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

Kazuo Niwano, Masashi Arai, Koichi Tomaru, Tsuyoshi Uchiyama, Yoshio Ohyama, Masahiko Kurabayashi

Abstract—Beraprost sodium (BPS), an orally active prostacyclin analogue, has been reported to be beneficial in the treatment of primary pulmonary hypertension and obstructive peripheral arterial disease. Although BPS was originally described for its effects on platelet aggregation and vasodilatory response, the effect on endothelial cells has been poorly understood. In this study, we examined the effects of BPS on the eNOS gene expression in mouse aorta and cultured human and bovine aortic endothelial cells. Treatment of these cells with BPS increased the eNOS expression as assessed by Northern blots, Western blots, and NO production by NO-specific fluorescence (DAF2-DA) and by the Griess method. Standard mRNA decay assays showed that BPS increases the stability of eNOS mRNA. In addition, BPS increased the promoter activity of the human eNOS gene, as determined by luciferase assays of the eNOS promoter gene. Progressive 5'-deletion and site-specific mutation analyses defined the BPS-responsive sequences as cAMP-responsive elements (CRE) located at −733 and −603. By using the oligonucleotide probe containing this CRE sequence in electrophoretic mobility shift assays, we showed that the phosphorylated form of CRE-binding protein is a major constituent of the complex in BPS-treated cells. Western blot analyses indicate that BPS but not endogenous prostacyclin phosphorylates CRE-binding protein. The presence of functional CRE sites within human eNOS promoter may represent a novel mechanism for regulating eNOS gene expression. (Circ Res. 2003;93:523-530.)

Key Words: endothelium ▪ prostaglandins ▪ nitric oxide ▪ nitric oxide synthase

Endothelial cells exert their vasoprotective functions through the biosynthesis and release of nitric oxide (NO), prostacyclin (PGI₂), prostaglandin E₂ (PGE₂), carbon monoxide (CO), and endothelium-derived hyperpolarizing factor. Because decreased bioavailability of NO is directly associated with cardiovascular events, including myocardial infarction and stroke, the level of NO production is considered a major determinant of endothelial performance.

PGI₂ is primarily generated in vascular endothelial cells and plays a crucial role in regulation of local vascular tone and platelet aggregation. PGI₂-induced relaxation of vascular beds is believed to be mediated by activation of the PGI₂ receptor, which leads to elevation of cAMP levels in vascular smooth muscle cells.

Beraprost sodium (BPS), which is a stable PGI₂ analogue, has been reported to be beneficial in the treatment of primary pulmonary hypertension and obstructive peripheral arterial disease. BPS mimics the biological properties of PGI₂, such as activation of adenylate cyclase and increasing intracellular cAMP levels, through activation of the PGI₂ receptor. Owing to its chemical characteristics, BPS is a more stable and more long-lived molecule and has higher affinity to PGI₂ receptor than natural PGI₂. The PGI₂ receptor is expressed in endothelial cells, but the effects of BPS on endothelial cells have not been documented.

Endothelial nitric oxide synthase (eNOS) is an important regulatory enzyme that catalyzes production of NO from arginine. Production of NO in endothelial cells is regulated by several distinct mechanisms. First, eNOS activity is regulated by phosphorylation of its Ser-1177. Multiple protein kinases, including AMP-activated protein kinase, cAMP-dependent protein kinase (protein kinase A [PKA]), Akt/protein kinase B (protein kinase B), and calmodulin-dependent kinase II activate eNOS by phosphorylating its Ser-1177. In endothelial cells, protein kinase C (PKC) inhibits eNOS activity by inducing phosphorylation of its Thr-495 and dephosphorylation of its Ser-1177. Second, intracellular trafficking of eNOS to plasmalemmal caveolae contributes to regulation of eNOS activity. Caveolin, an integral membrane protein that is a key structural component of caveolae, directly interacts with eNOS and inhibits its activity.

Third, in endothelial cells, an increase in intracellular

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From the Second Department of Internal Medicine, Gunma University School of Medicine, Japan.

Correspondence to Masahiko Kurabayashi, MD, PhD, Second Department of Internal Medicine, Gunma University School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. E-mail mkuraba@med.gunma-u.ac.jp

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Ca2+ elicited by diverse extracellular signals leads to activation of eNOS through binding of Ca2+-calmodulin to eNOS.15 Fourth, eNOS expression is regulated at the posttranscriptional level. Exposure of cultured endothelial cells to tumor necrosis factor-α, hypoxia, or a high concentration of oxidized LDL decreases eNOS levels by shortening eNOS mRNA half-life.16–18 Finally, transcription of the eNOS gene is regulated at the transcriptional level through a variety of regulatory elements, including AP-1 and Sp-1.19–21,28,29

In the present study, we tested the hypothesis that BPS stimulates eNOS expression. We found that BPS increased eNOS expression both in vivo and in vitro. By analyzing the human eNOS promoter using cultured aortic endothelial cells, we identified a functional cAMP-responsive element (CRE) within the eNOS gene promoter. This CRE mediates the transcriptional increase in eNOS gene expression induced by BPS. In addition, we found that BPS increases eNOS mRNA stability.

Materials and Methods

Reagents

BPS was obtained from Toray Co, Kanagawa, Japan. Cell culture reagents and FCS were obtained from Life Technologies. PGI2, forskolin, dideoxy-forskolin, L-NAME (nonspecific NOS inhibitor), KT5720 (PKA inhibitor), 4-cyano-3-methylisoquinoline (4C3M, PKC inhibitor), wortmannin (phosphatidylinositol-3 kinase inhibitor), protein phosphatase-1 (PP1, tyrosine kinase inactivator), PD98059 (MEKI inhibitor), and SB203580 (p38 mitogen-activated protein kinase inhibitor) were purchased from Dai-ichi Chemical Co.22

Animals

Male ICR mice (30 g, 4 weeks old, n = 30) were given either distilled water or distilled water containing 12 mg/kg per day BPS.23 All protocols involving experimental animals were in accordance with the institutional guidelines for animal care of the Gunma University School of Medicine. BPS did not affect the weight of the mice. At various times after the beginning of oral administration of BPS, mice were killed and their aortas were prepared for isolation of mRNA and protein.

Cell Culture

Bovine aortic endothelial cells (BAECs) were isolated from the intima of the descending bovine aorta, as described elsewhere.24,25 They were cultured at 37°C in RPMI-1640 medium supplemented with 10% FCS, 50 μM penicillin, and 50 μg/mL streptomycin. Cells from the third passage were used for experiments. The cells were plated on 10-cm plastic dishes at a density of 106 cells per dish.26,27 Human aortic endothelial cells (HAECs) (second passage) were purchased from Cell Application, Inc.26 They were cultured in basal medium, and the third or forth passages of cells were used for experiments. The cells were plated on type I collagen-coated 10-cm plastic dishes at a density of 106 cells per dish. HAECs and BAECs were exposed to BPS, PGI2, forskolin, and dideoxy-forskolin under various conditions.

RNA Extraction and Northern Blot Analysis

Northern blot analysis was performed as described elsewhere.26,27 Total cellular RNA (15 μg) was isolated from BAECs and mouse aorta. Radiolabeling of the probes, a 384-bp fragment of bovine eNOS cDNA, a 535-bp fragment of mouse eNOS cDNA, a 233-bp fragment of human PAI-1 cDNA, and a 420-bp fragment of bovine ET-1 cDNA was performed according to the protocol of the labeling kit manufacturer (Amersham).26

mRNA Decay Assay

To determine whether increases in eNOS mRNA levels are attributable to activation of eNOS gene transcription or to increased eNOS mRNA stability, we performed a standard mRNA decay assay with actinomycin D (the transcriptional inhibitor) in the absence or presence of BPS.26 The total RNA was prepared at different time points thereafter. eNOS mRNA level at the time of addition of actinomycin D (0 hours) was set at 100%. In BPS-treated cells, the rate of eNOS mRNA decay was compared with control cells.

Promoter-Luciferase Vector and Luciferase Assay

The human eNOS gene promoter (nt −1600/nt +21 bp) is described elsewhere.28,29 5′-Deletion mutagenesis constructs of the eNOS gene promoter at nt −1395, −793, −743, −673, −613, −530, and −123 were produced by polymerase chain reaction. Constructs with site-specific mutations at −733 (TGCCACA) and −603 (AATCACA) were also produced. Potential responsive elements within the eNOS promoter were analyzed using previously published methods and the TFSERCH program.30

Protein Kinase Inhibitor Assay

We used various protein kinase inhibitors to determine which intracellular signal transduction pathways mediate the effects of BPS on the eNOS gene.26,27 The eNOS mRNA levels in protein kinase inhibitors with BPS were compared with the same protein kinase inhibitors without BPS. BPS-mediated eNOS mRNA expression did not increase, because a specific protein kinase inhibitor blocked BPS-mediated signal pathway.

Western Blot Analysis

Western blot analysis was performed as described elsewhere.27,31,32 Whole protein extracts (30 μg) were prepared from BAECs and mouse aorta. Mouse anti-eNOS monoclonal antibodies, rabbit anti-mouse eNOS polyclonal antibody (Transduction Laboratories), rabbit anti-CREB polyclonal antibody, and rabbit anti-phospho-CREB (Ser-133) polyclonal antibody (New England Biolabs) were visualized with anti-mouse or anti-rabbit IgG secondary antibody using the ECL system (Amersham) as described elsewhere.31,32

Nitric Oxide Production in BAECs

Production of intracellular NO was determined using DAF-2/DA (10 μmol/L). DAF-2/DA is a cell-permeable compound that is converted to DAF-2 by intracellular esterases in the presence of NO. A triazole derivative of DAF-2 emits light at 515 nm on excitation at 489 nm; the intensity of this light is proportional to the amount of NO present.22,23 Production of nitrite and nitrate in culture was measured using the Griess method, as described elsewhere.24

Electrophoretic Mobility Shift Assay

Nuclear extracts (20 μg) were prepared from BAECs as described elsewhere.27,29,32 The sequences of the sense strands of double-stranded oligonucleotides used as probes or competitors in the electrophoretic mobility shift assay (EMSA) were as follows, with the consensus motif underlined and mutations of wild-type sequences in boldface: CRE1, 5′-CCCCGGGAAACGTGGTGTACTGAATGACAGG-3′; CRE2, 5′-AAGGGATACCTATGGCGATCAAGGAGCA-3′; mCRE1, 5′-CCCCGGGAAACGTGGTGTACTGAATGACAGG-3′; and mCRE2, 5′-AAGGGATACCTATGGCGATCAAGGAGCA-3′.

Rabbit anti-CREB antibody and rabbit anti-phospho-CREB (Ser-133) antibody (Upstate Biotechnology) were used for super shift assays, as described elsewhere.32

Statistical Analysis

Data were expressed as mean ± SEM. Significance of differences within groups was assessed using one-way ANOVA. Significance of differences between groups was assessed using the Bonferroni test.
Results

BPS Increases eNOS mRNA and Protein Levels In Vivo and In Vitro

In mouse aorta, levels of both eNOS mRNA and protein significantly increased at 12 hours after BPS administration, and they remained elevated at 72 hours (Figure 1A). In cultured BAECs exposed to 5 μmol/L BPS for 12 hours, eNOS mRNA levels significantly increased whereas ET-1 and PAI-1 mRNA levels were not measurably changed (Figure 1B), suggesting that BPS selectively increased eNOS mRNA levels. Results showed that BPS exposure significantly increased eNOS mRNA levels in a dose- and a time-dependent manner. Also, levels of eNOS protein were significantly increased by BPS exposure in a time- and a dose-dependent manner (Figure 1C).

BPS, but not PGI₂, significantly increased eNOS mRNA levels in HAECs in a dose-dependent manner. Likewise, forskolin (adenylate cyclase activator), but not dideoxy-forskolin (an inactive analogue of forskolin), upregulated eNOS mRNA and protein levels in a dose-dependent manner, but the degree of induced eNOS expression was much less than that induced by BPS (Figures 2C and 2D). We obtained the same results from a series of parallel experiments performed using BAECs (data not shown).

BPS Increases Both eNOS mRNA Stability and Transcription of the eNOS Gene

Results of the mRNA decay assay are shown in Figure 3. The half-life of eNOS mRNA, but not GAPDH mRNA, was significantly increased by BPS (6.3 versus 12.4 hours).

The luciferase assay showed that BPS and forskolin increased luciferase activity driven by the sequence containing 1.6 kb of the 5′-flanking region of the human eNOS gene (which we refer to as −1600eNOS-Luc) in a dose-dependent manner in HAECs and BAECs.

Using several overlapping clones of the 5′-flanking region of the eNOS promoter, we mapped a BPS-responsive se-
sequence downstream of −743, relative to the transcription start site in BAECs (Figure 4B). The schematic diagram we constructed of the eNOS promoter region (between −1600 and +21) with the above-described regulatory elements is shown in Figure 4B. CRE sequences are located at −733 and −603; we refer to these as CRE1 and CRE2, respectively (Figure 4B).

**BPS-Induced eNOS mRNA Expression Is Mediated Through the PKA Pathway**

Results of the protein kinase inhibitor assay are shown in Figure 5. The PKA inhibitors KT5720 and 4C3M significantly attenuated BPS-induced increases in eNOS mRNA levels in BAECs. In contrast, wortmannin (phosphatidylinositol-3 kinase inhibitor), PP1 (tyrosine kinase inactivator), PD98059 (MEK1 inhibitor), and SB203580 (p38 mitogen-activated protein kinase inhibitor) had no measurable effects on BPS-induced increases in eNOS mRNA levels. Calphostin C (PKC inhibitor) increased levels of eNOS mRNA in the absence of BPS; thus, we could not determine the effects of calphostin C on BPS-induced increases in eNOS mRNA expression.

**Transcription of the eNOS Gene Is Activated Through PKA- and CRE-Mediated Pathway**

To determine whether the CRE sequence and the PKA pathway were involved in BPS-induced increases in transcription of the eNOS gene in BAECs, we tested the effects of the PKA inhibitors KT5720 and 4C3M on the activity of −1600eNOS-Luc. Treatment of cells with KT5720 or 4C3M almost completely abolished BPS-induced luciferase activity of −1600eNOS-Luc (Figure 6A).

Next, to test the importance of CRE sequences within the eNOS promoter, we introduced site-specific mutations within CRE1 and CRE2 and performed transient transfection assays in BAECs. The construct containing a mutation in CRE1 in the context of −743eNOS-Luc [which we designated as −733(mCRE1)eNOS-Luc] showed lower luciferase activity in the absence of BPS compared with the wild-type construct −743eNOS-Luc. More importantly, the BPS-induced increase in luciferase activity was markedly reduced in −733(mCRE1)eNOS-Luc. The construct −603(mCRE2)eNOS-Luc, which contains a mutation in CRE2 in the context of −613eNOS-Luc, was unresponsive to BPS (Figure 6B).

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**Figure 3.** BPS increases eNOS mRNA half-life in BAECs. A, Time course of eNOS and GAPDH mRNA levels with actinomycin D (5 μg/mL) with or without BPS (10 μmol/L). B, Densitometric analysis of eNOS mRNA levels, which were normalized to 28S RNA. Experiments were replicated 3 times with similar results.

**Figure 4.** BPS increases transcription of the eNOS gene in HAECS and in BAECs. A, Human eNOS gene promoter (−1600 bp/+21 bp) activities were monitored after 24 hours of various treatments of PGI2, BPS, forskolin, and dideoxy-forskolin (10 or 20 μmol/L) in HAECS and in BAECs. Luciferase activities are expressed relative to that of the eNOS gene promoter (−1600 bp/+21 bp) without various treatments, which is designated as 1.0. Data are expressed as mean±SEM (ANOVA; *P<0.05 vs control conditions [−], n=3 cells). B, 5′-Deletion analysis of the eNOS gene promoter with or without BPS (10 μmol/L) for 48 hours in BAECs. Luciferase activity is expressed relative to that of the eNOS gene promoter (−1600 bp/+21 bp) with vehicle, which is designated as 1.0. Mapping of the BPS-responsive elements is indicated. Data are reported as mean±SEM (ANOVA; *P<0.05 vs basal condition [BPS, −], n=6 cells).

**Figure 5.** Effects of protein kinase inhibitors on BPS-induced increases in eNOS mRNA levels in BAECs. eNOS and GAPDH mRNA levels are shown after 24 hours of treatment with calphostin C (Cal.C, 0.5 μmol/L), KT5720 (1 μmol/L), 4C3M (12 μmol/L), wortmannin (Wor, 100 μmol/L), PP1 (10 μmol/L), PD98059 (PD, 50 μmol/L), or SB203580 (SB, 10 μmol/L).
These results suggest that both CRE1 and CRE2 play an indispensable role in BPS-induced increases in eNOS promoter activity.

CREB Phosphorylation Is Induced by BPS

To determine the nuclear factors that bind to CRE1 and CRE2, we performed EMSAs using nuclear extracts from BAECs and labeled probes containing either CRE1 or CRE2. Incubation of nuclear protein with CRE1 or CRE2 gave rise to a prominent DNA/protein complex, C1 or C2, respectively. Both complexes were shown to be sequence-specific because their formation was inhibited by molar excess of unlabeled wild-type oligonucleotides but not by oligonucleotides containing mutations within the CRE sequence (mCRE1 and mCRE2, respectively) (Figure 7A). The supershift assay showed that both C1 and C2 contain CREB, and binding of the phosphorylated form of CREB was significantly increased by BPS treatment (Figures 7A and 7B). In addition, in Western blot analysis, the phosphorylation of CREB induced by BPS was first detected at 0.5 hours after BPS treatment, peaked at 1 hour, and gradually declined thereafter. This change in phosphorylation of CREB was somewhat different from that observed in cells treated with forskolin (a direct activator of adenylate cyclase), in which CREB was more rapidly phosphorylated and dephosphorylated (Figure 7C). Phosphorylation of CREB induced by BPS was mediated by PKA, because KT5720 and 4C3M, but not calphostin C, completely blocked the effects of BPS (Figure 7D).

BPS Stimulates NO Production Through the PKA Pathway

The DAF2/DA assay showed that BPS significantly increased levels of intracellular NO, whereas L-NAME decreased intracellular NO production in BAECs (Figure 8A). In addition, nitrite and nitrate levels in culture medium were significantly increased by BPS, whereas L-NAME decreased nitrite and nitrate production (Figure 8C). BPS-induced increase in NO, nitrite, and nitrate was attenuated by the PKA inhibitors KT5720 and 4C3M (Figures 8B and 8C).

Discussion

This study was designed to investigate the effects of BPS on eNOS expression and its underlying molecular mechanisms. We demonstrated that BPS increases the steady-state levels of eNOS mRNA and protein in mouse aorta and in cultured HAECs and BAECs whereas PGI2 had little effect on the eNOS gene expression. We also demonstrated that BPS...
Mechanisms of cAMP-Mediated NO Production

The finding that BPS increases production of NO in endothelial cells deserves particular attention. In contrast to the factors that dilate vessels via endothelial NO production (e.g., shear stress, acetylcholine, and bradykinin), many classic cAMP-mediated vasodilators (including isoproterenol, PGI₂, adrenomedullin, and adenosine) are widely believed to exert their vasorelaxant effects via receptor-mediated activation of adenylate cyclase, formation of cAMP, and activation of PKA (and deactivation of myosin light chain kinase in vascular smooth muscle cells).  Consequently, it is not clear whether BPS activates phosphodiesterase (PDE) to deplete cyclic AMP, which would prevent further cAMP-mediated vasodilation. However, growing evidence indicates that inhibition of eNOS impairs the relaxation response to these vasodilators. Thus, cAMP-mediated vasodilation is generally considered to be endothelium-independent. However, growing evidence indicates that inhibition of eNOS impairs the relaxation response to these vasodilators. Consequently, it has been suggested that complex interactions between NO and cAMP in blood vessels are involved in control of vascular tone. Zhang et al recently described the effects of cAMP-mediated vasodilators on NO production in aortic microvessels. They showed that forskolin, isoproterenol, and 8-bromo-cAMP increased release of nitrite from endothelium via phosphorylation of eNOS rather than expression of eNOS mRNA or protein. Although we did not address the effects of BPS on eNOS activity, it is likely that cAMP-mediated NO production is regulated by diverse intracellular mechanisms whose contributions vary depending on the experimental conditions.

In contrast to studies reporting no effects of cAMP-PKA pathways on the eNOS transcription in HUVECs, we found the significant effects on eNOS transcription in HUVECs and BAECs. Our observation may be attributed to the use of the aortic endothelial cells of low passage numbers, because we failed to see the BPS response when we used the higher passage number of endothelial cells.

We examined the mechanisms of BPS-mediated eNOS gene expression. Despite the role of cAMP in inducing NO production, few studies have demonstrated cAMP-mediated transcriptional regulation of the eNOS gene. Although a sequence similar to CRE has been identified in the human eNOS promoter, no functional studies of this sequence have been reported. Indeed, we demonstrated that BPS activated transcription of the eNOS promoter/reporter gene via CRE sequences located at −733 and −603. We verified that BPS induces phosphorylation of CREB in Western blot analysis and EMSA using antiphosphorylated CREB (Ser-133) antibody. These results imply that induction of NO biosynthesis can be modulated by a variety of extracellular stimuli that activate cAMP/PKA signal transduction.

Interpretation of the effects of BPS on eNOS expression in mice aorta is limited by the possibility that eNOS expression levels in aorta vary depending on hemodynamic conditions, which are affected by several factors, including water intake, blood pressure, and body weight. The primary focus of the present study was to determine the molecular mechanism of BPS-induced increases in NO production in endothelial cells; this study was not designed to determine whether BPS-induced eNOS expression is secondary to alteration of hemodynamic conditions. Thus, one must be careful when extrapolating the in vitro effects of BPS on endothelial cells to the vasculature in vivo.

It has been reported that eNOS is phosphorylated at Ser-1177 by AMP-activated protein kinase and Akt/protein kinase B, which are serine/threonine protein kinases that can
be phosphorylated and activated by cAMP.\textsuperscript{12} The present results show that additional regulatory mechanisms control eNOS expression via cAMP signaling. To date, functional CRE has been found in a few endothelial genes, including thrombomodulin and tissue-type plasminogen activator.\textsuperscript{35,36} Thrombomodulin and tissue-type plasminogen activator can play a vasoprotective role by inhibiting the coagulation cascade and enhancing fibrinolysis, respectively. Given the present results and these previous observations, we speculate that CREB plays a key role in development of a wide range of vasoprotective effects. Indeed, a recent study of ours suggests that downregulation of eNOS expression by inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α is eliminated by phosphorylation of CREB (data not shown).

**Clinical and Physiological Implications**

It is worthwhile to note that PGI\textsubscript{2} has no effect on eNOS expression in HAECS and BAECs, although PGI\textsubscript{2} analogue BPS has significant effect. This result is probably attributable to the short-lived or unstable property of PGI\textsubscript{2} in culture medium compared with BPS. Thus, the NO-producing properties of endogenous PGI\textsubscript{2} warrant additional evaluation in vivo. Although continuous intravenous infusion of PGI\textsubscript{2} has been proven to be effective for the treatment of pulmonary hypertension, this treatment is associated with serious complications arising from the complex delivery system. Recent clinical trials indicate that BPS improves exercise capacity and symptoms in patients with pulmonary arterial hypertension.\textsuperscript{10} This evidence may be formally ascribed to the ability of BPS to dilate the vessels and to inhibit the platelet aggregation and thrombus formation. On the basis of the results of this study, however, it is tempting to speculate that an increase in NO production may contribute to the beneficial effects of BPS in clinical settings.

**Conclusions**

The present results indicate that expression of the eNOS gene is increased by BPS at the transcriptional and posttranscriptional levels in vascular endothelial cells and that BPS-induced increase in eNOS transcription is selectively mediated by interaction between CRE and CREB. Identification of the regulatory mechanisms by which cAMP increases NO production in endothelial cells may provide new insight into the role of cAMP-elevating drugs in patients with vascular disease. The clinical efficacy of BPS may be partly mediated by stimulation of PGI\textsubscript{2} signaling and NO pathways in endothelial cells.

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