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# Role of AP1 Element in the Activation of Human eNOS Promoter by Lysophosphatidycholine

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**Abstract** Human endothelial nitric oxide synthase (eNOS) plays a crucial role in maintaining blood pressure homeostasis and vascular integrity. It, therefore, is very essential to elucidate the regulation of it. In the current study, a red fluorescent protein (RFP) reporter system containing human eNOS promoter was first constructed, being characteristics of real time morphologic and quantitative analysis for the same sample. It was observed by DNA sequence deletion that 68% of the basal activity of the promoter was controlled by the region from −1 to −166 bp, and 32% of it was dependent on the region from −1,033 to −1,600 bp. The mutation of SSRE element (−999∼−994 bp) and wild-type SSRE decoy oligodeoxynucleotides (ODN) did not alter the basal activity and the stimulating activity by lysophosphatidycholine (LPC). The mutation of upstream AP1 element (−1,530∼−1,524 bp) did not affect the basal activity, but resulted in near 30% reduction in the stimulating activity by LPC. Moreover, wild-type AP1 decoy ODN also remarkably attenuated it. It was proved by EMSA analysis that LPC indeed enhanced the activity of AP1 transcriptional factor binding to AP1 element. However, the role of AP1 was dependent on the presence of SP1, which was proved by the combining mutation of AP1 with SP1. The mutation of downstream AP1 element (−662∼−656 bp) had no influence on the basal and stimulating activities by LPC. These results strongly suggest that the main functional region of the promoter is from −1 bp to −166 bp, that the upstream AP1 participates in the activation of the promoter by LPC on the premise of the presence of SP1, and that the downstream AP1 and SSRE do not involve the basal and stimulating activity by LPC. J. Cell. Biochem. 98: 872–884, 2006. 

**Key words:** endothelial nitric-oxide synthase; activator protein 1 element; shear stress response element; lysophosphatidycholine; regulation

Human endothelial nitric oxide synthase (eNOS) not only participates in regulating blood pressure and vascular permeability, preventing white blood cell adhesion and platelet aggregation, but also concerns some pathological processes such as shock, hypertension, atherosclerotic heart disease, diabetes, ischemia strock, vascular stenosis, and occlusion [Ziegler et al., 1998; Houston et al., 1999; Bivalacqua et al., 2000; Gan et al., 2000]. It plays a pivotal role in maintaining blood pressure homeostasis and vascular integrity. The strong evidence has shown that overexpression of human *eNOS* gene within the vascular endothelium in transgenic mice attenuates both cardiac and pulmonary dysfunction and dramatically improves survival during severe congestive heart failure [Jones et al., 2003]. It, therefore, is very significant to elucidate its regulation mechanism by which expression of *eNOS* gene is increased or decreased. An effective and

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dynamic eNOS reporter system is required to reach this goal. Actually, a human eNOS promoter-drived luciferase reporter gene construct was early reported [Karantzoulis-Fegaras et al., 1999; Laumonnier et al., 2000; Anderson et al., 2004], but it has some serious shortages as follows: A: Influencing factors are not easy to control. B: Transfected cells have to be lysed for analysis so as not to make dynamic observation on the same cells disposed. C: Quantitative and morphologic analysis is impossibly made at the same time. Fortunately, a novel red fluorescent protein (RFP) was originally isolated from an Indo-Pacific sea anemone-relative, *Discosoma sp*. It would be complementary to the homologous green fluorescent protein (GFP) from Aequorea, and it has much higher extinction coefficient and quantum yield than various fluorescent proteins previously reported plus excellent resistance to pH extreme and photobleaching [Matz et al., 1999; Baird et al., 2000; Garcia-Parajo et al., 2001]. It is especially suitable to make real-time and dynamic observation of some gene transcriptional activity in the same cells disposed. In the view of these, RFP reporters containing the full-length or different regions of human eNOS promoter coupled to a RFP reporter gene were constructed, and their expression regularity in mammalian cells and their response to different stimuli were observed in detail.

On the other hand, research reports about functional characteristic of human eNOS promoter are less, maybe for the promoter is traditionally considered to be regulated just by mechanistic stimulus such as sheer stress and for the transcriptional activity of the promoter is very weak so that it is not easy to investigate. The promoter was early cloned and fused to a luciferase reporter gene to reveal regions of the promoter important for basal transcription in bovine aortic endothelial cells, finding that the basal activity of the promoter was mainly dependant on the regions from −1,033 to −779 bp and from −494 to −166 bp [Zhang et al., 1995]. Hereafter, no paper has concerned the relationship between the region and function of the promoter, but just some papers involve SP1 and GATA2 elements in the promoter. Zhang et al. found that SP1 element controlled 85% basal transcription activity of the promoter, but German et al. [2000] did that cell-specific eNOS expression in airway epithelium was dependent on GATA2. Another research group showed that a major transcriptional effect was identified for SP1/SP3 and GATA2 transcription factors, for which binding sites were located respectively at −103 and −230 bp upstream from the major transcription start site [Laumonnier et al., 2000]. And it should be emphasized that these researches are mainly limited to the basal transcription activity of the promoter. Moreover, it was proved that the promoter still contains some other *cis*-elements, such as activator protein 1 (AP1) and a shear stress response element (SSRE) besides described above, but their role in regulation of the promoter activity remains unreported. It is these reasons that attract us to find out roles of AP1 and SSRE elements, and interrelations among AP1, SSRE, and SP1 elements in an activation of the promoter by lysophosphatidycholine (LPC) using the RFP reporter system. Our novel findings suggest that the basal activity of the promoter is mainly dependant on the region from −166 to −1 bp, that the upstream AP1 participates in the activation of the promoter by LPC on the premise of the presence of SP1, and that the downstream AP1 and SSRE do not involve the basal activity and the stimulating activity by LPC.

## MATERIALS AND METHODS

### Construction of a eNOS Promoter-RFP Reporter Gene Vector

The RFP reporter containing a full-length of human eNOS promoter was constructed. In brief, human genomic DNA was extracted from human umbilical vein endothelial cells with 3S Genomic DNA Isolation Kit (BBST). 5′-flanking full-length of the promoter sequence at the nucleotide position from −1,600 to +1 bp (Gen Bank™, Accession Number: AF387340) was obtained by polymerase chain reaction (PCR) with the genomic DNA as a template, in which a forward primer is 5′-GA**AGATC-T**ATCTGATGCTGCCTGTCACCTTGACCCT-GAG-3′ with *Bgl*II site (boldface underlined), and a reverse primer is 5′-ATT**AAGCTT**T-GCCTGCTCCAGCAGAGCCCTGGCCTTTTC-3′ with *Hind*III site (boldface underlined). PCR product purified from 1% agarose gel was digested with *Bgl*II and *Hind*III (TaKaRa) and fused into a promoterless RFP expression vector, pDsRed 1-1 (Clontech). The construct is designated as pDseNOSRed, which was

identified to be correct by double restriction endonuclease digestion, PCR, and DNA sequencing.

### DNA Sequence Deletion

The RFP reporters containing different regions of human eNOS promoter were constructed, respectively. For generation of DNA deletion, the following forward primers, each with a *Bgl*II site (boldface underlined), were subjected to PCR reaction with pDseNOSRed construct containing the full-length of the promoter as a template: 5′-CC**AGATCT**CCGTTTCTTTCTTAAACT-3′ for F1033 (+1∼−1,033 bp); 5′-CG**AGATCT**-GAGGTGAAGGAGAGAAC-3′ for F494 (+1∼−494 bp); 5′-CA**AGATCT**GTGGAGCT-GAGGCTTT-3′ for F166 (+1∼−166 bp). Their reverse primers with a *Hind*III site are the same as 5′-ATT**AAGCTT**TGCCTGCTCCAGCAGA-GCCCTGGCCTTTTC-3′. The PCR products were purified by 1% agarose gel, digested with *Bgl*II and *Hind*III, and subcloned, respectively, into the *Bgl*II and *Hind*III sites of pDsRed1-1 vector. They were identified to be right by double restriction endonuclease digestion, PCR and DNA sequencing, being referred to as pDsF1033Red, pDsF494Red, and pDsF166Red, respectively.

### DNA Site-Directed Mutagenesis

For generation of site-directed mutants of upstream AP1 (−1,530∼−1,524 bp composed of TGAGTCA sequence), downstream AP1 (−662∼−656 bp composed of TGAGTCA sequence), SSRE (−999∼−994 bp composed of GGTCTC sequence), and Sp1 (−101∼−92 bp composed of GGGGCGGGGC sequence) elements in human eNOS promoter, site-directed mutation PCR was performed by MutanBEST Kit (TaKaRa) according to its instruction with pDseNOSRed as a template. Then, combining mutants of upstream AP1 with downstream AP1, of SP1 with upstream AP1 or downstream AP1 or both of them, respectively, and of SP1 with SSRE were done by the same approach. Forward and reverse primers for SP1 mutation were 5′-ATA**G**A**G**A**CT**G**GGC**GAGGGCCAG-CACT-3′ and 5′-CCCATACACAATGGGACAG-GAACAAG-3′, for the upstream AP1 mutation were 5′-TGAG**GA**ATCCTTGGTCATGCAC-ATT-3′ and 5′-TTTTCCTAGGTCCCAGTT-AATGGAGC-3′, for the downstream AP1 mutation were 5′-TGAG**GA**ATGGGGGTGT-GGGGGTT-3′ and 5′-AGTGGGGGACACAA-AAGAGCAGGA-3′, and for SSRE mutation were 5′-A***G*****CAG**TCGAAATCACGAGGCTTC-G-3′ and 5′-CAGAGACTGAGAGAAAGTTTAA-GA-3′ (the *cis*-elements are underlined, the mutation bases appear in boldface). The PCR products were isolated from 1% agarose gel, purified by plasmid purification kit (QIAGEN), blunted with blunting kination enzyme, extracted by Phenol/chloroform, and subjected to ligation with T4 DNA ligase. All constructs were confirmed by sequencing the mutation site, then denominated pDsSP1mRed (containing the mutation of SP1), pDsAP1m1Red (containing the mutation of the upstream AP1), pDsAP1m2Red (containing the mutation of the downstream AP1), pDsAP1m12Red (containing the combining mutation of the upstream AP1 with the downstream AP1), pDsSSREmRed (containing the mutation of SSRE), pDsSP1SSREmRed (containing the combining mutation of SP1 with SSRE), pDsSP1AP1m1Red (containing the combining mutation of SP1 with the upstream AP1), pDsSP1AP1m2Red (containing the combining mutation of SP1 with the downstream AP1), and pDsSP1AP1m12Red (containing the combining mutation of SP1 with the upstream AP1 and the downstream AP1), respectively.

### Cell Culture, Transient Transfection, and Stimulation

ECV304 or HEK293, or NIH3T3 cells grew in DMEM (Gibco) containing 10% or 5% FBS (Hyclone) in 24-microwell plates. To compare the activities among different regions of human eNOS promoter, ECV304 cells were chosen according to the paper [Cieslik et al., 2001] and transfected with Lipofectamine Reagent Kit (Invitrogen) for 5 h by addition of 0.6 μg of pDseNOSRed, pDs F1033Red, pDsF494Red, pDsF166Red, promoterless pDsRed1-1, and pDsRed1-N1 (Clontech) containing $P_{CMV\ IE}$-drived RFP, respectively. To confirm the role of AP1 and SSRE elements, and the interrelation among AP1, SSRE, and SP1 in regulation of the promoter, the cells were transfected with Lipofectamine Reagent Kit for 5 h by addition of 0.6 μg of pDseNOSRed, pDsSP1mRed, pDsAP1m1Red, pDsAP1m2Red, pDsAP1m12Red, pDsSSREmRed, pDsSP1SSR Em Red, pDsSP1AP1m1Red, pDsSP1 AP1m2Red, and pDsSP1AP1m12Red, respectively. In the

stimulated groups, an administered dosage for lipopolysaccharide (Lps) (Sigma) was 100 ng or 500 ng/well, for tumor necrosis factor-α (TNF-α) (Sigma) 3 ng or 10 ng/well and for transforming growth factor-β (TGF-β) (Sigma) 1 ng or 10 ng/well. The cells were treated with them 24 h and 48 h after the transfection. In the group treated with LPC (Sigma), the transfection medium was removed and replaced withcomplete medium overnight. Then, the cells were washed, incubated in medium containing 0.5% FBS for 16 h, and done in fresh medium containing 5% FBS in the presence or absence of 50 μM LPC. The promoter activity was measured at indicated time. The transfection efficiency was normalized by an approach to co-transfect 0.2 μg of pEGFP-N1 vector as an internal control with the target constructs described above.

### AP1, SSRE, and SP1 Decoy Oligodeoxynucleotides

Sense and antisense strands of AP1, SSRE, and SP1 decoy ODN as well as their mutant decoy ODN were designed and then synthesized by TaKaRa company. AP1 decoy sequences were 5′-TGAGTCATGAGTCATGAGTCA-3′ and 5′-TGACTCATGACTCATGACTCA-3′, and its mutant decoy sequences were 5′-TGAGGAATGAGGAATGAGGAA-3′ and 5′-TTCCTCATTCCTCATTCCTCA-3′, respectively. SP1 decoy sequences were 5′-GGGGCGGGGCGGGGCGGGGCGGGGCGGGGC-3′ and 5′-GCCCCGCCCCGCCCCGCCCCGCCCCGCCCC-3′, and its mutant decoy sequences were 5′-GAGACTGGGCGAGACTGGGCGAGACTGGGC-3′ and 5′-GCCCAGTCTCGCCCAGTCTCGCCCAGTCTC-3′, respectively. SSRE decoy sequences were 5′-GGTCTCGGTCTCGGTCTC-3′ and 5′-GAGACCGAGACCGAGACC-3′, and its mutant decoy sequences were 5′-GCAGTCGCAGTCGCAGTC-3′ and 5′-GACTGCGACTGCGACTGC-3′, respectively. Each sense-antisense pair was annealed by heating to 95°C and decreasing the temperature by 5°C increments every 15 min. After 3 h, the reaction mixture was held at a base temperature of 4°C. pDseNOSRed was co-transfeted with wild-types or mutants of AP1, SP1, and SSRE decoy ODNs, respectively, into ECV304 cells growing in 24-microwell plates.

### Electrophoretic Mobility Shift Assay

The harvested cells were suspended in cold PBS containing 0.5 mM PMSF, spun down, resuspended in 400 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.0 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 2.0 μg/ml aprotinin, 2.0 μg/ml leupeptin, 1.0 μg/ ml pepstatin, 1.0 mM PMSF), and placed on ice for 15 min. Afterwards, the cells were added by 25 μl 10% Nonidet P-40. They were violently rocked for 30 s and centrifuged at 12,000 rpm below 4°C for 20 min. Then, the deposit was resuspended in 100 μl buffer B (20 mM HEPES, pH 7.9, 400 mM KCl, 1.0 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 2.0 μg/ml aprotinin, 2.0 μg/ml leupeptin, 1.0 μg/ ml pepstatin, 1.0 mM PMSF), rocked on ice for 45 min and centrifuged at 12,000 rpm below 4°C for 20 min, and finally the supernatant, that is, nuclear extract (NE), was applied to electrophoretic mobility shift assay (EMSA). Protein concentrations were determined by Bradford assay, using bovine serum albumin (BSA) as a standard.

Double strand DNA probes of AP1 were synthesized according to the promoter sequence in human umbilical vein endothelial cells. The upstream and downstream sequences of Ap1 probe are 5′-CCTAGGAAAAAATGAGTCATCCT-3′ and 5′-AGGATGACTCATTTTCCTAGG-3′. AP1 oligodeoxynucleotide probe was labeled with [α-$^{32}$P]dATP by terminal deoxynucleotidyl transferase (TaKaRa). NE (5.0 μg) in binding buffer containing 20 mM Tris-HCL, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5mM DTT, 2.0mM $MgCl_2$, 1.5 μg of poly (dI-dC), 7.5% glycerol and 0.5 mM PMSF was incubated with a cold oligodeoxynucleotide probe on ice for 10 min to confirm the specificity of binding to [α-$^{32}$P]dATP-labeled AP1 probe. Then, [α-$^{32}$P]dATP-labeled AP1 probe (10,000–25,000 cpm) was added and incubated at room temperature for 30 min. The mixture was subjected to non-denaturing gel electrophoresis at 9 V/cm on 6% polyacrylamide gel with buffer containing 1 × TBE and 0.5% Nonidet P-40. The gel was vacuum-dried, followed by autoradiography at −70°C overnight in a cassette with an intensifying screen.

### RFP Reporter Gene Assay

The transfected cells were observed under inverted fluorescence microscope (Nikon TE300) at each interval of 12 h, with an excitation wavelength of 550 nm. Red fluorescence-emitting cells in each microwell were scanned at random under low power visual field (magnification 100×) using CCD sensor (Penguin

150CL) that was connected with a computer. More than 10 low power visual fields for each group were scanned for avoiding the bias from RFP expression variations in cells. Then, optical density (OD) of red fluorescence, which represents eNOS promoter activity, was determined using a fluorescence analysis software, Image-Pro Plus (Mediacy). Green fluorescence emitted by GFP was determined with an excitation wavelength of 488 nm 36 h after the transfection.

### Statistical Analysis

Results shown represent mean ± SD. Statistical analysis was performed by one-way ANOVA test and by Student-Neuman–Keuls test.

## RESULTS

### Expression of RFP Reporter Containing the Promoter in Different Cells

As showed in Figure 1, human eNOS promoter full-length-drived RFP reporter might efficiently express in different mammalian cell lines including ECV304, HEK293, and NIH3T3 cells. And the RFP expression efficiency in ECV304 cells was further verified to be 25.2% by flow cytometry (Elite, Coulter Corp.).

### Expression Regularity of RFP Reporter Containing the Promoter

After identified to be correct by double restriction enzyme digestion, PCR and sequencing, pDseNOSRed, pDsF1033Red, pDsF494Red, and pDsF166Red vectors were effectively expressed in ECV304 cells. The red fluorescence emitted by RFP uniformly dispersed all over the cells, appearing 48 h after transfection, reaching its peak at 96 h, and gradually disappearing 168 h after. The quantity and intensity in RFP expression drived by human eNOS promoter were eminently lower than those in RFP expression drived by $P_{\mathrm{CMV\ IE}}$ in pDsRed 1-N1 vector as a positive control. In the cells transfected by pDsRed1-1 as a negative control, no RFP expression was found. The RFP expression regularity was similar among pDseNOSRed, pDsF1033Red, pDsF494Red, and pDsF166Red groups.

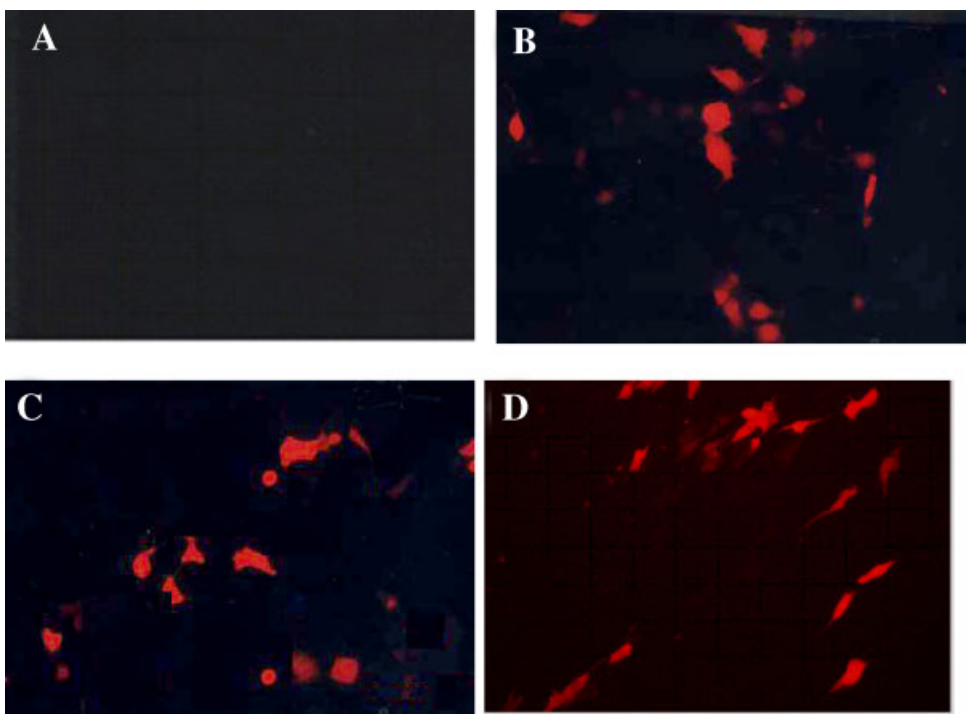


**Fig. 1.** Expressions of pDseNOSRed vector containing human eNOS full-length promoter in different cell lines (**A**∼**D**: 200×, 96 h after transfection). A: ECV304 or HEK293, or NIH3T3 cells were transfected by blank vector, pDsRed1-1. B: ECV304 cells were transfected with pDseNOSRed vector. C: HEK293 cells were transfected with pDseNOSRed vector. D: NIH3T3 cells were also transfected with pDseNOSRed vector. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Response of RFP Reporter Containing the Promoter to Different Stimuli

Compared with the basal activity of human eNOS promoter in pDseNOSRed group ($1{,}116.50 \pm 279.07$), 500 ng of LPS, 3 ng of TNF-α, and 10 ng of TNF-α all resulted in dramatic decreases of the promoter activity which were $775.54 \pm 188.55$ ($P < 0.05$), $817.29 \pm 359.94$ ($P < 0.05$), and $722.01 \pm 498.00$ ($P < 0.01$), respectively. By contrast, 1 ng of TGF-β and 10 ng of TGF-β might upregulate the promoter activities which were $1{,}425.52 \pm 312.22$ ($P < 0.05$) and $1{,}521.53 \pm 445.15$ ($P < 0.01$), respectively. In contrast to the control, 50 μM of LPC also upregulated the promoter transcription level ($1{,}776.11 \pm 370.05$), which appeared obvious statistical difference ($P < 0.01$). Although 100 ng of LPS induced the promoter activity to decrease, there was no marked difference, compared with the control as indicated in Figure 2.

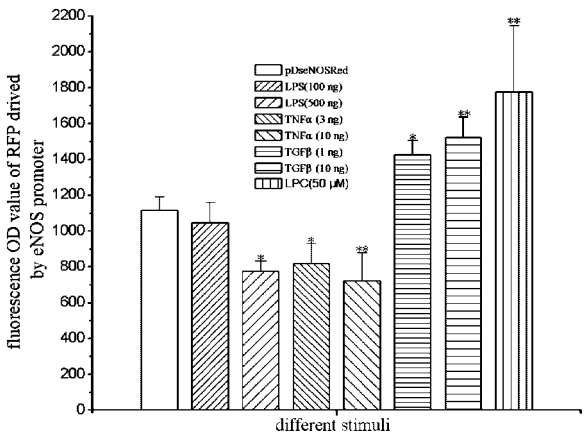


**Fig. 2.** Effects of different stimuli on human eNOS promoter transcriptional activity. ECV304 cells were transfected with the RFP reporter containing the full-length of human eNOS promoter, pDseNOSRed, then treated with 100 ng or 500 ng LPS, 3 ng or 10 ng TNF-α, and 1 ng or 10 ng TGF-β, respectively, 24 h and 48 h after the transfection. In the group treated with LPC, the transfection medium was removed and replaced with complete medium overnight. Then, the cells were washed, incubated in medium containing 0.5% FBS for 16 h, and done in fresh medium containing 5% FBS in the presence or absence of 50 μM LPC, after which the procedure is the same as other stimuli used above. The stimulated cells were subjected to fluorescence quantitative analysis 24∼48 h after the stimulation. The OD value of RFP expression drived by the promoter is normalized by co-transfecting pEGFP-N1 vector. Results represent mean ± SD of four independent experiments, in which more than 15 low power visual fields for each experiment were scanned at random. $^{*}P < 0.05$, $^{**}P < 0.01$ versus pDseNOSRed group.

### Functional Regions of the Promoter

ECV304 cells were transfected with RFP reporter constructs containing the full-length or the different regions of human eNOS promoter including pDseNOSRed, pDsF1033Red, pDsF494Red, and pDsF166Red vectors, followed by fluorescence quantitative analysis 96 h after transfection. The results showed that the transcription activities of F1033, F494, and F166 regions of the promoter were $763.42 \pm 383.86$, $775.35 \pm 326.71$, and $821.18 \pm 358.08$, respectively, which were prominently lower than that of the full-length of the promoter ($1{,}116.50 \pm 279.07$, $P < 0.05$) but no obvious difference existed among them (Fig. 3), suggesting that 68% of the basal activity of the promoter is decided by the region from −1 to −166 bp and 32% of it is dependent on the region from −1,033 to −1,600 bp.

### Effect of AP1 and SSRE Elements on the Promoter Activity

In contrast to the control, the basal activity of human eNOS promoter was not influenced by the mutation of the upstream AP1 (in pDsAP1m1Red group) or the downstream AP1 (in pDsAP1m2Red group), or their combination (in pDsAP1m12Red group). Compared with LPC group, the mutation of the downstream AP1 (in pDsAP1m2Red + LPC group) did not influence the stimulating activity of the promoter by LPC, but the mutation of the

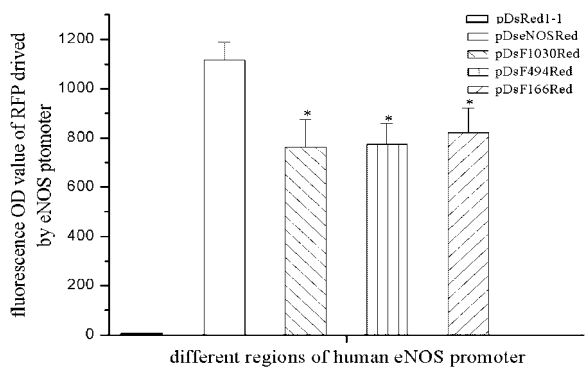


**Fig. 3.** Functional regions of human eNOS promoter. ECV304 cells were transfected with pDseNOSRed, pDsF1033Red, pDsF494Red, and pDsF166Red vectors, respectively and done with pDsRed1-1 as a negative control, followed by fluorescence quantitative analysis 96 h after transfection. The OD value of RFP expression drived by the promoter is normalized by co-transfecting pEGFP-N1 vector. Results represent mean ± SD of five independent experiments, in which more than 15 low power visual fields for each experiment were scanned at random. $^{*}P < 0.05$ versus pDseNOSRed group.

upstream AP1 (in pDsAP1m1Red + LPC group and in pDsAP1m12Red + LPC group) resulted in near 30% reduction in the stimulating activity by LPC ($P < 0.05$, Fig. 4B). Moreover, 5.0 pM of wild-type AP1 decoy ODN [in AP1w ODN (5.0 pM) + LPC group] also remarkably attenuated the stimulating activity by LPC as compared with LPC group ($P < 0.05$), which was completely reversed by 5.0 pM of its mutant [in AP1m ODN (5.0 pM) + LPC group], but did not affect the basal activity as compared with the control (Fig. 4C). These data indicate that the upstream AP1 participates in the activation of the promoter by LPC. Because the mutation of the upstream AP1 did not entirely offset the induction of the stimulating activity by LPC, it is suggested that there must be other *cis*-elements to function also in the induction.

The mutation of SP1 caused not only the basal activity, but also the stimulating activity by LPC to disappear almost completely, compared with the control and LPC group, respectively ($P < 0.01$, Fig. 5B). Consistent with this result, 5.0 pM of wild-type SP1 decoy ODN also markedly decreased the basal activity and the stimulating activity by LPC as compared with the control and LPC group, respectively ($P < 0.01$, Fig. 5C), which was reversed by 5.0 pM of its mutant. If the mutation of the upstream AP1 was combined with the mutation of SP1, the role of the upstream AP1 in increasing the stimulating activity by LPC was abolished, suggesting that AP1 functions in the presence of SP1 (Fig. 5B).

Compared with the control and LPC group, respectively, the mutation of SSRE had no effect on both the basal activity and the stimulating activity by LPC (Fig. 6B), and 0.5 pM and 5.0 pM of wild-type SSRE decoy ODN did not alter them, suggesting that SSRE does not involve the basal activity and the stimulating activity by LPC (Fig. 6C).

### AP1 DNA Binding Activity Induced by LPC

To confirm an activity of AP1 transcriptional factor binding with AP1 element in human eNOS promoter stimulated by LPC at different intervals, the nuclear extract was subjected to EMSA analysis. AP1 DNA binding activity swiftly reached the peak 30 min after treatment with LPC, lasting to 4 h, beginning to decrease after 6 h. The result indicates that LPC may enhance AP1 DNA binding activity, further offering the strong evidence for AP1 element to exert an influence on the activation of the promoter by LPC (Fig. 7).

## DISCUSSION

Human eNOS promoter-drived luciferase reporter gene construct was early reported [Karantzoulis-Fegaras et al., 1999; Laumonnier et al., 2000; Anderson et al., 2004], but transfected cells have to be lysed for analysis so as not to make dynamic observation of both quantity and morphology on the same cells disposed at the same time. A RFP reporter gene, however, just offsets these shortages. In the view of this, RFP reporters drived by the full-length and the different regions of the promoter were established in this study. All the RFP reporter constructs, pDseNOSRed, pDsF1033Red, pDsF494Red, and pDsF166Red, might be remarkably expressed in different mammalian cell lines, including ECV304, HEK293, and NIH3T3 cells, and responded well to different stimuli including LPC, LPS, TGF-β, and TNF-α. Furthermore, the transient expression of the RFP reporters appeared well regularity, which was observed in detail. These results show that the RFP reporters may accurately offer real time morphologic and quantitative analysis for the promoter activity. Besides, the RFP fluorescence intensity in a great part of the cells was markedly weaker than the positive control, suggesting that human eNOS promoter is not a strong promoter.

Zhang et al. [1995] constructed different regions of the promoter into pGL2 vector with luciferase reporter gene, finding that the region from −1,033 to −779 bp resulted in an approximately 40% reduction in the basal promoter activity, and that the region from −494 to −166 bp reduced the promoter activity by 40–50%. Until now, no papers published have involved the functional region. In this study, the results different from above were obtained. The sequence deletion from −1,600 to −1,033 bp of upstream in the promoter transcriptional initiation caused 32% reduction, but progressive deletions of −1,034∼−495 bp and −494∼−167 bp did not influence the basal activity of the promoter, suggesting that 32% of the basal activity is associated with the region from −1,600 to −1,034 bp, whereas 68% of it is dependent on the region from −166 to −1 bp. It is emphasized that this reporter system can

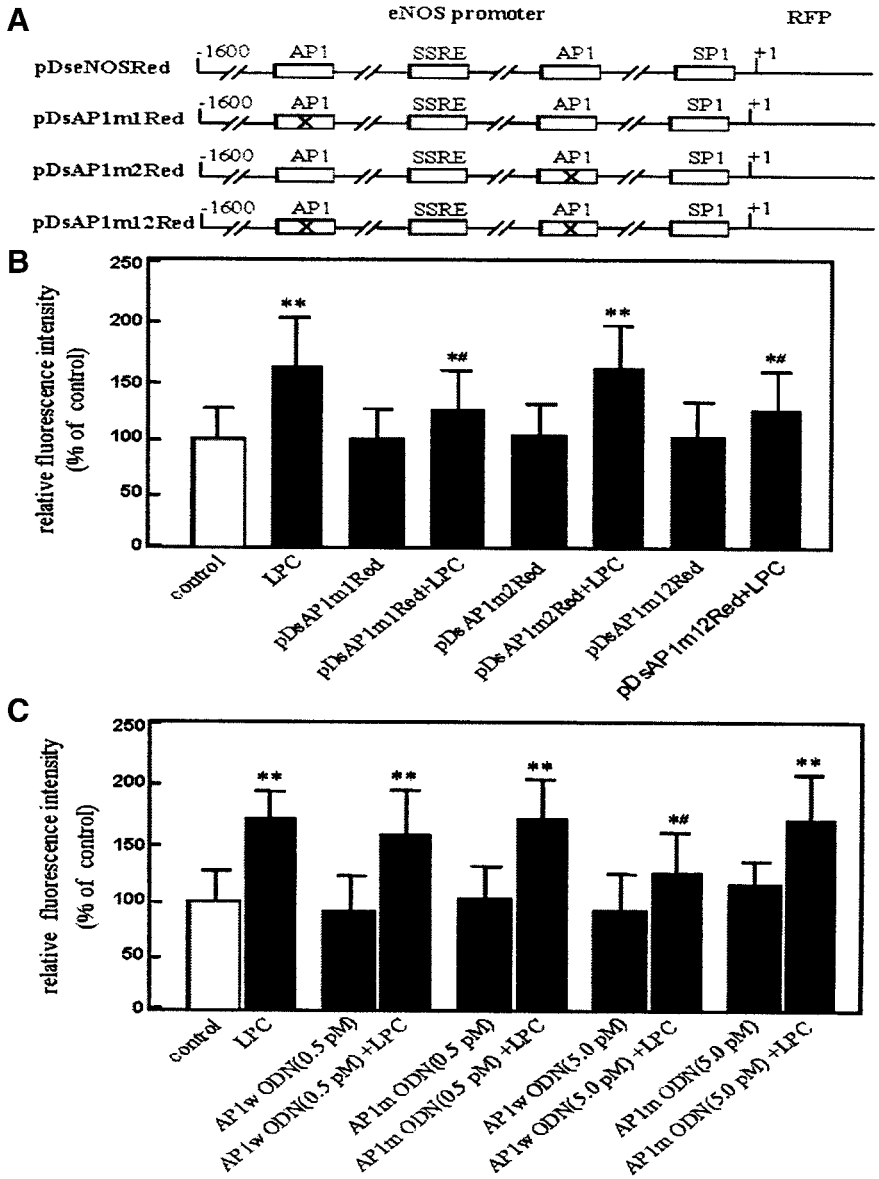


**Fig. 4.** Effects of AP1 element on human eNOS promoter activity. To find out a role of AP1 element in human eNOS promoter activity induced by LPC, ECV304 cells were transfected with the RFP reporter containing the full-length of human eNOS promoter, pDseNOSRed, with AP1 mutants, and then co-transfected with the RFP reporter plus AP1 decoy ODN. **A**: The mutants including pDsAP1m1Red, pDsAP1m2Red, and pDsAP1m12Red are shown below the wild-type eNOS promoter (−1,600 to +1 bp) reporter construct, pDseNOSRed, and an X in the boxes represents the mutation of AP1 site. **B**: The cells were transfected with the RFP reporter as a control, with the upstream AP1 mutant as pDsAP1m1Red group, with the downstream AP1 mutant as pDsAP1m2Red group, and with the combining mutant of the upstream AP1 and the downstream AP1 as pDsAP1m12Red group. The cells transfected with the RFP reporter or with the AP1 mutants were stimulated with 50 μM LPC as LPC group, pDsAP1m1Red + LPC group, pDsAP1m2-Red + LPC group, pDsAP1m12Red + LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. **C**: The control and LPC group were treated as described above. The cells were co-transfected with the RFP reporter plus 0.5 pM of wild-type AP1 decoy ODN as AP1w ODN (0.5 pM) group, plus 0.5 pM of AP1 decoy ODN mutant as AP1m ODN (0.5 pM) group, plus 5.0 pM of wild-type AP1 decoy ODN as AP1w ODN (5.0 pM) group, and plus 5.0 pM of AP1 decoy ODN mutant as AP1m ODN (5.0 pM) group, respectively. The cells co-transfected with the RFP reporter and the AP1 decoy ODN were stimulated with 50 μM LPC as AP1w ODN (0.5 pM) + LPC group, AP1m ODN (0.5 pM) + LPC group, AP1w ODN (5.0 pM) + LPC group, and AP1m ODN (5.0 pM) + LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. The relative fluorescence intensity of RFP expression in each group is normalized by co-transfecting pEGFP-N1 vector, but a percent of that in the control group. Results represent mean ± SD of four independent experiments, in which 13–16 low power visual fields for each experiment were scanned at random. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control; $^{\#}P < 0.05$ versus LPC group.

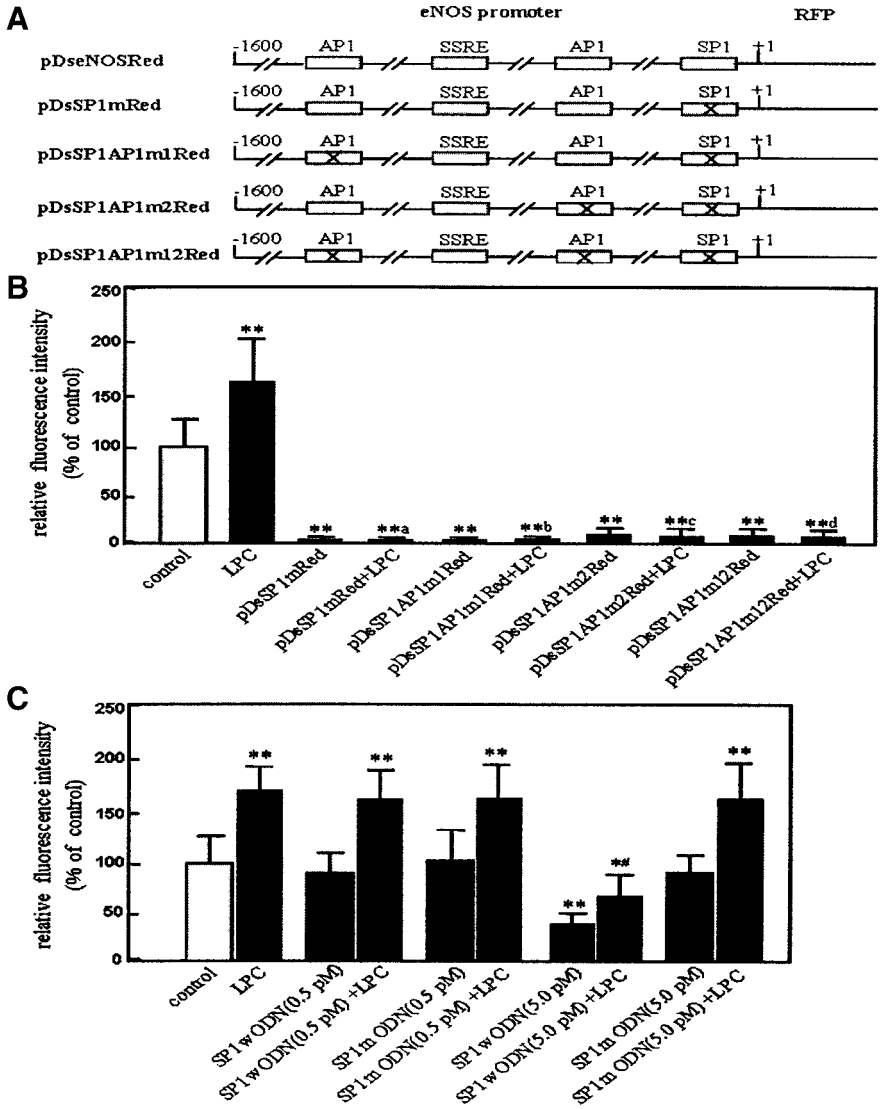


**Fig. 5.** Interrelation between AP1 and SP1 elements in human eNOS promoter activity. To find out an interrelation of AP1 and SP1 elements in human eNOS promoter activity induced by LPC, ECV304 cells were transfected with the RFP reporter containing the full-length of human eNOS promoter, pDseNOSRed, with SP1 mutant and with the combining mutants of AP1 and SP1, and then co-transfected with the RFP reporter plus SP1 decoy ODN. **A**: The mutants including pDsSP1mRed, pDsSP1AP1m1Red, pDsSP1AP1m2Red, and pDsSP1AP1m12Red are shown below the wild-type eNOS promoter (−1,600 to +1 bp) reporter construct, pDseNOSRed, and an X in the boxes represents the mutation of SP1 or AP1 site. **B**: The cells were transfected with the RFP reporter as a control, with SP1 mutant as pDsSP1mRed group, with the combining mutant of the upstream AP1 and SP1 as pDsSP1AP1m1Red group, with the combining mutant of the downstream AP1 and SP1 as pDsSP1AP1m2Red group, and with the combining mutant of the upstream AP1, the downstream AP1 and SP1 as pDsSP1AP1m12Red group, respectively. The cells transfected with the RFP reporter, with the SP1 mutant, and with the combing mutants of both AP1 and SP1 were stimulated with 50 μM LPC as LPC group, pDsSP1mRed + LPC group, pDsSP1AP1m1Red + LPC group, pDsSP1AP1m2Red + LPC group, and pDsSP1AP1m12Red + LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. **C**: The control and LPC group were treated as described above. The cells were co-transfected by the RFP reporter plus 0.5 pM of wild-type SP1 decoy ODN as SP1w ODN (0.5 pM) group, plus 0.5 pM of SP1 decoy ODN mutant as SP1m ODN (0.5 pM) group, plus 5.0 pM of wild-type SP1 decoy ODN as SP1w ODN (5.0 pM) group, and plus 5.0 pM of SP1 decoy ODN mutant as SP1m ODN (5.0 pM) group, respectively. The cells co-transfected with the RFP reporter and the wild-type SP1 decoy ODN or with the RFP reporter and the SP1 decoy ODN mutant were stimulated with 50 μM LPC as SP1w ODN (0.5 pM) + LPC group, SP1m ODN (0.5 pM) + LPC group, SP1w ODN (5.0 pM) + LPC group, and SP1m ODN (5.0 pM) + LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. The relative fluorescence intensity of RFP expression in each group is normalized by co-transfecting pEGFP-N1 vector, but a percent of that in the control group. Results represent mean ± SD of six independent experiments, in which 13–21 low power visual fields for each experiment were scanned at random. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control; $^{\#}P < 0.01$ versus LPC group; $^{a}P > 0.05$ versus pDsSP1mRed group; $^{b}P > 0.05$ versus pDsSP1AP1m1Red group; $^{c}P > 0.05$ versus pDsSP1AP1m2Red group; $^{d}P > 0.05$ versus pDsSP1AP1m12Red group.

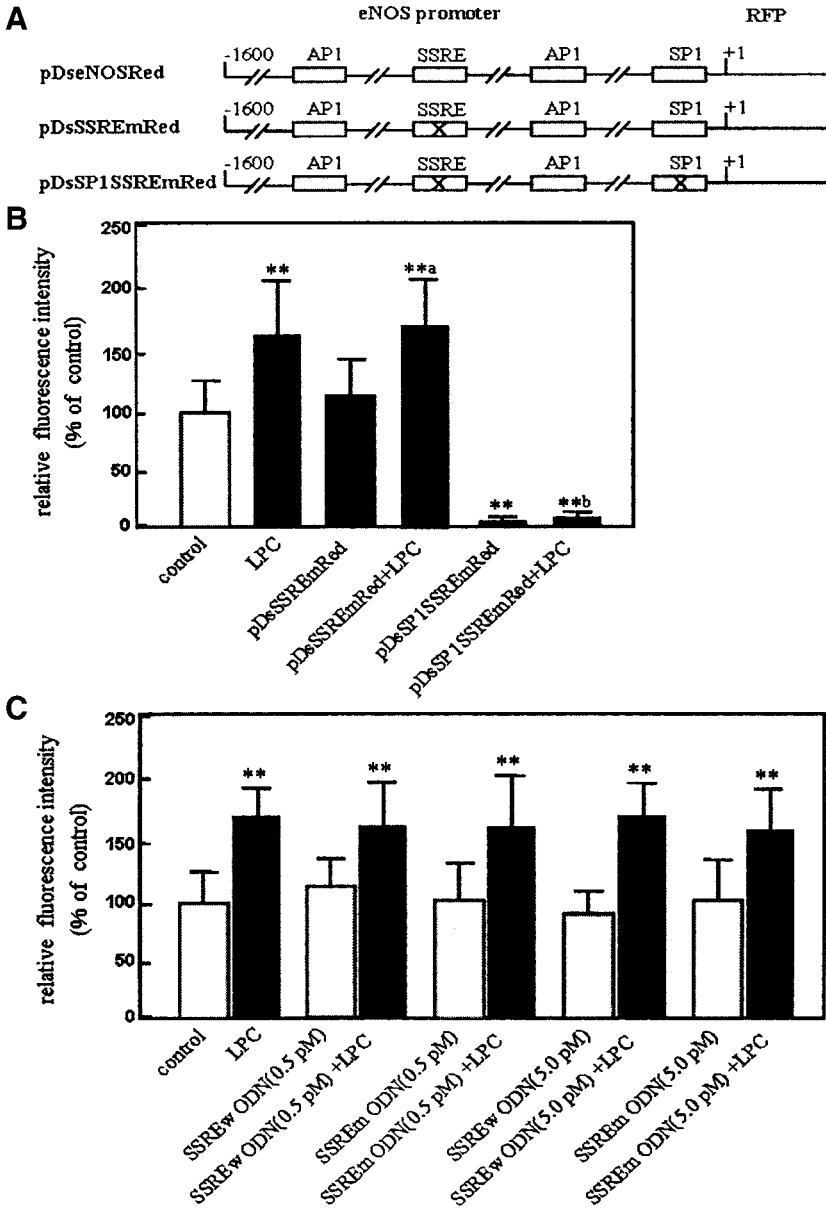


**Fig. 6.** Effects of SSRE element on human eNOS promoter activity. To find out a role of SSRE element in human eNOS promoter activity induced by LPC, ECV304 cells were transfected with the RFP reporter containing the full-length of human eNOS promoter, pDseNOSRed, with SSRE mutant, and then co-transfected with the RFP reporter plus SSRE decoy ODN. **A**: The mutants including pDsSSREmRed and pDsSP1SSREmRed are shown below the wild-type eNOS promoter (−1,600 to +1 bp) reporter construct, pDseNOSRed, and an X in the boxes represents the mutation of SP1 or SSRE site. **B**: The cells were transfected with the RFP reporter as a control, with SSRE mutant as pDsSSREmRed group, and with the combining mutant of SSRE and SP1 as pDsSP1SSREmRed group. The cells transfected with the RFP reporter or with the SSRE mutant were stimulated with 50 μM LPC as LPC group, pDsSSREmRed+LPC group and pDsSP1SSREmRed +LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. **C**: The control and LPC group were treated as described above. The cells were co-transfected by the RFP reporter plus 0.5 pM of wild-type SSRE decoy ODN as SSREw ODN (0.5 pM) group, plus 0.5 pM of SSRE decoy ODN mutant as SSREm ODN (0.5 pM) group, plus 5.0 pM of wild-type SSRE decoy ODN as SSREw ODN (5.0 pM) group, and plus 5.0 pM of SSRE decoy ODN mutant as SSREm ODN (5.0 pM) group, respectively. The cells co-transfected with the RFP reporter and the wild-type SSRE decoy ODN or with the RFP reporter and the SSRE decoy ODN mutant were stimulated with 50 μM LPC as SSREw ODN (0.5 pM) + LPC group, SSREm ODN (0.5 pM) + LPC group, SSREw ODN (5.0 pM) + LPC group, and SSREm ODN (5.0 pM) + LPC group, respectively, followed by fluorescence quantitative analysis 24∼48 h after the stimulation. The relative fluorescence intensity of RFP expression in each group is normalized by co-transfecting pEGFP-N1 vector, but a percent of that in the control group. Results represent mean ± SD of four independent experiments, in which 15–20 low power visual fields for each experiment were scanned at random. $^{**}P < 0.01$ versus the control; $^{a}P > 0.05$ versus LPC group; $^{b}P > 0.05$ versus pDsSP1SSREmRed group.

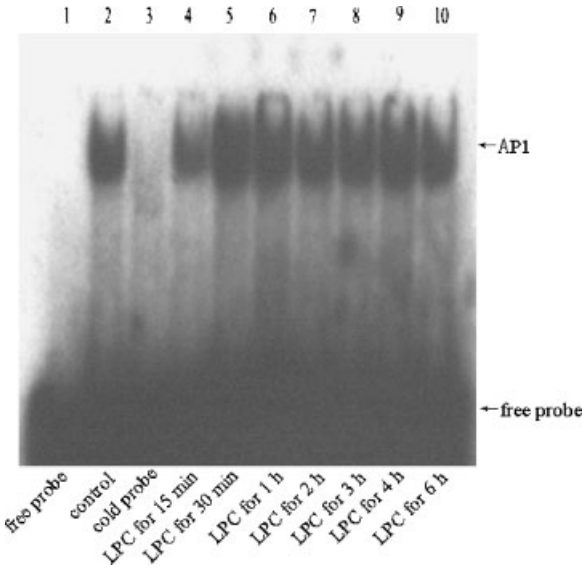


**Fig. 7.** Stimulation of AP1 DNA binding activity by LPC at different intervals. The nuclear extracts were prepared from ECV304 cells treated without or with LPC at different intervals. The extracts were incubated with $^{32}$P-labeled AP1 oligodeoxynucleotide probe and subjected to nondenaturing gel electrophoresis followed by autoradiography. 1, free probe; 2, ECV304 cells untreated with LPC were used as a control; 3, AP1 band was almost competed out by 100-fold molar excess of cold probe; 4∼10, the cells were treated with 40 μM of LPC for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 6 h, respectively. AP1 binding is indicated by the *upper arrow*, and free probe is shown by the *lower arrow*. A representative result of three independent experiments is shown.

accurately report the promoter activity not only by real time morphologic analysis, but also by quantitative analysis. On the other hand, we mutated SP1 element to result in almost all disappearance of the basal and stimulating activities of the promoter. Moreover, wild-type SP1 decoy ODN might also make them to go down markedly. It is known that SP1 element just exists in the region from −166 to −1 bp. Therefore, these results further support that the promoter activity is mainly dependent on the region from −166 to −1 bp.

The specific mutation of GATA2 site (−228∼−223 bp composed of TTATCA sequence) reduced the basal promoter activity by 25%, and that the mutation of Sp1 resulted in 85% reduction in the basal promoter activity [Zhang et al., 1995]. Other laboratories showed that SP1 and GATA2 elements play an important part in controlling the promoter activity [Karantzoulis-Fegaras et al., 1999; Laumonnier et al., 2000]. But cell-specific eNOS expression in airway epithelium was found mainly to be dependent on GATA2 [German et al., 2000]. It is easy through DNA sequence analysis to find out that the region from −1,600 to −1,034 bp, which decides 32% of the promoter activity, mainly contains AP1 and sterol regulatory element 1 (SRE1) elements, and that the another region from −166 to −1 bp, which decides 68% of the promoter activity, mainly contains SP1 element. But, it is not known whether 32% of the basal activity of the promoter is controlled by AP1 and SRE1 elements and whether they are associated with the activation of the promoter by LPC. Our results showed that the site-directed mutagenesis of the upstream AP1 element did not affect the basal activity, but resulted in near 30% reduction in the stimulating activity by LPC. Moreover, wild-type AP1 decoy ODN also remarkably attenuated it. It was proved by EMSA analysis that LPC indeed enhanced the activity of AP1 transcriptional factor binding to AP1 element. But the role of AP1 was dependent on the presence of SP1, which was proved by the combining mutation of AP1 with SP1. However, the mutation of the downstream AP1 element did not influence the basal activity and the stimulating activity by LPC. SSRE element was not associated with the basal activity and the stimulating activity by LPC, either. In addition, the consistent outcomes were obtained by our many repeated experiments to prove that the mutation of SP1 element led to almost all disappearance (over 98%) of the basal activity and the stimulating activity by LPC, which is much more than that reported previously. Consequently, we speculate that SP1 element may be a premise for other *cis*-elements in the promoter to function whenever any stimuli. On the other hand, $T^{-786} \rightarrow C$ mutation resulted in a significant reduction in the promoter activity and the patients with the mutation were predisposed to coronary spasm [Nakayama et al., 1999; Yoshimura et al., 2000]. $Glu^{-298} \rightarrow Asp$ and $T^{-786} \rightarrow C$ polymorphisms of human *eNOS* gene were associated with the presence and severity of angiographically defined coronary artery disease in Italian population and also with a higher risk of multivessel coronary artery disease in Caucasians [Colombo et al., 2003; Rossi et al., 2003a]. $T^{-786} \rightarrow C$ promoter polymorphism and its interaction with exon 7 $Glu^{-298} \rightarrow Asp$ affected endothelium-dependent vasodilation in mild- to moderate- hypertensive patients and normotensive Caucasian

subjects [Rossi et al., 2003b]. For these reasons, functional characteristics of human eNOS promoter are extremely complex, and especially interrelation among *cis*-elements remains to be elucidated.

Notwithstanding many research groups have shown that flow fluid shear stress may induce the promoter activity [Busses and Fleming, 1998; Geller and Billia, 1998; Traub and Berk, 1998; Wedgwood et al., 2003], it was reported that TGF-β [Saura et al., 2002], TNF-α [Anderson et al., 2004], LPC [Cieslik et al., 1998], and anoxia [Chicoine et al., 2002; Coulet et al., 2003] may also upregulate or downregulate eNOS gene expression. A lot of papers demonstrated that LPS might induce iNOS gene expression, but no papers indicated an effect of it on eNOS gene expression. LPS was recently found to stimulate the diphosphorylation of SP1 protein to downregulate DNA-binding activity of SP1 [Ye and Liu, 2002]. Because SP1 plays a pivotal role in controlling the promoter activities, it is speculated by us that LPS may influence eNOS gene expression. Hence, LPS, TNF-α, TGF-β, and LPC were chosen as stimuli in order to observe the RFP reporters to respond to them. Our result proved first from transcription level that LPS markedly downregulated the promoter activity, which is consistent with the most recent reports that iNOS-mediated nitric oxide enhancement [Tsao et al., 2002] and that eNOS-mediated nitric oxide reduction were led to from post-translation level by in vivo injection of LPS [Hallemeesch et al., 2003]. For this reason, our result undoubtedly complements the traditional viewpoint that *eNOS* gene is constitutively expressed by the vascular endothelial cells and is not induced by LPS that is a strong stimulus to iNOS. Besides, LPC is generated from oxidized low-density lipoprotein [Parthasarathy et al., 1985] or from inflammatory cells as a result of phospholipase $A_2$ action [Asaoka et al., 1993]. It possesses a variety of proinflammatory and proatherogenic properties [Sato et al., 1998; Engelmann et al., 1999; Morimoto et al., 2001]. And bacterial LPS is the major outer surface membrane component present in almost all Gram-negative bacteria, which is intimately involved through interactions among Toll-like receptor 4, LPS binding protein, CD14, MD2, and MyD88 in a variety of severe pathologic processes, such as inflammation, septemia, tissue and organ injury as well as septic shock [Alexander and Rietschel, 2001; Alikhani et al., 2003]. We, therefore, consider that LPC and LPS are more suitable as stimuli for the investigation on up- and downregulation mechanism of human eNOS gene expression for the prevention and therapy of cardiovascular diseases.

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