Hypoxia-Induced Endothelial NO Synthase Gene Transcriptional Activation Is Mediated Through the Tax-Responsive Element in Endothelial Cells

Jiho Min, Yoon-Mi Jin, Je-Sung Moon, Min-Sun Sung, Sangmee Ahn Jo, Inho Jo

Abstract—Although hypoxia is known to induce upregulation of endothelial NO synthase (eNOS) gene expression, the underlying mechanism is largely unclear. In this study, we show that hypoxia increases eNOS gene expression through the binding of phosphorylated cAMP-responsive element binding (CREB) protein (pCREB) to the eNOS gene promoter. Hypoxia (1% O₂) increased both eNOS expression and NO production, peaking at 24 hours, in bovine aortic endothelial cells, and these increases were accompanied by increases in pCREB. Treatment with the protein kinase A inhibitor H-89 or transfection with dominant-negative inhibitor of CREB reversed the hypoxia-induced increases in eNOS expression and NO production, with concomitant inhibition of the phosphorylation of CREB induced by hypoxia, suggesting an involvement of protein kinase A/pCREB-mediated pathway. To map the regulatory elements of the eNOS gene responsible for pCREB binding under hypoxia, we constructed an eNOS gene promoter (−1600 to +22 nucleotides) fused with a luciferase reporter gene [pGL2-eNOS(−1600)]. Hypoxia (for 24-hour incubation) increased the promoter activity by 2.36±0.18-fold in the bovine aortic endothelial cells transfected with pGL2-eNOS(−1600). However, progressive 5′-deletion from −1600 to −873 completely attenuated the hypoxia-induced increase in promoter activity. Electrophoretic mobility shift, anti-pCREB antibody supershift, and site-specific mutation analyses showed that pCREB is bound to the Tax-responsive element (TRE) site, a CAMP-responsive element–like site, located at −924 to −921 of the eNOS promoter. Our data demonstrate that the interaction between pCREB and the Tax-responsive element site within the eNOS promoter may represent a novel mechanism for the mediation of hypoxia-stimulated eNOS gene expression. (Hypertension. 2006;47:1189-1196.)

Key Words: nitric oxide synthase ■ nitric oxide ■ endothelium ■ gene regulation ■ vasodilation ■ signal transduction

In response to hypoxia, systemic arteries vasodilate, allowing more blood to be delivered to peripheral tissues. This vasodilation occurs within seconds after hypoxia and is maintained for several hours.¹ The molecular basis of hypoxic vasodilation is still somewhat unknown, although potassium channel activation, decreased ATP levels, increased intracellular acidification, decreased release of vasoconstrictors, such as endothelin and thromboxane, and increased release of vasodilators, such as adenosine, prostacyclin, and NO, have all been suggested as key components.²,³

NO plays an important role in vasodilation, and in endothelial cells, NO is catalyzed from the amino acid L-arginine and molecular oxygen by endothelial NO synthase (eNOS). The activity⁴ and expression⁵–¹¹ of eNOS have been found to increase in response to hypoxia, but conflicting studies have observed a decrease¹²,¹³ or lack of response.¹⁴ The discrepancies between these studies can potentially be explained by differences in either the duration of exposure to hypoxia or the endothelial cell type used.

Coulet et al¹¹ reported recently that 2 contiguous hypoxia-responsive elements (HREs) located at −5375 to −5366 were identified in the eNOS gene promoter, and that transcription factor hypoxia-inducible factor (HIF)-2α preferentially bound to these elements under hypoxia, leading to the activation of eNOS gene transcription. However, several other studies using a construct of a shorter 5′-region flanking the eNOS gene (1600-bp), which is devoid of HRE, also clearly showed an increased transcriptional activity by hypoxia,⁹,¹¹ implying that mechanism(s) other than the HIF/HRE-mediated signaling pathway may be involved. To date, there has been only 1 study showing that enhanced eNOS gene transcription was mediated by redox-sensitive activating protein 1 signaling pathways.⁹

In this study, we searched for other element(s) of the eNOS promoter that are responsible for hypoxia-induced transcriptional activation. We identified a Tax-responsive element (TRE) located from −924 to −921 within the eNOS promoter and showed that phosphorylated cAMP-responsive
element (CRE) binding (CREB) protein (pCREB) preferentially binds to this response element under hypoxia and activates eNOS gene transcription.

Materials and Methods

Materials

H-89 was purchased from Calbiochem. Minimum essential medium, Hanks’ balanced salt solution, newborn calf serum, penicillin–streptomycin, L-glutamine, trypsin-EDTA solution, and other plastic wares for cell culture were obtained from Gibco-BRL. Antibodies against eNOS and HIF-1α were purchased from Transduction Laboratories and BD Biosciences, respectively. Antibodies against pCREB (phosphorylated at Ser133) and CREB were purchased from Cell Signaling Technology, Inc.

Genomic DNA Extraction and Plasmid Construction

Genomic DNA was prepared from human blood leukocytes. Human blood leukocytes were obtained from Ansan Health Study,15 and all of the procedures for human research were approved by the Institutional Review Board of Ansan Health Study. Using genomic DNA as a template, a DNA fragment of the 5’-flanking region of the eNOS gene (~1600 to +22 nucleotides) was ampliﬁed by PCR and designated eNOS (~1600). The sense and antisense primers containing KpnI and XhoI sites, respectively, for PCR ampliﬁcation are shown as follows: sense, 5′-TTGACCCCTGAGGATGCCAGT-3′; antisense, 5′-TGTGGCTGCCTCGACGAGCCAGGC-3′. Furthermore, 5′-deletion DNA fragments of the eNOS promoter at −1400, −962, −873, and −428 were also ampliﬁed by PCR and designated eNOS (~1400), eNOS (~962), eNOS (~873), and eNOS (~428), respectively. Each sense primer is shown as follows: eNOS (~1400), sense 5′-CTTGACCTGAGGATGCCAGT-3′; eNOS (~962), sense 5′-AAGTGGACCTGAGGATGCCAGT-3′; eNOS (~873), sense 5′-GAGTGGCTGAGGATGCCAGG-3′; and eNOS (~428), sense 5′-ATCCAGCATACAGAATAAC-3′. The same antisense sequences were used to construct all of the 5′-deletion DNA fragments. In a separate experiment, site-directed mutagenesis (A→G at −922) of eNOS (~922) of the eNOS promoter was designed. NO released by BAECs was measured by the Griess method with a minor modiﬁcation.

Cell Culture, Transfections, and Hypoxia Treatment

Bovine aortic endothelial cells (BAECs) were isolated and cultured as described.16 BAECs were transfected with (3 μg each) pGL2-eNOS (~1600), pGL2-eNOS (~1400), pGL2-eNOS (~962), pGL2-eNOS (~873), and pGL2-m-eNOS (~962) using Lipofectin (Invitrogen Inc) according to the manufacturer’s instructions. In a separate experiment, BAECs were also transfected with 3 μg of dominant-negative inhibitor of CREB, termed A-CREB.17 BAECs or transfecants were then subjected to either hypoxia or normoxia for the indicated times. Protocols for cell culture, transfections, luciferase assays, and hypoxia treatments are described in detail in the online data supplement (available online at http://www.hypertensionaha.org).

Measurement of NO Release

Measurement of NO release is described in detail in the online data supplement.

Electrophoretic Mobility Shift Assay

The method for electrophoretic mobility shift assay (EMSA) is described in detail in the online data supplement. The sequences of the sense strands of double-stranded oligonucleotides used as probes and competitors in the EMSA were as follows, with the consensus motifs of CRE and TRE3 italicized: The CRE probe (sense strand) used was 5′-CCGGAGGCCTGAGTCACTGAAATGCA-3′ (Figure 3C). At this moment, it should be noted that the CRE sequence indicated in the eNOS gene is not a “classical” palindromic DNA sequence (TGACGTC) of CRE, but it has been widely used as a putative CRE sequence in the previous studies.18,19 In a separate experiment, a positive control for CRE probe with a classical palindromic DNA sequence of CRE (italicized) used was 5′-AGAGTTGCTCAGCTAGAGCTCAGT-3′. The wild-type TRE3 probe used was 5′-CCAGGGCTCTAGACACAGACAGAC-3′ (Figure 4C), and mutant TRE3 (mTRE3, with mutation in boldface) was 5′-CCAGGGCTCTAGACACAGACAGAC-3′ (Figure 4C). For the mutation of TRE3 sequence, the site-specific mutation (A→G) at −922 of the eNOS promoter was directed from the discovery of eNOS gene single-nucleotide polymorphic sites.21,22

Western Blot Analysis

Western blot analysis is described in detail in the online data supplement.

Real-Time RT-PCR

Measurement of the levels of eNOS mRNA is described in detail in the online data supplement.

Statistical Analysis

All of the data are presented as mean (SD) with n indicating the number of experiments. Statistical signiﬁcance was determined by the Student t test for 2 points. Differences were deemed statistically significant at *P < 0.05.

Results

Hypoxia Increases eNOS Protein Level and NO Production

Hypoxia (1% O2 concentration), as evidenced by the increase in HIF-1α expression (Figure 1A), increased the NO produc-
tion by BAECs in a time-dependent manner (Figure 1B). The maximal increase in NO levels (2.78±0.14-fold of the control) was observed after 24-hour treatment, and this increase subsequently fell over the next 48-hour period. Therefore, unless otherwise stated, all of the subsequent experiments were performed using this condition (1% O2 concentration for 24-hour treatment). Western blot analysis revealed that this increase was accompanied by an increase in eNOS protein expression (Figure 1B).

Increase in eNOS Protein Level and NO Production by Hypoxia Is Mediated Through pCREB-Mediated Pathway

Hypoxia is reported to increase the phosphorylation of CREB. This result, considered in context with a previous finding that beraprost sodium (BPS), an orally active prostacyclin analogue, increased eNOS gene transcription by pCREB-mediated pathways, prompted us to test whether these hypoxia-stimulated increases in eNOS expression and NO production are also mediated by pCREB-CRE signaling pathways. As shown in Figure 2A, hypoxia increased the phosphorylation of CREB with no alteration of CREB expression. The maximal increase in CREB phosphorylation was observed after 24-hour treatment, showing a similar time-dependent pattern to that of eNOS expression. To determine whether the increase in CREB phosphorylation mediates hypoxia-induced increases in eNOS protein level and NO production, we first used H-89, a specific protein kinase A (PKA) inhibitor. PKA is well known to phosphorylate CREB in a variety of cells. As shown in Figure 2B, pretreatment with H-89 (10 μmol/L for 1-hour treatment) significantly decreased CREB phosphorylation by hypoxia. This drug, however, did not alter CREB expression. Furthermore, H-89 significantly attenuated hypoxia-induced increases in eNOS expression and NO production. To further clarify a role of pCREB in this mechanism, we next transfected A-CREB, a dominant-negative inhibitor of CREB, into BAECs. A-CREB, devoid of Ser133 phosphorylation site, is well known to inhibit CRE-mediated transcription by DNA binding of pCREB to the specific promoter sequence of several target genes. Recently, A-CREB was also reported to inhibit the phosphorylation of CREB, leading to a decrease in CRE-mediated steroidogenic acute regulatory protein gene transcription induced by insulin-like growth factor 1. Parallel to the results with H-89, we found that transfection of A-CREB also decreased CREB phosphorylation by hypoxia (Figure 2C). Furthermore, this transfection significantly decreased hypoxia-stimulated eNOS expression and NO production by hypoxia. Taken together, our data suggest that the stimulatory effect of hypoxia on eNOS expression and NO production in BAECs is mediated at least in part by CREB phosphorylation.

Figure 2. Hypoxia increases phosphorylation of CREB by PKA-dependent signaling pathway. BAECs were prepared and treated with hypoxia as described in Figure 1. The amount of pCREB was measured by Western blot analysis with antibody specific for CREB phosphorylated at Ser133 and normalized to total CREB level (A). In some experiments, BAECs were pretreated with 10 μmol/L H-89 for 1 hour (B), or transfected with a dominant-negative inhibitor of CREB, A-CREB (C), and the pCREB and NO were measured (B and C). The control cells were treated with vehicle 0.01% dimethyl sulfoxide (DMSO) (B) or empty pcDNA3.1 (Mock) vector (C) only. Data are presented as described in Figure 1 (n=4 to 6).
CRE Within −1.6-kb eNOS Gene Promoter Is Not Involved in Transcriptional Activation of eNOS Gene by Hypoxia

To test whether hypoxia increased the eNOS expression via the interaction between pCREB and CRE within the eNOS promoter, we first examined the transcriptional activation of eNOS by hypoxia. In agreement with previous reports, we clearly found that hypoxia (for 24-hour treatment) increased the amount of eNOS mRNA (Figure 3A), suggesting that the stimulatory effect of hypoxia on eNOS gene expression occurs at least at the level of transcription. We next constructed pGL2-eNOS(−1600), as described in the Methods section, to explore the role of the interaction between pCREB and CRE in the transcriptional activation of the eNOS gene. As shown in Figure 3B, hypoxia clearly increased the promoter activity (2.36±0.18-fold of the control) in cells transfected with pGL2-eNOS(−1600). Based on a Genomatix database search (http://genomatix.de), we identified 1 putative CRE site located from −733 to −727 of the eNOS promoter (Figure 3B). However, using pGL2-eNOS(−873) containing this putative CRE site, no increase in promoter activity was observed in response to hypoxia, suggesting that this CRE site is not involved in hypoxia-stimulated eNOS transcription. To further corroborate this finding, we explored EMSA to find whether the pCREB induced by hypoxia bound to the CRE. As shown in Figure 3C, CRE site is not involved in hypoxia-stimulated eNOS gene transcription. The relative eNOS mRNA expression levels obtained by real-time RT-PCR analysis were calculated as the ratio of eNOS expression in hypoxia-treated cells to those in normoxic cells, and both were normalized to GAPDH mRNA level. Results are mean±SD. Statistically significant at *P<0.01 (n=3; A). Left, eNOS promoter-luciferase deletion constructs. BAECs were transfected with each construct, and were exposed to normoxia or hypoxia for 24 hours. Right, relative activity of each of the constructs in the BAECs. Luciferase activity was measured by the Luciferase Assay System, normalized to total the cellular protein, and expressed relative to that of pGL2-eNOS(−1600) without hypoxia treatment. Results are mean±SD and are statistically significant at **P<0.01 (n=4; B). Sense sequence of the double-stranded probe for the CRE site in the eNOS gene promoter region used in the EMSA is shown; consensus motif of CRE is italicized (C). Nuclear extracts from the normoxic and hypoxic BAEC were incubated with radiolabeled probe containing the CRE sequence and subjected to EMSA and supershift assay using anti-pCREB antibody (Ab). Normal protein–DNA complexes and pCREB or CREB–DNA complexes are indicated by an arrow (D).
3D, the supershift experiment using anti-pCREB antibody revealed that pCREB did not bind to the CRE site. This result is valid, because we found that pCREB clearly bound to the positive control. We also found that this binding was specific, because it was inhibited in a dose-dependent manner by a 10- and 50-molar excess of unlabeled CRE (from positive control or from eNOS gene) oligonucleotides. These data suggest that pCREB may not bind to the CRE in the eNOS promoter. A positive control contains a “classical” palindromic DNA sequence (TGACGTCA) of CRE, whereas CRE in the eNOS gene is very similar in sequence to the classical CRE with only 1 nucleotide (A) deleted (TGCGTCA).

**TRE Site Located From −924 to −921 of the eNOS Promoter Is Identified as a pCREB Binding Sequence Responsible for Hypoxia-Stimulated eNOS Gene Transcription**

To map the region(s) of the eNOS promoter responsible for hypoxia-stimulated eNOS transcription, we further searched a Genomatix database. In addition to CRE, we found that there are 4 more putative sequences, so-called TREs, for CREB binding (Figure 4A). Progressive 5'-deletion from −1600 to −962 [pGL2-eNOS(−1400) and pGL2-eNOS(−962)] did not significantly alter the increased eNOS promoter activity by hypoxia (Figure 4B). However, further deletion from −962 to −873 [pGL2-eNOS(−873)] completely attenuated the increased promoter activity, whereas deleting down to −428 [pGL2-eNOS(−428)] did not reduce activity further (Figures 3B and 4B). This result suggests an important role for the TRE3 site in hypoxia-stimulated eNOS gene transcription. To further corroborate these findings, nuclear extracts after hypoxia were incubated with wild-type (TRE3) and mutant double-stranded oligonucleotides (mTRE3) corresponding with the TRE3 region and analyzed by EMSA (Figure 4C). The sequence of mTRE3 was adopted from the well-known single-nucleotide polymorphic sequence identified by several genomic epidemiology studies.20,21 As shown in Figure 4D, incubation with TRE3 resulted in significantly retarded complexes (left); more prominent retarded complex was found when incubated with extracts from hypoxic cells. The supershift experiment using anti-pCREB antibody demonstrated that pCREB bound to the TRE3 probe. The binding of pCREB to the TRE3 was specific, because this binding was significantly inhibited in a dose-dependent manner by a 10- and 50-molar excess of unlabeled wild-type TRE3 oligonucleotides. In contrast, competition and supershift assays showed that pCREB did not bind to the mTRE3 probe (right), although incubation with mTRE3 resulted in a more prominent retarded complex compared with incubation with TRE3. These results defined the hypoxia-responsive sequence as TRE3 located at −924 to −921 of the eNOS promoter. The importance of TRE3 for the observed effects was further supported by a finding that the construct pGL2-m-eNOS(−962), which contains mTRE3 in the context of pGL2-eNOS(−962), was unresponsive to hypoxia (Figure 4E). Furthermore, cotransfection of A-CREB significantly inhibited the luciferase activity of pGL2-eNOS(−962) induced by hypoxia, whereas no alteration in luciferase activity of pGL2-m-eNOS(−962) was observed, suggesting that pCREB bound to the TRE3 sequence, leading to an increase in eNOS gene transcription.

**Discussion**

Our study demonstrates that hypoxia increases NO production by activating pCREB-mediated eNOS gene transcription. Promoter analysis of the eNOS gene mapped the hypoxia-responsive sequence to TRE, located at −924 to −921. These data are further confirmed by EMSA, supershift assay, and site-specific mutation analysis. We believe that this is the first solid evidence that eNOS gene expression is induced in response to hypoxia through the interaction between pCREB and TRE.

Our current finding, that hypoxia increases pCREB-mediated eNOS gene transcription, deserves particular attention, because cAMP-mediated vasodilation has been generally considered to be eNOS independent.28–30 However, it has been reported recently that cAMP increased NO production via either phosphorylation of eNOS19,31 or expression of eNOS,18 suggesting a direct effect of cAMP on eNOS modulation. A particularly interesting finding from a previous study, which relates to our current study, was that BPS, an orally active prostacyclin analogue, increased the promoter activity of the eNOS gene through the interaction between pCREB and CREs.18 In this previous study, PKA was found to phosphorylate CREB, and PKA inhibitors completely blocked the effect of BPS on eNOS gene transcription and NO production. This PKA-mediated, pCREB-stimulated eNOS gene transcription was fairly reproduced by our current data using H-89 treatment and A-CREB transfection (Figure 2B and 2C). However, it should be noted that the phosphorylation of CREB was first detected at 6 hours after hypoxia, peaked at 24 hours, and gradually declined thereafter (Figure 2A), whereas BPS increased pCREB at a much earlier time point (30 minutes) and peaked at 1 hour.18 Furthermore, the authors of the previous report defined 2 contiguous CRE sequences at −733 and −603 as a pCREB binding site. Interestingly, however, this result was not reproduced by our current study, because we did not observe any of the interactions of pCREB with CREs that they had defined (Figure 3D). Instead, we found that pCREB induced by hypoxia treatment bound to the TRE site. At present, the reason for these incompatible results is not fully understood, although it may be attributable to different experimental conditions: hypoxia treatment versus BPS treatment.

Although the involvement of TRE, the human T-cell leukemia virus type 1 (HTLV-1), a CRE-like site, in the regulation of hypoxia-stimulated eNOS gene transcription is of great interest, a detailed underlying mechanism is not clearly understood at the present time. It was reported previously that Tax, a unique oncoprotein encoded by a viral genome, interacts with HTLV-1 long terminal repeat indirectly via binding to CREB, which binds to TRE.32,33 Furthermore, Tax was known to stabilize CREB/DNA complexes and to enhance the binding affinity of CREB to TRE sites.32,34 Biochemical experiments have demonstrated that, in the presence of Tax, CREB binds to the TRE in a phosphorylation-independent manner,35,36 thereby serving as a cofactor for Tax activation of HTLV-1 transcription. However, in the
Figure 4. The TRE site located from −924 to −921 of the eNOS promoter is identified as a pCREB binding sequence responsible for hypoxia-stimulated eNOS transcription. Based on a Genomatix database search, 5 putative sequences for CREB binding (4 TREs and 1 CRE) within the human eNOS promoter are found; consensus motifs of CRE and TREs are italicized (A). Relative luciferase activity of each of the constructs in the BAECs after transfection was determined, and data are presented as described in Figure 3 (n=3; B). Sense sequences of the double-stranded probes for TRE3 and mTRE3 sites in the eNOS gene promoter region used in the EMSA are shown. The mutation site (A→G at −922) in bold is directed by a well-known SNP site identified by several genomic epidemiology studies (C). EMSA and supershift assay were done with TRE3 or mTRE3 probes as described in Figure 3 (D). The relative luciferase activities of pGL2-eNOS(−962) and pGL2-m-eNOS(−962) in the BAECs after transfection with or without A-CREB were determined, and data are presented as described in Figure 3 (n=3; E).
absence of Tax, as shown in uninfected mammalian cells, CREB binds to the CRE sites only after its phosphorylation via the cAMP-dependent PKA signaling pathway. In this regard, it was expected that pCREB should bind to the CRE within the eNOS promoter. Unexpectedly, however, our data showed that pCREB induced by hypoxia bound to the TRE but not CRE (Figures 3D and 4D). These data strongly suggest that Tax may not be involved in our current system. Although CREB was reported to bind equally to the TRE at normoxia and hypoxia in virus-infected cells, to our knowledge, our study is the first to show that pCREB binds to the TRE in uninfected mammalian cells. Nonetheless, it is worthwhile to test whether there are other unknown Tax-like protein(s) in relation to TRE-mediated eNOS gene regulation in BAECs; such a test, however, is beyond the scope of our study.

The finding in our study of a specific TRE site responsible for hypoxia-stimulated eNOS gene transcription had not been observed by 2 recent reports. At present, the reasons for these incompatible results have yet to be clarified. Authors of 2 previous reports stated in their reports that they constructed and used an eNOS promoter sequence that was obtained either from GeneBank Accession No. AC09246611 or from 2 previously published reports. Interestingly, however, it was found that the eNOS promoter sequence presented from GeneBank or previously published reports was a mutant eNOS gene sequence containing a well-known single nucleotide polymorphism (SNP) site (A→G mutation at −922). In this regard, it should be noted that this SNP loses the TRE consensus motif. We further tested our result by EMSA and found that pCREB did not bind to the mutant TRE3 site (Figure 4D), suggesting that the consensus motif (TGAC) of the TRE3 region in the eNOS gene promoter might play an important role in interacting with pCREB. From this result, our data demonstrate, for the first time, that an A→G SNP site at −922 of the eNOS gene may be another important functional locus for regulating eNOS gene expression, in addition to a T→C mutation at −786. Several studies have found previously that the repressed transcription of the eNOS gene is linked to a T→C SNP at −786, which results in an increased risk of cardiovascular disease. Thus, a T→C mutation at −786 has already been recognized as the most important functional locus for eNOS gene regulation. Although this SNP was reported to be in linkage disequilibrium with 2 other SNPs (A→G SNP at −922 and T→A SNP at −1468), further study will be needed to clarify this issue.

**Perspectives**

Our results shed light on the molecular mechanisms underlying hypoxia-stimulated eNOS gene transcription, which implicates pCREB-TRE mediation. This study extends our understanding of the mechanisms involved in NO regulation by other cardiovascular risk factor(s). It was reported previously that cigarette smoking, known as an important risk factor in cardiovascular disease, decreased eNOS gene expression in endothelial cells and decreased the amount of lung cAMP, an upstream molecule of PKA/pCREB-mediated signaling pathway. The identification of TRE site in the eNOS gene, thus, may hold a promise in unraveling cigarette smoking–induced vascular dysfunction. Finally, our results underscore the potential of the TRE site as a novel therapeutic target for the design of drugs aimed at improving endothelial function in NO dysregulation–related cardiovascular disease, such as hypertension.

**Acknowledgments**

This work was supported in part by the Korea National Institute of Health intramural research grant (348-6111-213-000-207) awarded to I.J. and by the Biomedical Brain Research Center grant from the Ministry of Health and Welfare (0405NS01-0704-0001) awarded to S.J.

**References**


18. Niwano K, Arai M, Tomaru K, Uchiyama T, Ohyama Y, Kurabayashi M. Transcriptional stimulation of the eNOS gene by the stable prostacyclin analogue beraprost is mediated through cAMP-responsive element in...
vascular endothelial cells: Close link between PGI\textsubscript{2} signal and NO pathways. Circ Res. 2002;93:523–530.
Hypoxia-Induced Endothelial NO Synthase Gene Transcriptional Activation Is Mediated Through the Tax-Responsive Element in Endothelial Cells

Jiho Min, Yoon-Mi Jin, Je-Sung Moon, Min-Sun Sung, Sangmee Ahn Jo and Inho Jo

Hypertension. 2006;47:1189-1196; originally published online May 1, 2006; doi: 10.1161/01.HYP.0000222892.37375.4d

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/47/6/1189

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2006/05/01/01.HYP.0000222892.37375.4d.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Methods

Cell Culture, Transfections, and Hypoxia Treatment

Bovine aortic endothelial cells (BAEC) were isolated exactly as previously described\textsuperscript{1} and maintained in MEM supplemented with 5\% NCS at 37\(^\circ\)C under 5\% CO\textsubscript{2} – 20\% O\textsubscript{2} air. The endothelial cells were evaluated by their typical cobblestone configuration when viewed by light microscopy and by a positive indirect immunofluorescence test for the von Willebrand factor VIII complex. Cells between passages 5 and 7 were used for this experiment. Before various treatments, BAEC were further incubated for 24 hours in MEM supplemented with 0.5\% NCS.

For transfection, BAEC were transferred to 6-well plates and allowed to grow until they were approximately 60\% confluent, at which time they were transfected with pGL2-eNOS(−1600), pGL2-eNOS(−1400), pGL2-eNOS(−962), pGL2-eNOS(−873), pGL2-eNOS(−428), and pGL2-m-eNOS(−962) (3 \(\mu\)g each) using Lipofectin (Invitrogen Inc., Carlsbad, CA) according to the manufacturer’s instructions. In a separate experiment, BAEC were also transfected with 3 \(\mu\)g dominant-negative inhibitor of CREB, termed A-CREB,\textsuperscript{2} using Lipofectin. In all transfection experiments, BAEC were cotransfected with 3 \(\mu\)g \(\alpha\)-actin-driven \(\beta\)-galactosidase expression plasmid for normalization of transfection efficiency. After transfection, the transfectants were further grown for 24 hours, washed three times with magnesium- and calcium-free phosphate-buffered saline (PBS), and lysed directly on each plate by the addition of 100 \(\mu\)L of 1 \(\times\) reporter lysis buffer (Promega Co., Madison, WI). After complete lysis, the lysates were centrifuged at 14,000 \(\times\) g for 1 min, and 10 \(\mu\)L of supernatant were
subjected to the luciferase assay. Luciferase activity of the transfectants was measured using a Luciferase Assay System (Promega) as previously described, and α-actin-driven β-galactosidase activity was measured using a β-Galactosidase Enzyme Assay System (Promega). All data were normalized as relative light units/β-galactosidase activity.

For hypoxia treatment, BAEC cultured at 80% confluency were subjected to either hypoxia or normoxia for indicated times, respectively. The hypoxia was achieved as previously described using a hypoxia chamber (COY Laboratory Products, Inc., Hypoxic Glove Box, MI), which maintained a humidified atmosphere with a low oxygen tension (5% CO₂ – 1% O₂).

**Measurement of NO Release**

NO production by BAEC was measured as the nitrite (a stable metabolite of NO) concentration in cell culture supernatants, as described in previous studies, with minor modifications. Briefly, after the cells were treated with 1% O₂ for the indicated times, the culture medium was changed to the Kreb's solution (pH 7.4, 1.5 mL/60 mm dish), which contained 118 mmol/L NaCl, 4.6 mmol/L KCl, 27.2 mmol/L NaHCO₃, 1.2 mmol/L MgSO₄, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, and 11.1 mmol/L glucose, and was equilibrated for 1 hour at 37°C. At the end of the incubation, 200 μL of each supernatant (in Kreb's solution) were carefully transferred into a 96-well plate with the subsequent addition of 100 μL of Griess reagent (50 μL of 1% sulfanilamide containing 5% phosphoric acid and 50 μL of 0.1% N-(1-naphthyl)ethylenediamine). After the development of color at room temperature for 15 min, the absorbance was measured on
a microplate reader at a wavelength of 530 nm. Each sample was assayed in triplicate wells, and a calibration curve was plotted using known amounts of sodium nitrate solution. With this protocol, the measured values represent the amounts of NO produced by the cells during the 1 hour incubation in the Kreb's solution following hypoxic-condition treatment of a specified duration. Therefore, subsequent NO production was solely dependent on eNOS activity at the end of these treatments.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts from BAEC with or without hypoxia treatment were isolated by the method of Lee et al.6 The sequences of the sense strands of double-stranded oligonucleotides used as probes and competitors in the EMSA were as follows, with the consensus motifs of CRE and TRE3 underlined, and mutation of wild-type TRE3 sequence in boldface: The CRE probe (sense strand, with consensus motif underlined) used was 5’-CGGGAAGCGTGCGTCACTGAATGAC-3’ (Figure 3C). At this moment, it should be noted that the CRE sequence indicated in the eNOS gene is not a “classical” palindromic DNA sequence (TGACGTCA) of CRE but it has been widely used as a putative CRE sequence in the previous studies.7,8 In a separate experiment, a positive control for CRE probe with a classical palindromic DNA sequence of CRE (underlined) used was 5’-AGAGATTGCCTGACGTCAGAGGCTAG-3’.9 The wild-type TRE3 probe used was 5’-CCAGCCCCTCAGATGACAGAACTACAAAC-3’ (Figure 4C) and mutant TRE3 (mTRE3, with mutation sequence in boldface) was 5’-CCAGCCCCTCAGATGGCACAGAACTACAAAC-3’(Figure 4C). For the mutation of TRE3 sequence, the site-specific mutation (A →G) at −922 of the eNOS promoter was directed from the discovery of eNOS gene single nucleotide polymorphic sites.10,11
Three µg of nuclear extract were then incubated at 20°C for 15 min with 1 µg of poly(dI-dC) (Amersham Pharmacia Biotech., Uppsala, Sweden) plus the [P³²]ATP-labeled oligonucleotide (0.1 pmol, ~15,000 c.p.m.). In the competition studies, excess wild-type and mutant double-stranded oligonucleotides were added in 10-fold molar excess prior to the addition of the [P³²]ATP-labeled probe. The binding reaction was carried out in solution containing 20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 5 mmol/L MgCl₂, 10% glycerol, and 1 mmol/L dithiothreitol. The reaction mixtures (final volume, 20 µL) were then directly loaded onto 5% non-denaturing polyacrylamide gels (29:1, acrylamide:bisacrylamide) containing 2.5% glycerol in 1 × low ionic strength buffer (7 mmol/L Tris-HCl, pH 7.5, 3 mmol/L sodium acetate, and 1 mmol/L EDTA) that had been pre-electrophoresed for 20 min. After electrophoresis (120 V for 2.5 hours at room temperature), the gels were dried and autoradiographed with an intensifying screen. Monoclonal anti-pCREB antibody was also added to the reaction mixture to supershift pCREB.

**Western Blot Analysis**

For western blot analysis, cells in the absence or presence of various chemicals were washed with ice-cold PBS and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, and 1 mmol/L Na₃VO₄) containing 1 × Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN). Protein concentrations were determined with the BCA protein assay kit (Sigma). Equal quantities of protein (30 µg) were separated on sodium dodecyl
sulfate-polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibody directed against eNOS (1:1000), HIF-1α (1:1000), CREB (1:1000), and pCREB (1:1000), followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence reagents (ECL, Amersham, DE). The level of actin expression was used as an internal control.

**Real-Time Reverse Transcription–PCR**

The levels of eNOS mRNA were quantified by real-time reverse transcription (RT)-PCR using a SYBR® Green PCR Master Mix (ABI 7700, Applied Biosystems, CA) as previously described\(^\text{12}\), with minor modifications. In brief, the total RNA from BAEC cultured under hypoxia or normoxia for 24 hours was extracted using TRIzol reagent (Gibco-BRL) according to the manufacturer’s instructions. Reverse transcription was first performed to synthesize cDNA using total RNA (0.5 µg), oligo-dT (16mers) (Bioneer Co., Daejeon, Korea) and dNTPs (1 mmol/L), 4.5 units of AMV reverse transcriptase (Promega Co.), and 20 units of RNase inhibitor (Promega Co.). cDNA corresponding to 50 ng of RNA was then added to the SYBR-Green AmpliTaq® Master Mix and 0.9 µmol/L of each specific primer in a total volume of 50 µL. The primer pairs for eNOS cDNA are as follows: sense 5’-ATCTCCGCCTCGCTCATG-3’ and antisense 5’-TCGGAGCCATACAGGATTGTC-3’. Amplification of GAPDH cDNA was also performed in a separate tube to enable normalization using the following primers: sense 5’-AATTCATGGCACCCTGCAAG-3’ and antisense 5’-TCTCGCTCCTGGAAGATGGT-3’. PCR was carried out in a real-time PCR cycler (ABI 700, Applied Biosystems, CA). The thermal cycling condition was 48°C for 30
min, 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. At the end of each phase at 60°C, the fluorescence was measured and used for quantitation. Each sample was analyzed in triplicate.

References


Transcriptional stimulation of the eNOS gene by the stable prostacyclin analogue beraprost is mediated through cAMP-responsive element in vascular endothelial cells: Close link between PGI2 signal and NO pathways. *Circ Res.* 2002;93:523-530.


