-AAAGATCTTCCATCTCCCTCCTG-3′) and EN3H (5′-GGGAAGCTTGTTACTGTGCGTCCACTCTG-3′). The PCR product was purified from the agarose gel digested with BglII/HindIII and cloned into the promoterless luciferase reporter vector pGL3. 5′-Deletion mutants of wild-type were also prepared by PCR and constructed into pGL-3 by a similar procedure.

Site-directed Mutagenesis—A 4-base mutation at the Sp1 site at
position −104/−90 was created by PCR using synthetic oligonucleotides: 5′-GGGATAGGGTCACTACGGGGCCGAC-3′ (mutated at positions −100, −98, −96, and −95) and EN3H as sense and antisense primers, respectively. The PCR product was purified and used with oligonucleotide EN1322G to produce the 1.3-kilobase GH fragment which was recloned into the pGL3 vector. Site-directed mutations in PEA-3 sites were constructed by similar procedures using the following primers: M1 (5′-CCCCCTTCTGAAATCCAGCGGGCC-3′) (mutated at positions −34, −33, −31, −30, −29, and −27) and EN3H; M2 (5′-CCCCCTTCTGAAATCCAGCGGGCC-3′) (mutated at positions −29, −27, −26, and −25) and EN3H. The PCR products were purified and used with oligonucleotide EN1322G to produce the 1.3-kilobase mutant fragments that were recloned into the pGL3 vector. We also produced a S/M1 double mutant using Bmp1 restriction endonuclease and T4 DNA ligase.

Cell Culture and Transient Expression—Human umbilical vein endothelial cells (HUVEC) were cultured in Medium 199 containing 20% fetal bovine serum (FBS) and 50 μg/ml endothelium mitogen in six-well plates. Passage 1 HUVEC were used in all experiments. Transient expression by lipofection was performed as described (18). In brief, HUVEC were incubated in serum-free medium containing a mixture of 10 μl of Lipofectin and 2 μg of plasmid constructs at 37 °C for 5 h. Medium was removed, and cells were incubated with fresh complete medium overnight. Cells were then washed and incubated with Medium 199 containing 0.5% FBS for 16 h. The medium was then removed and replaced with medium containing 5% FBS in the presence or absence of 100 μM Lyso-PC at 37 °C for 6 h. The cells were harvested and lysed with lysis buffer. The promoter activity was determined by luciferase assay in a luminometer (Analytical Luminescence Laboratories, Monolight model 2010) as described (19).

Construction of DNA Probe—The eNOS promoter construct GH was obtained by digesting with HindIII. The smaller fragment of Nos-III promoter was obtained by PCR using primers: P3 (AAAGATCTCGGC-GCTGGAGCTGAGGTCTTA) and EN3H. DNA was end-labeled with [γ-32P]ATP by Klenow polymerase. Unincorporated nucleotides were removed by Bio-Spin chromatography columns. End labeling oligonucleotide probes, Sp1 and PEA-3, were labeled with [γ-32P]ATP by T4 polynucleotide kinase.

Preparation of Nuclear Extract (NE)—Cells were cultured in complete medium (Medium 199, 20% FBS, 50 μg/ml endothelium mitogen). 16 h before experiment, the medium was changed to 0.5% FBS. Cells were treated with 100 μM LPC (in 5% FBS), and after 3 h of incubation, cells were harvested. Harvested cells were suspended in cold phosphate-buffered saline containing 0.5 mM PMSF, spun down at 400 g, washed with 3/4 package cell volume with buffer A and spun down again and resuspended in two package cell volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 300 mM sucrose, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aproitin, 0.5 mM PMSF), and allowed to stay on ice for 10 min. Cells were centrifuged at 6500 rpm for 20 s, washed with 3/4 package cell volume with buffer A and spun down again. Extraction buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 2 mM EDTA, pH 8.0, 25% glycerol, 1 μg/ml leupeptin, 1 μg/ml aproatin, 0.5 mM PMSF) was added to the nuclei at 1/3 package cell volume. Nuclei were passed 10 times through a 23-gauge needle and stirred on ice for 30 min. The nuclear debris was pelleted for 5 min at 12,000 rpm. The supernatant was dialyzed isovolumetrically in buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 1 mM DTT, 0.2 mM EDTA, pH 8.0, 20% glycerol, 1 μg/ml leupeptin, 0.5 μg/ml aproatin, 0.5 mM PMSF). Protein concentrations were determined using Bradford assay (Coomassie protein assay reagent: Pierce), using bovine serum albumin as a standard.

Electrophoretic Mobility Shift Assay (EMSA)—5.0 μg of nuclear extract in binding buffer containing 50 mM HEPES, pH 7.9, 1 mM EDTA, 100 mM KCl, 20% glycerol, 2.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF, 1.5 μg of poly(dI-dC) was incubated with competing oligonucleotides for 15 min on ice. 32P-Labeled probe (10,000 cpm) was added and incubated at room temperature for 10 min. The mixture was electrophoresed at 12.5 V/cm on 5% polyacrylamide gel in 0.25 × TBE. The gel was vacuum-dried and autoradiographed for 2 h at −70 °C with an intensifying screen.

Serine/Threonine Protein Phosphatase (PP) Assays—Assays for PP2A, PP2B, and PP2C were based on determining the amount of free phosphate generated in reaction by measuring the absorbance of a molybdate-malachite green-phosphate complex. 10 μg of nuclear extract proteins obtained from cells treated with or without Lyso-PC (100 μM) was incubated on 96-well plates in the presence or absence of an inhibitor (5 μM OA), together with a peptide substrate RRApTVA and appropriate buffer for 30 min at 30 °C. After incubation, the molybdate

RESULTS

Involvement of Sp1 and PEA-3 Sites in Lyso-PC-induced Promoter Activity—We have shown previously that a 5′-flanking fragment from nucleotide −165 to +22 confers basal eNOS promoter activity (17). To determine lyso-PC-induced promoter activity, we transfected into HUVEC a series of 5′-deletion mutants of the 1.3-kilobase GH fragment of eNOS gene and incubated the transfected cells in medium containing 100 μM lyso-PC or vehicle in the presence of 5% FBS. The results show that the region from −265 to +22 that confers the

FIG. 1. Functional analysis of the basal and lyso-PC-induced promoter activity conferred by 5′-flanking DNA fragments of eNOS gene. A, serial 5′-deletion mutants were constructed into pGL3 and expressed in cultured human umbilical vein endothelial cells. C− denotes negative control, and C+ denotes positive control. B, luciferase activity conferred by GH and its 5′-deletion mutants. The data are mean ± S.D. of three to five experiments.

Complex dye was added and incubated for an additional 30 min at room temperature to allow for color development. Reaction was read at 630 nm with a plate reader. This assay system detects PP2A, PP2B, and PP2C activities by using different buffers: for PP2A, 50 mM imidazole pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin; for PP2B, 50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, 10 mM MgCl2, 0.4 mM CaCl2, 50 μM calmodulin; and for PP2C, 50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, 5 mM MgCl2, 0.1 mg/ml bovine serum albumin.

RESULTS

Involvement of Sp1 and PEA-3 Sites in Lyso-PC-induced NOS-III Promoter Activity—We have shown previously that a 5′-flanking fragment from nucleotide −165 to +22 confers basal eNOS promoter activity (17). To determine lyso-PC-induced promoter activity, we transfected into HUVEC a series of 5′-deletion mutants of the 1.3-kilobase GH fragment of eNOS gene and incubated the transfected cells in medium containing 100 μM lyso-PC or vehicle in the presence of 5% FBS. The results show that the region from −265 to +22 that confers the
basal activity is responsive to lyso-PC induction (Fig. 1). In this region, there is a GC box located at $2^{104}$ to $2^{90}$ and PEA3 sites between $2^{113}$ to $2^{149}$ (Fig. 2). Further 5'-deletion of this region to remove the putative Sp1 binding sites resulted in a marked diminution of basal and lyso-PC-induced promoter activity. To evaluate the potential involvement of Sp1 and PEA3 sites in basal and lyso-PC-induced promoter activity, we constructed a Sp1 mutant (Sp1-M or S) contains a four-nucleotide mutation, PEA3 mutant 1 (M1) contains a six-nucleotide mutation located both in the sense and antisense PEA3 motifs, and PEA3 mutant 2 (M2) contains a four-nucleotide alteration in the sense motif (Fig. 2). These mutants were inserted into the promoterless luciferase expression vector pGL3 and transiently expressed in HUVEC by Lipofectin. Results from 3–5 experiments are shown in Fig. 3. Sp1 mutation reduced basal and lyso-PC-induced promoter activities more than 95% (Fig. 3). Residual activities were noted in basal and lyso-PC-treated cells, and the activity in the lyso-PC-treated cells was about twice that of the basal activity (Fig. 3). PEA3 M1 and M2 mutations decreased the basal promoter activity to 68 and 53% of the wild-type activity, respectively, and severely curtailed the lyso-PC-induced promoter activity (Fig. 3). Both basal and lyso-PC-induced promoter activities were almost entirely abolished by combined S and M1 mutations (Fig. 3). These results indicate that activation of transcription by lyso-PC requires Sp1 sites at $2^{104}$ to $2^{90}$ and PEA3 sites at $2^{40}$ to $2^{24}$.

Lyso-PC Increased Binding of Sp1-related Proteins—Binding of Sp1-related proteins to this minimal promoter region was determined by EMSA, using [α-32P]dATP-labeled fragment $(-1322/+22)$ of WT, S, M1, M2, and S/M1 were constructed into pGL3 vector and expressed in HUVEC. The results are mean ± S.D. of three to five experiments. □, −LPC; ■, +LPC.

**Fig. 2.** Nucleotide sequence of wild-type (WT), mutated Sp1 (Sp-1 M or S), two site-directed mutants of PEA3 sites M1 and M2, and double mutant S/M1. This figure shows only nucleotides from $-110$ to $-20$ of fragment $-1322$ to $+22$ at the 5'-flanking region of eNOS gene.

**Fig. 3.** Functional analysis of promoter activity. DNA fragment $(-1322/+22)$ of WT, S, M1, M2, and S/M1 were constructed into pGL3 vector and expressed in HUVEC. The results are mean ± S.D. of three to five experiments. □, −LPC; ■, +LPC.

**Fig. 4.** Determination of binding of nuclear extract proteins to [α-32P]dATP-labeled probe $(-165/+22)$ by EMSA. Lane 1 is a free probe. Lanes 2–6 show the binding activity of NE from non-lyso-PC-treated cells; lanes 7–11, lyso-PC-treated cells. Competitors Sp1 and PEA3 denote pretreatment of NE with a 25–50-fold molar excess of Sp1 or PEA3 consensus oligonucleotides. The arrow refers to the major Sp1-DNA complex, and the bracket refers to two Ets-DNA complexes.
evaluate the Sp1 binding activity in the lyso-PC stimulated NE, the Sp1 consensus sequence was labeled and incubated with NE from resting and lyso-PC-treated cells. A single Sp1-DNA complex band was noted, and this band was enhanced by lyso-PC (Fig. 5, compare lane 2 with lane 7). Competition with molar excesses of unlabeled oligonucleotides showed that the binding activity of lyso-PC-treated NE is about 5-fold higher than the binding activity of basal NE. By contrast, there was no apparent change in binding of NE proteins to PEA3 consensus sequence by lyso-PC treatment (data not shown). These data suggest that lyso-PC causes a change in the binding activity of Sp1-related proteins in NE.

Identification of Sp and Ets Binding Proteins—Four Sp proteins have been characterized. Specific antibodies against each class of proteins were used in a supershift assay to determine the exact Sp protein that is responsible for the binding activity detected in NE. The Sp-DNA complex band was supershifted only with antibodies against Sp1 (Fig. 6A, lanes 4 and 5). Antibodies to Sp2, Sp3, and Sp4 did not cause any shift of the band (Fig. 6A, lanes 6–11). The supershifted band of lyso-PC-stimulated NE was denser than that of basal NE consistent with an enhancement of Sp1 binding to its canonical Sp1 sequence on the promoter region. Supershift assays were also performed to identify the Ets protein that binds to labeled PEA3 consensus oligonucleotides. Pretreatment of NE obtained from control and lyso-PC-treated cells with antibodies to Ets 1, erg 1, Elk1, or PEA3 resulted in a marked reduction of the Ets-DNA complex compared with the control (Fig. 6B). As has been reported previously (20), antibodies to Ets proteins did not cause a shift of the complex, probably due to the interference of these antibodies with the binding of Ets proteins to DNA. These results suggest that the PEA3 sites on the promoter region were promiscuous for binding by a broad spectrum of Ets family proteins.

Effect of Okadaic Acid and Sodium Orthovanadate on Sp1 Binding Activity and Lyso-PC-induced eNOS Promoter Activity—To determine whether PP are involved in modifying Sp1 binding activity in lyso-PC-treated cells, NEs obtained from these cells were pretreated at room temperature for 30 min with okadaic acid, a serine PP inhibitor, or vanadate, a tyrosine PP inhibitor, and the Sp1 binding activity in NE was evaluated by EMSA using labeled Sp1 consensus oligonucleotides as the probe. Okadaic acid reduced the NE Sp1 binding activity in lyso-PC-treated cells in a concentration-related manner, and the maximal suppression was noted at 50 nM okadaic acid (Fig. 7A). Sodium orthovanadate reduced the NE Sp1 binding activity in lyso-PC-treated cells in a concentration-related manner, and the maximal suppression was noted at 50 μM monosodium orthovanadate (Fig. 7B).
7A). By contrast, vanadate at 50 \( \mu \text{M} \) had no apparent effect on blocking lyso-PC-induced Sp1 binding activity (Fig. 7B). To ascertain that the okadaic acid effect occurs in vivo, HUVEC were incubated in medium containing 50 nM okadaic acid in the presence or absence of lyso-PC for 3 h. Nuclear extracts from these cells were prepared and used in EMSA. The results indicate that okadaic acid reduced the lyso-PC-induced Sp1 binding activity to the basal level (Fig. 7C, compare lane 4 with lanes 2 and 1). Okadaic acid had no effect on the basal Sp1 binding activity.

The effect of okadaic acid on lyso-PC-induced eNOS promoter activity was evaluated by preincubating HUVEC transfected with eNOS promoter GH with 50 nM okadaic acid, and 1 h later, lyso-PC was added and incubated for 6 h. Okadaic acid reduced the lyso-PC-induced promoter to the basal promoter activity (Fig. 8). It has been shown that okadaic acid at low concentrations (10–50 nM) selectively inhibits PP2A activity (21). Our results are consistent with a role of PP2A in controlling lyso-PC-induced Sp1 binding activity and eNOS promoter activity.

Increase in Nuclear Extract PP2A Activity by Lyso-PC—The effect of lyso-PC on PP2A activity was evaluated by assaying PP2A as well as PP2B and PP2C activities in nuclear extracts of HUVEC treated with and without lyso-PC (100 \( \mu \text{M} \)). Lyso-PC significantly increased the PP2A activity (Fig. 9A), which was suppressed by OA (Fig. 9A). By contrast, lyso-PC had no effect on PP2B or PP2C activity (Fig. 9B).

DISCUSSION

Lyso-PC is a main component of oxidized low density lipoprotein (22). The level of lyso-PC is highly elevated in human atherosclerotic tissues (23). It has been shown that lyso-PC increases chemotaxis for monocytes and induces endothelial adhesive molecules such as intercellular adhesive molecule-1 and vascular cell adhesive molecule-1, which mediate monocyte and neutrophil adhesion to vascular endothelium (24). Hence, lyso-PC has been considered to play an important role in monocyte accumulation in the arterial walls. Furthermore, lyso-PC induces the expression platelet-derived growth factor A and B subunits and heparin-binding epidermal growth factor from endothelial cells (25, 26). These growth factors promote smooth muscle cell migration and proliferation. These findings have led to the conclusion that lyso-PC is a key mediator of atherosclerosis (22). Work from this laboratory provides evidence to indicate that the expression of NOS-III and cyclooxygenase-2 are stimulated by lyso-PC (27), thereby increasing the synthesis of NO and prostacyclin, which act synergistically to block monocyte adhesion and smooth muscle cell proliferation; platelet activation and aggregation; and pathological vasoconstriction. This leads to a postulate that vascular injurious agents such as lyso-PC create a yin-yang situation in which it induces the expression of vasoprotective genes to counteract the vascular damaging genes. It is interesting to note that lyso-PC induces the expressions of those diverse genes with two distinct types of kinetics: 1) rapid, transient as in heparin-binding epidermal growth factor and cyclooxygenase-2 gene induction and 2) delayed, sustained as in eNOS and intercellular adhesive molecule-1 gene induction. It is unclear whether these two types of genes are transcriptionally regulated by a common mechanism. In fact, the mechanism by which lyso-PC induces any of these genes had not been reported previously. This report is the first to shed light on the promoter regulation of eNOS gene by lyso-PC. Our results indicate that lyso-PC enhances the transcriptional activation of this housekeeping gene primarily by increasing the Sp1 binding activity via a PP2A-dependent reaction. Els binding to its cognate site on the promoter region is also involved in basal and lyso-PC-induced eNOS transcription.

Sp1 is a ubiquitous transcriptional activator mediating basal
and regulated gene expression. Sp1 binding to its cognate motif depends not only on nuclear Sp1 levels but also on posttranslational modification of the Sp1 molecule. Recent studies indicate that Sp1 binding activity is influenced by phosphorylation: phosphorylation decreases, whereas dephosphorylation by the action of phosphatases increases Sp1 binding activity (28, 29). A recent study on the acetyl-CoA carboxylase gene indicates that glucose induces this gene transcription by a mechanism involving dephosphorylation of Sp1 by protein phosphatase 1 and 2A (28). Our data, which show suppression of lyso-PC-induced Sp1 binding activity by okadaic acid, but not vanadate, and a selective increase in PP2A activity by lyso-PC, which was inhibited by okadaic acid, are consistent with a role of PP2A in modifying Sp1 binding activity. Results from this study led us to postulate that the Sp1 is phosphorylated at the basal cellular state, and lyso-PC activates PP2A, which in turn dephosphorylates Sp1, resulting in an increased Sp1 binding activity.

Signal pathways for lyso-PC-induced transcriptional activation have not been fully established. A recent study suggests the involvement of stress-activated protein kinase/c-Jun amino-terminal kinase pathway that leads to AP1 activation and binding to its cognate site (30). It is unclear whether JNK and/or other mitogen-activated protein kinase pathways are involved in increased Sp1 binding activity. It is possible that lyso-PC may increase Sp1 binding activity by activating protein phosphatases via the mitogen-activated protein kinase or other kinase pathways. This is being investigated in our laboratory.

Our results indicate that a PEA3/Ets sequence in the promoter/enhancer region of eNOS gene is involved in basal and lyso-PC-induced promoter activity. Nuclear Ets-related proteins contain a large family of polypeptides sharing an ETS domain (31). They bind to purine-rich sequences with a GGA core. The 5’- and 3’-flanking sequences of the GGA core confer relative specificity for Ets family proteins. The GGA-containing sequences on eNOS lack a specificity for any given class of Ets-related proteins. This is in agreement with our experimental data showing promiscuity of binding for several representatives of Ets family proteins. Ets proteins are involved in cellular response to external stresses. They generally serve as co-activators for transcriptional activators including Sp1 (32, 33). Several reports have suggested that PEA3 and Sp1 sequences are often colocalized on the promoter/enhancer region and binding of Ets and Sp1 to their respective sites causes a synergistic activation of a number of genes (33). As regards the activation of eNOS gene, our data are in agreement with the proposition that Ets serves as a co-activator for Sp1. Binding of Ets alone resulted in minimal promoter activation, whereas it augmented Sp1-mediated transcriptional activation. It is possible that Ets interacts with components of the general transcription factors in the preinitiation complex through which it may enhance recruitment of the preinitiation complex to the promoter region.

Acknowledgments—We thank Zhifei Zu for technical assistance and Susan Mitterling for excellent assistance in preparing this manuscript.

REFERENCES
1. Moncada, S., Palmer, R. J., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
2. Lowenstein, C. J., and Snyder, S. H. (1992) Cell 70, 705–707
3. Nathan, C. (1992) FASEB J. 6, 3051–3064
4. Marletta, M. A. (1993) J. Biol. Chem. 268, 12231–12234
5. Bredt, D. S., and Synder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 682–685
6. Steuer, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., and Nathan, C. F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7773–7777
7. Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D’Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992) J. Biol. Chem. 267, 13274–13278
8. Nathan, C., and Xie, Q.-W. (1994) J. Biol. Chem. 269, 13725–13728
9. Wu, K. K. (1995) Adv. Pharmacol. 33, 179–207
10. Nadaud, S., Philippe, M., Arnal, J. F., Michel, J. B., and Soubrier, F. (1996) Circ. Res. 79, 857–863
11. Sessa, W. C., Pritchard, K., Seyedl, N., Wang, J., and Hintze, T. H. (1994) Circ. Res. 74, 349–353
12. Arnet, U. A., McMillan, A., Dinerman, J. L., Ballermann, B., and Lowenstein, C. J. (1996) J. Biol. Chem. 271, 15069–15073
13. Weiner, C. P., Lisazoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5212–5216
14. Zsembowicz, A., Tang, J.-L., and Wu, K. K. (1995) J. Biol. Chem. 270, 17006–17010
15. Hirata, K., Miki, N., Kuroda, Y., Sakoda, T., Kanashima, S., and Yokoyama, M. (1995) Circ. Res. 76, 958–962
16. Marsden, P. A., Heng, H. H., Scherer, S. W., Stewart, R. L., Hall, A. V., Shi, X.-M., Tsui, L. C. and Shappert, K. T. (1993) J. Biol. Chem. 268, 17478–17488
17. Tang, J.-L., Zsembowicz, A., Xu, X.-M., and Wu, K. K. (1995) Biochem. Biophys. Res. Commun. 214, 673–680
18. Felgner, P. L. (1991) Methods Mol. Biol. 7, 81–89
19. DeWet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., Suleramani, S. (1987) Mol. Cell. Biol. 7, 725–737
20. Wang, L. G., Liu, X. M., Li, Z. R., Denstman, S., and Bloch, A. (1994) Cell Growth Differ. 5, 1243–1251
21. Favre, B., Turowski, P., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 17398–17404
22. Parthasarathy, S., Streinbrucher, U. P., Barnett, J., Witztum, J. L., and Steinberg, D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3000–3004
23. Portman, O. W., and Alexander, M. (1969) J. Lipid Res. 10, 158–165
24. Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Yui, S., Yamaizaki, M., Schichiri, M., and Horiuchi, S. (1994) J. Biol. Chem. 269, 31340–31345
25. Kume, N., and Gimbrone, M. A. (1992) J. Clin. Invest. 89, 907–911
26. Kume, N., Cybulski, M. I., and Gimbrone, M. A., Jr. (1992) J. Clin. Invest. 90, 1138–1144
27. Zsembowicz, A., Jones, S. L., and Wu, K. K. (1995) J. Clin. Invest. 96, 1688–1692
28. Daniel, S., Zhang, S., DePaoli-Roach, A. A., and Kim, K. H. (1996) J. Biol. Chem. 271, 14092–14097
29. Armstrong, S. A., Barry, D. A., Leggett, R. W., and Mueller, C. R. (1997) J. Biol. Chem. 272, 13489–13495
30. Fang, X., Gibson, S., Flowers, M., Furui, T., Baet, R. C., Jr., and Mills, G. B. (1997) J. Biol. Chem. 272, 13683–13689
31. Wasylyk, B., Hahn, S. L., and Giovane, A. (1993) Eur. J. Biochem. 211, 7–18
32. Khachigian, L. A., Williams, A. J., and Collins, T. (1995) J. Biol. Chem. 270, 27679–27686
33. Geggone, A., Bosselut, R., Bailly, R.-A., and Ghysdael, J. (1993) EMBO J. 12, 1169–1178