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Prostaglandin E_2 stimulates angiogenesis by activating the nitric oxide/cGMP pathway in human umbilical vein endothelial cells

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Abbreviations: Akt, protein kinase B; COX, cyclooxygenase; DB-cAMP, dibutyryl cyclic AMP; DB-cGMP, dibutyryl cyclic GMP; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; ODQ, 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalon-1-one; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PKI, myristoylated protein kinase A inhibitor amide 14-22; Wort, wortmannin

Abstract

Prostaglandin E_2 (PGE_2), a major product of cyclooxygenase, has been implicated in modulating angiogenesis, vascular function, and inflammatory processes, but the underlying mechanism is not clearly elucidated. We here investigated the molecular mechanism by which PGE_2 regulates angiogenesis. Treatment of human umbilical vein endothelial cells (HUVEC) with PGE_2 increased angiogenesis. PGE_2 increased phosphorylation of Akt and endothelial nitric oxide synthase (eNOS), eNOS activity, and nitric oxide (NO) production by the activation of cAMP-dependent protein kinase (PKA) and phosphatidylinositol 3-kinase (PI3K). Dibutyryl cAMP (DB-cAMP) mimicked the role of PGE_2 in angiogenesis and the signaling pathway, suggesting that cAMP is a down-stream mediator of PGE_2. Furthermore, PGE_2 increased endothelial cell sprouting from normal murine aortic segments, but not from eNOS-deficient ones, on Matrigel. The angiogenic effects of PGE_2 were inhibited by the inhibitors of PKA, PI3K, eNOS, and soluble guanylate cyclase, but not by phospholipase C inhibitor. These results clearly show that PGE_2 increased angiogenesis by activating the NO/cGMP signaling pathway through PKA/PI3K/Akt-dependent increase in eNOS activity.

Keywords: cyclic AMP; endothelial cells; endothelial nitric oxide synthase; prostaglandin E_2

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vessels, which enables the delivery of oxygen and nutrients and is strongly regulated in many physiological and pathological conditions. The angiogenic process is complex and involves several discrete steps such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (Folkman *et al.,* 1992; Hanahan *et al.,* 1996). Extensive interest has focused on the elucidation of the cellular and molecular mechanisms involved in the angiogenic process of endothelial cells in response to angiogenic stimuli.

Nitric oxide (NO), synthesized from L-arginine by the catalytic reaction of nitric oxide synthase (NOS), is a gaseous molecule with an astonishingly wide range of physiological and pathophysiological activities, including the regulation of vessel tone and angiogenesis in wound healing, inflammation, ischemic cardiovascular diseases, and malignant diseases. Many studies have revealed the predominant role of endothelial NOS (eNOS) in both vasculogenesis and angiogenesis (Fukumura *et al.,* 2001). In addition, successes in gene therapy, together with the administration of an eNOS-specific inhibitor (Chen *et al.,* 1997; Gratton *et al.,* 2003), suggest that the regulation of eNOS-dependent NO production might be a key factor for the control of physiological or pathological neovascularization.

Prostaglandins (PGs)[1], small lipid molecules, function as lipid mediators in inflammatory processes,

tumor growth, vascular function, and angiogenesis. Their synthesis is initiated from arachidonic acid by the catalytic reaction of constitutive cyclooxygenases-1 (COX-1) and inducible COX-2 to PGG/H2, generating five primary bioactive prostanoids: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxan A_2 by cell-specific isomerases and synthases (Narumiya *et al.,* 1999; Breyer *et al.,* 2001; Harris *et al.,* 2002). PGE_2 is a major product of COX and regulates wound repair (Joyce *et al.,* 1994), cell migration (Joyce *et al.,* 1995), and angiogenesis (BenEzra, 1978; Form *et al.,* 1983). PGE_2 also mediates mitogenesis (Baylink *et al.,* 1996), growth and metastasis of tumors (Yano *et al.,* 1997), and gene transcription (Simonson *et al.,* 1994). These biological actions of PGE_2 are mediated through ligation of four distinct G protein-coupled receptors [E-prostanoid $(EP)_1$, EP_2, EP_3, and EP_4] (Breyer *et al.,* 1999). EP_1 is coupled to intracellular Ca^{2+} mobilization (Regan *et al.,* 1994), EP_2 and EP_4 increase intracellular cAMP concentration (Honda *et al.,* 1993; Regan *et al.,* 1994), and EP_3 mainly inhibits intracellular cAMP accumulation (Sugimoto *et al.,* 1992). Therefore, the biologic functions mediated by PGE_2 may be particularly diverse in different tissues and cells, because these receptors are cell-type specifically expressed.

Extensive studies have indicated that PGE_2 production by COX-2 expression can increase angiogenesis in endothelial cells (Narumiya *et al.,* 1999; Breyer *et al.,* 2001); however, the cellular and molecular mechanisms are poorly understood. In the present study, we investigated the molecular mechanism and signaling pathway by which PGE_2 regulates angiogenic process in cultured human endothelial cells (HUVECs). Our results clearly show that PGE_2 plays a significant role in facilitating angiogenesis by activating the NO/cGMP signaling pathway through PKA/PI3K/Akt-dependent increase in eNOS activity.

Materials and Methods

Chemicals and antibodies

The following agents were purchased: PGE_2, wortmannin, and myristoylated protein kinase A inhibitor amide 14-22 (PKI) from Calbiochem (San Diego, CA); 1H-(1,2,4)-oxadiazole[4,3-a]quinoxalon- 1-one (ODQ) from Tocris (Ellisville, MO); growth factor-reduced Matrigel from BD biosciences (Frankin Lakes, NJ); M199 medium, penicillin, streptomycin, and heparin from Invitrogen life technologies (Carlsbad, CA); antibodies for phospho- $Akt^{(Ser473)}$, Akt, phospho-$eNOS^{(Ser1177)}$, and eNOS from New England Biolabs (Beverly, MA). All other reagents were purchased from Sigma (St. Louis, MO), unless indicated otherwise.

Cell culture

HUVECs were isolated from human umbilical cord veins by collagenase treatment as described previously (Jaffe *et al.,* 1973) and used in passages 3-6. The cells were grown in M199 medium supplemented with 20% fetal bovine serum (FBS; Cambrex, Walkersville, MD), 100 U/ml penicillin, 100 g/ml streptomycin, 3 ng/ml bFGF (Upstate, Waltham, MA), and 5 U/ml heparin at 37°C under 5% CO_2/95% air.

Cell proliferation

Endothelial cell proliferation was determined by DNA synthesis. Briefly, HUVECs were seeded at a density of 2.5×10^4 cells/well in gelatin-coated 24-well plates. Cells were incubated in growth media and allowed to attach for 24 h. Cells were washed twice with M199 and incubated for 6 h with M199 containing 1% FBS. Cells were stimulated with PGE_2, dibutyryl-cAMP (DB-cAMP) or dibutyryl-cGMP (DB-cGMP) for 30 h and followed by the incubation with 1 Ci/ml [^{3}H]thymidine for 6 h. High molecular mass [^{3}H]-labeled radioactivity was precipitated using 5% trichloroacetic acid at 4°C for 30 min. After washing twice with ice-cold H_2O, [^{3}H]-labeled radioactivity was solublized in 0.2 N NaOH containing 0.1% sodium dodecyl sulfate and determined by liquid scintillation counter.

Tube formation assay

The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel was performed as previously described (Lee *et al.,* 1999; Hur *et al.,* 2005). Twenty-four well culture plates were coated with Matrigel according to the manufacturer's instructions. HUVECs were incubated in M199 containing 1% FBS for 6 h, plated onto the layer of Matrigel at a density of 2.5×10^5 cells/well, and followed by the addition of PGE_2, DB-cAMP or DB-cGMP with or without each inhibitor. Matrigel cultures were incubated at 37°C for 20 to 26 h. Tube formation was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. The degree of tube formation was quantified by measuring the length of tubes in 5 randomly chosen low-power fields ($\times$ 100) from each well using the Image-Pro Plus v4.5 (Media Cybernetics, San Diego, CA).

Cell migration assay

Migration assays were performed as previously

described (Lee *et al.,* 1999). Briefly, the chemotactic motility of HUVECs was assayed using Transwell with 6.5 mm diameter polycarbonate filters (8 m pore size). The lower surface of the filter was coated with 10 g of gelatin. The fresh M199 media (1% FBS) containing PGE_2, DB-cAMP, or DB-cGMP were placed in the lower wells. HUVECs were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS. Each inhibitor was incubated with the cells for 30 min at room temperature before seeding. One hundred l of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37°C for 4 h. Cells were fixed and stained with hematoxylin and eosin. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. Chemotaxis was quantified by counting the cell that migrated to the lower side of the filter in low-power fields ($\times$ 100). All fields were counted for each assay.

Western blotting

HUVECs were serum-starved in M199 (1% FBS) for 6 h prior to addition of activators or inhibitors. Cells were scraped off the plates and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate). Cell lysates (50 g protein) were electrophoresed on SDS-PAGE gel and transferred to polyvinyldifluoride membrane. Membranes were incubated with antibodies against eNOS, phospho-eNOS$^{(Ser1177)}$, Akt, and phospho-Akt$^{(Ser473)}$ as previously described (Kim *et al.,* 1997). After incubation, the corresponding secondary antibody signals were detected by the enhanced chemiluminescence reagents.

Measurement of nitrite plus nitrate and eNOS activity

Production of nitrite plus nitrate (NOx) was measured by ozone-chemiluminescence method. Culture media from HUVECs were collected and assayed for NOx using a chemiluminescent NO analyzer (Antek instruments, Houston, TX). Activity of eNOS was assayed by L-[^{14}C]citrulline production from L-[^{14}C] arginine as described previously (Barbacanne *et al.,* 1999). In brief, cell lysates (100 l) were incubated in 50 mM Tris-HCl buffer, pH 7.5, containing the cofactors 100 nM calmodulin, 2.5 mM $CaCl_2$, 1 mM NADPH, 10 M tetrahydrobiopterin, 1 mM dithiotreitol, and the substrate, 1 Ci L-[^{14}C]arginine for 15 min at 37°C. After the incubation period, the reaction was quenched by the addition of 1 ml of stop buffer (20 mM HEPES, 2 mM EDTA and 0.2 mM EGTA, pH 3). The reaction mixture was applied to a 1 ml column containing Dowex AG 50WX-8 resin (Na^+ form, Bio-Rad, Hercules, CA) that had been pre-equilibrated with the stop buffer. L-[^{14}C]citrulline was eluted twice with 0.5 ml of stop buffer and the radioactivity was determined by liquid scintillation counting.

Aortic ring sprouting assay

Aortas were excised immediately after sacrifice from 5-6 week-old male Sprague Dawley rats (Orient, Sungnam, Korea) and eNOS-deficient and wide type mice. The aortas were transversely cut by 1 mm-thickness and were embedded in growth factor-reduced Matrigel. PGE_2 and each inhibitor were added to the wells in a final volume of 200 l of human endothelial serum-free media (Invitrogen Life Technologies, Carlsbad, CA). After 5 days, microvessel outgrowth was photographed under a phase microscope. Results were scored from 0 (least positive) to 5 (most positive).

Statistical analysis

The data are presented as means $\pm$ standard deviation (SD) of at least three separate experiments in triplicate. Comparisons between two groups were analyzed using the Student's *t*-test, and significance was established at a P value < 0.05.

Results

PGE_2 stimulates cell proliferation, migration, and tube formation in HUVECs

Angiogenesis requires the three essential processes of proliferation, migration, and tube-like structure formation of endothelial cells. To examine whether PGE_2 enhances endothelial cell proliferation, HUVECs were stimulated with various concentrations of PGE_2 in the presence of 1 Ci/ml [^{3}H]thymidine for 6 h and DNA synthesis was determined. HUVEC proliferation was increased in a dose-dependent manner by PGE_2 treatment (Figure 1A). We next tested whether PGE_2 would induce endothelial cell migration. Treatment of HUVECs with PGE_2 for 4 h significantly increased the migration in a concentration-dependent manner (Figure 1B). We also determined whether PGE_2 regulates tube-like structure formation of endothelial cells on Matrigel-coated plates. Treatment of the cells with PGE_2 resulted in a dose-dependent increase in tube formation (Figure 1C). These findings suggest that PGE_2 induced proliferation, migration, and tube formation in *in vitro* cultured HUVECs.

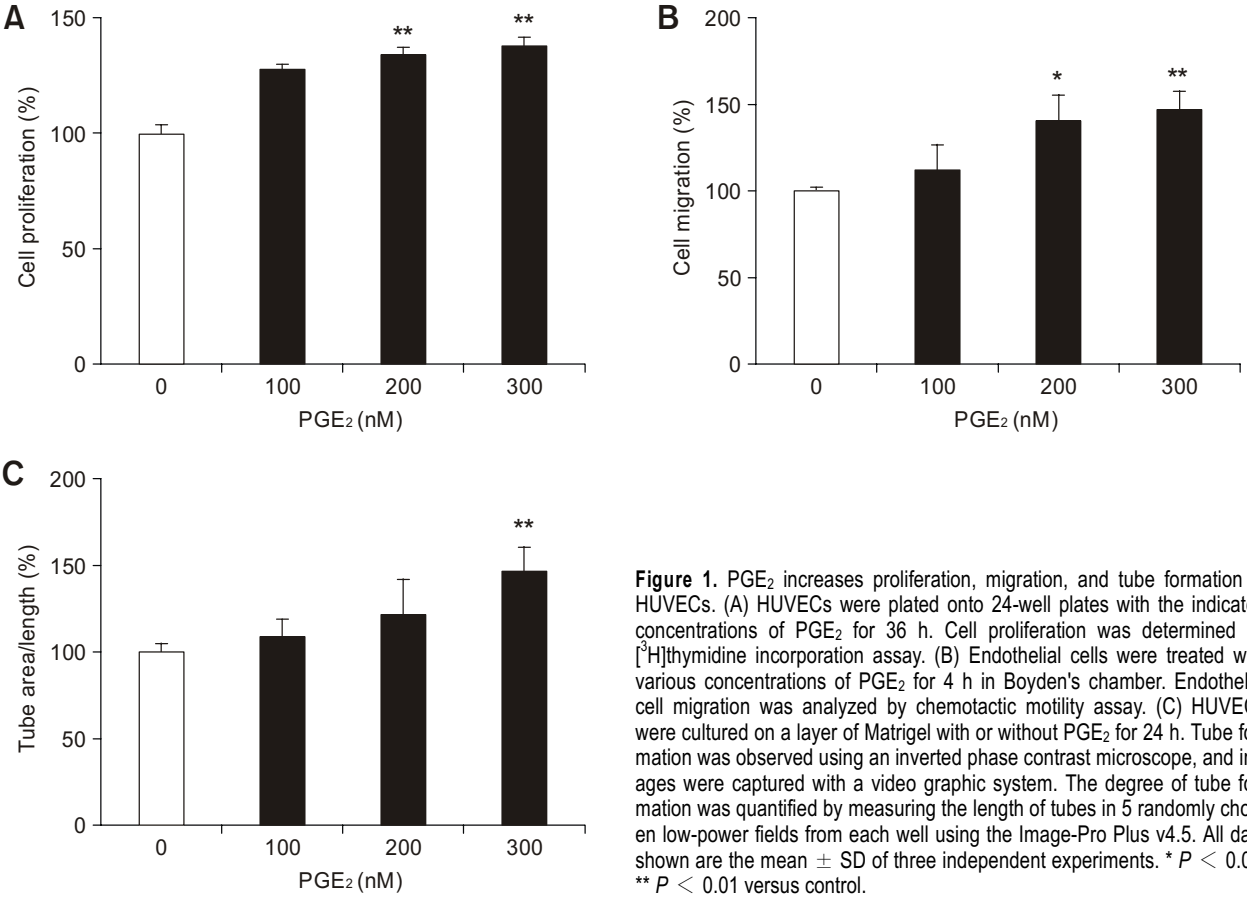

Figure 1. PGE_2 increases proliferation, migration, and tube formation in HUVECs. (A) HUVECs were plated onto 24-well plates with the indicated concentrations of PGE_2 for 36 h. Cell proliferation was determined by [^{3}H]thymidine incorporation assay. (B) Endothelial cells were treated with various concentrations of PGE_2 for 4 h in Boyden's chamber. Endothelial cell migration was analyzed by chemotactic motility assay. (C) HUVECs were cultured on a layer of Matrigel with or without PGE_2 for 24 h. Tube formation was observed using an inverted phase contrast microscope, and images were captured with a video graphic system. The degree of tube formation was quantified by measuring the length of tubes in 5 randomly chosen low-power fields from each well using the Image-Pro Plus v4.5. All data shown are the mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus control.

PGE_2 and cAMP induce angiogenesis via the PKA- and PI3K/Akt-dependent signaling pathways

PGE_2 binds EP_2 and EP_4 and activates adenylate cyclase, leading to an increase in intracellular cAMP formation (Regan *et al.,* 1994; Katsuyama *et al.,* 1995; Breyer *et al.,* 2001). As previously reported (Dormond *et al.,* 2002), we found that EP_2 and EP_4 receptors were expressed in HUVECs and that EP_4 was more abound than EP_2 (data not shown), suggesting that cAMP may be involved in PGE_2-mediated regulation of angiogenesis. The major target molecule of cAMP is PKA, which mediates its effects through phosphorylation of specific substrates (Walsh *et al.,* 1994; Schwartz, 2001). It has also been shown that cAMP can activate the PI3K/Akt pathway (Li *et al.,* 2000), which is involved in the angiogenic process (Walsh and Van Patten, 1994). To test whether the angiogenic effect of PGE_2 on endothelial cell migration, proliferation, and tube formation would be regulated by the PKA inhibitor PKI and the PI3K inhibitor wortmannin, HUVECs were treated with PGE_2 following pretreatment with PKI or wortmannin for 30 min. Treatments with PKI and wortmannin inhibited PGE_2-induced increases in migration, proliferation, and tube formation of HUVECs to the control level (Figure 2A-C). However phospholipase C inhibitor U-73122 did not inhibit PGE_2-mediated *in vitro* angiogenic process (Figure 2A-C). We next examined the effect of cAMP on angiogenesis. Treatment of HUVECs with the membrane permeable cAMP analogue, DB-cAMP, increased cell migration, proliferation, and tube formation of endothelial cells, and these increases were significantly inhibited by PKA and PI3K specific inhibitors (Figure 2D-F). These results suggest that both PKA and PI3K/Akt are involved in the intracellular signaling pathway of PGE_2- and cAMP-mediated angiogenesis.

PGE_2 stimulates cAMP-dependent activation of Akt and eNOS

Since activation of Akt increases NO production by phosphorylating Ser^{1177} of eNOS (Walsh and Van Patten, 1994), which is an important factor in regulating angiogenesis, we determined whether PGE_2 regulates both phosphorylation of Akt and eNOS using specific antibodies against phospho-$Akt^{(Ser473)}$ and phospho-$eNOS^{(Ser1177)}$. Western blot

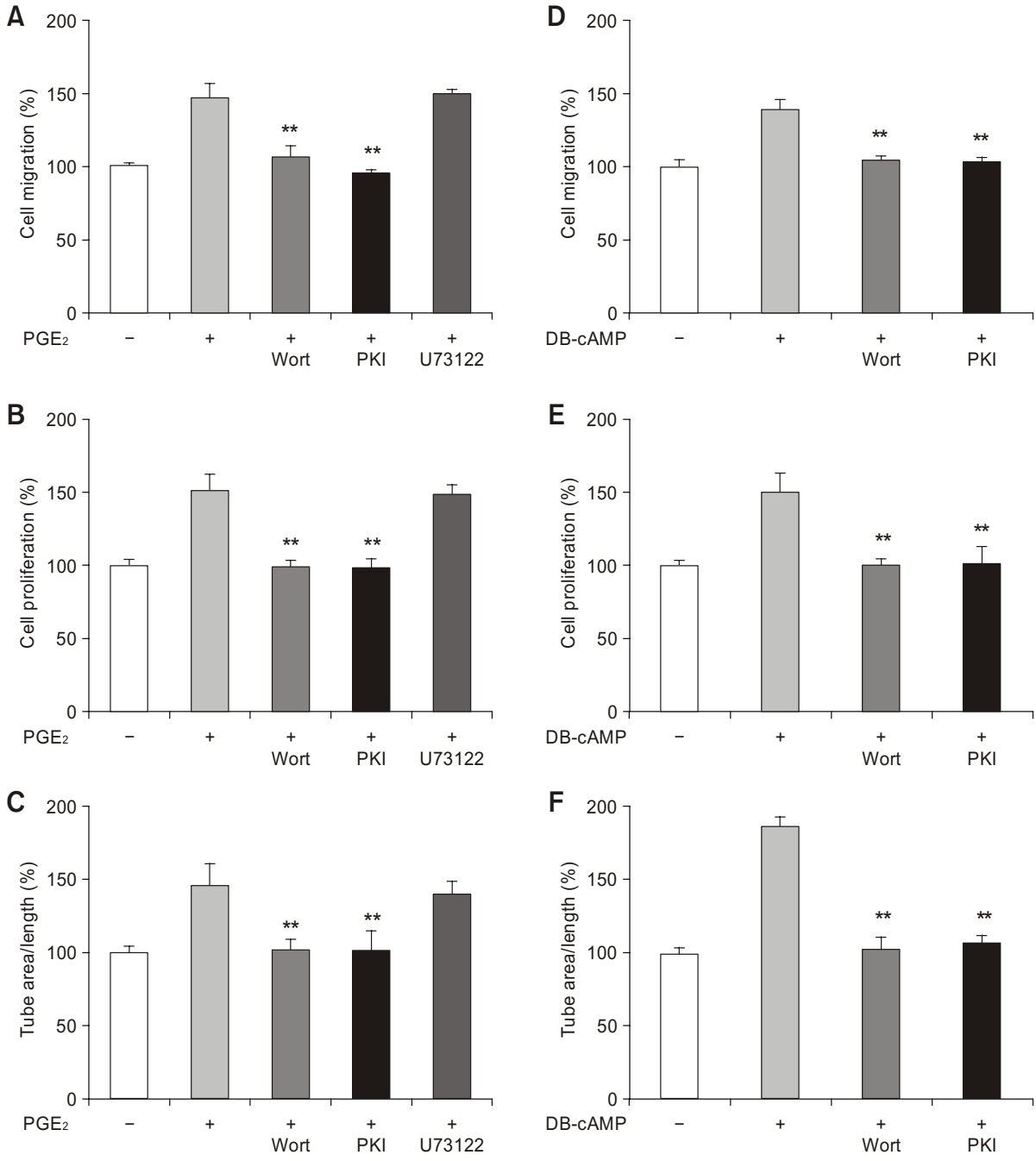

Figure 2. Involvement of both PKA and PI3K/Akt in PGE_2-mediated angiogenesis in HUVECs. Endothelial cells were pretreated with PI3K inhibitor (100 nM wortmannin), PKA inhibitor (3 M PKI), or phospholipase C inhibitor (1 M U73122) for 30 min before exposure to PGE_2 (300 nM) or DB-cAMP (50 M). (A and D) After 4 h, cell migration was assayed using Transwell with 6.5 mm diameter polycarbonate filters. (B and E) Cell proliferation was determined by [^{3}H]thymidine incorporation assay for 36 h. (C and F) After 24 h, tube formation was determined on Matrigel-coated wells. Data shown are the mean ± SD of three independent experiments. $^{**}P < 0.01$ versus control.

analyses revealed that treatment of HUVECs with PGE_2 increased phosphorylation of Akt and eNOS in a time-dependent manner (Figure 3A). These phosphorylations were apparent 15 min after PGE_2 treatment and reached a maximum by 45 min. Similarly, treatment with DB-cAMP increased Akt and eNOS phosphorylation, which appeared after 10 min of DB-cAMP treatment and reached a maximum by 20 min (Figure 3B), indicating that Akt and eNOS phosphorylation of HUVECs exposed to DB-cAMP oc-

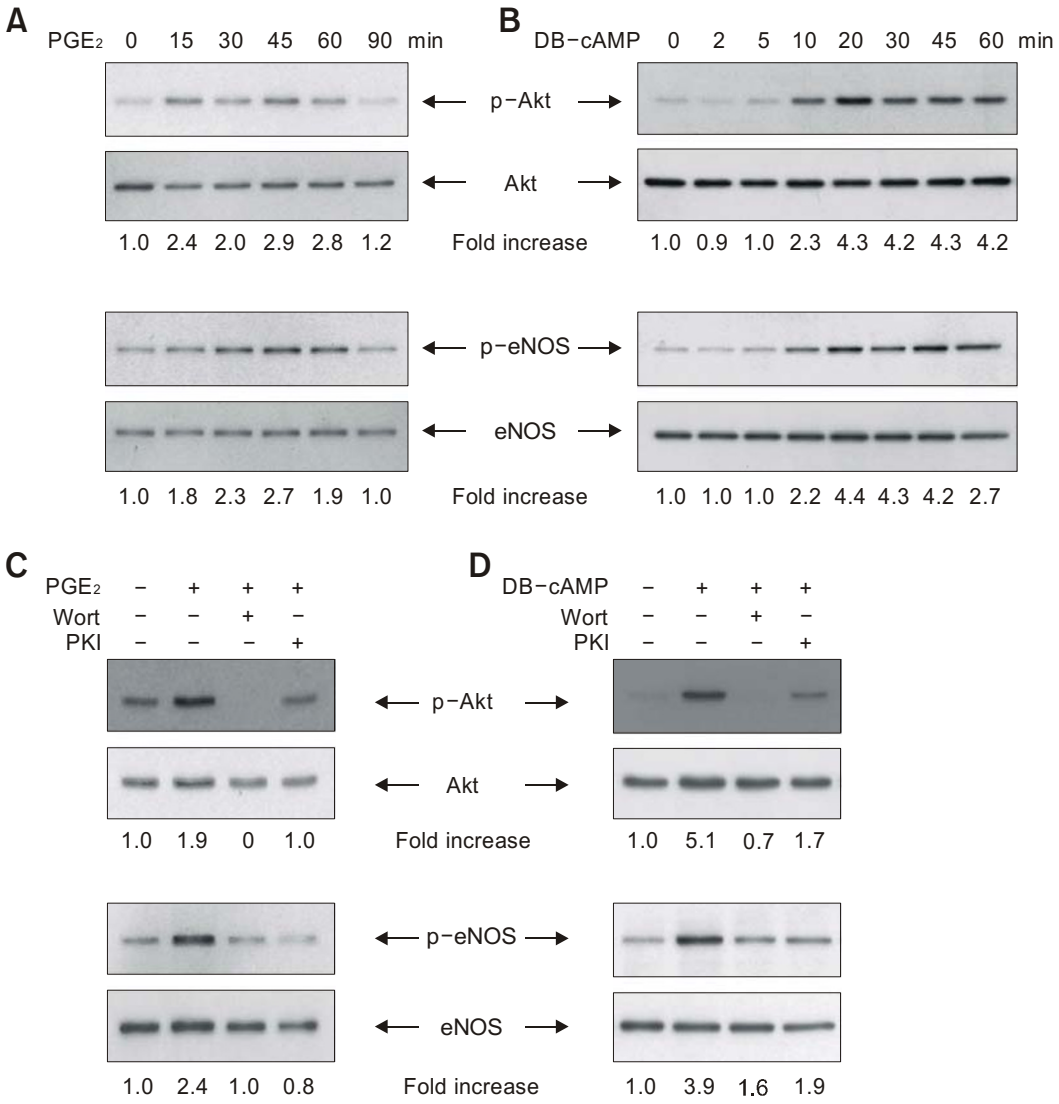

Figure 3. PGE_2 induces phosphorylation of Akt and eNOS through PKA- and PI3K-dependent manners. HUVECs were incubated with PGE_2 (300 nM) (A) or DB-cAMP (50 M) (B) for the indicated time. Phospho-Akt and phospho-eNOS were determined by Western blot analyses using specific antibodies for phospho-Akt$^{(Ser473)}$ and phospho-eNOS$^{(Ser1177)}$. The protein levels of Akt and eNOS were determined in the same membranes by Western blot analysis using antibodies for Akt and eNOS. (C and D) HUVECs were pretreated with PKI (3 M) and wortmannin (100 nM) for 30 min before exposure to PGE_2 (300 nM) or DB-cAMP (50 M). The protein levels of phospho-Akt, phosphor-eNOS, Akt, and eNOS were determined by Western blot analyses using specific antibodies for each protein. Blots shown are representatives of at least three independent experiments.

curred little earlier than that of PGE_2-treated cells. We next examined whether the PGE_2-dependent phosphorylation of Akt and eNOS would be regulated by PKA- and PI3K-dependent mechanisms. HUVECs were treated with PGE_2 or DB-cAMP following pretreatment with PKI or wortmannin and then phosphorylation levels of Akt and eNOS were analyzed by Western blotting. Treatment of the cells with PKI and wortmannin significantly blocked PGE_2- and DB-cAMP-dependent increases in phosphorylations of Akt and eNOS (Figure 3C, D). These results indicate that PGE_2 induced angiogenesis by phosphorylating Akt and eNOS through the cAMP/PKA/PI3K signaling pathway.

PGE_2 increases both eNOS activity and NO production

Previous studies have shown that Ser^{1177} phosphorylation of eNOS increases eNOS activity and NO production (Chen *et al.,* 1999; Michell *et al.,* 1999; Michell *et al.,* 2001). We examined whether

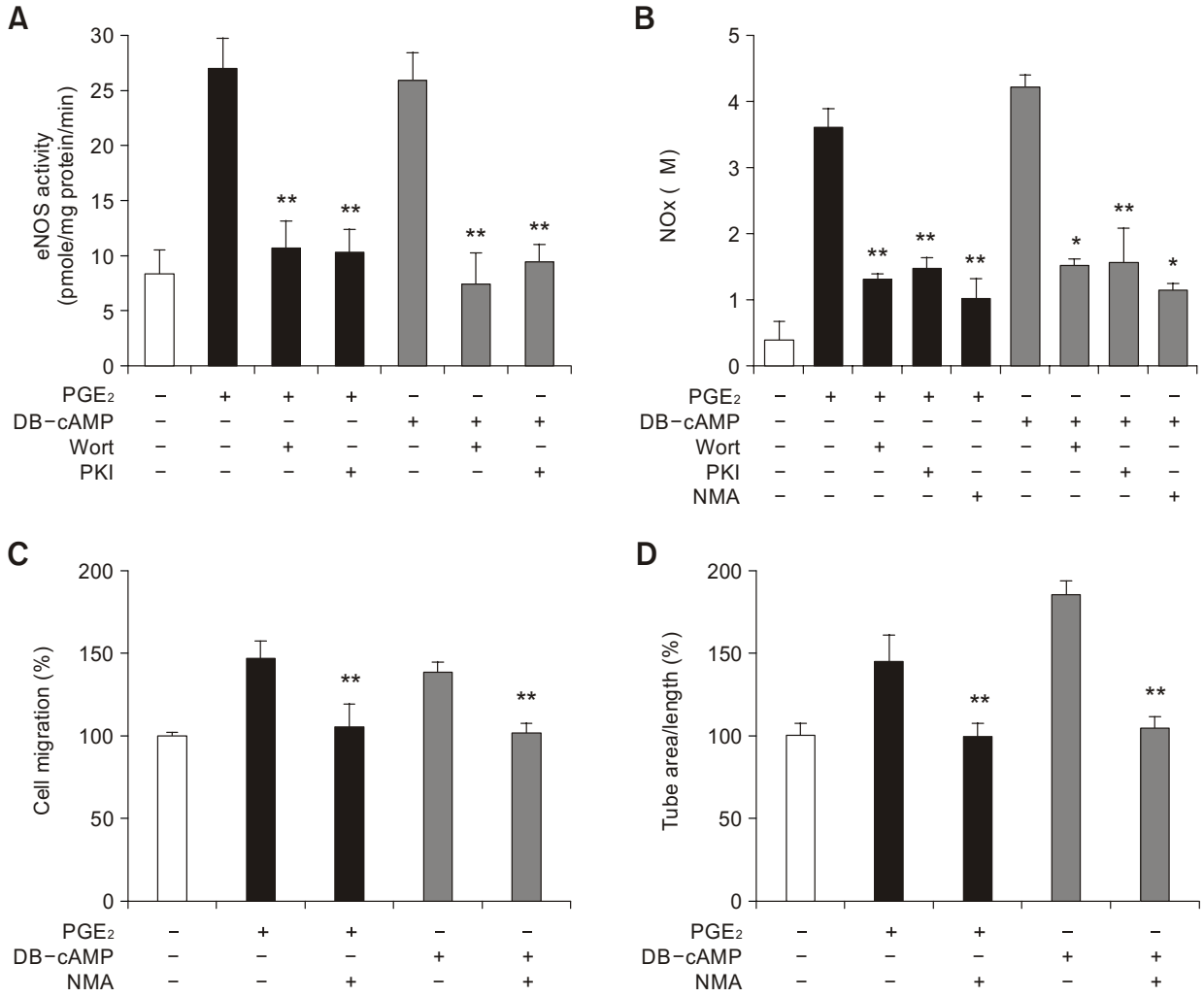

Figure 4. PGE_2 increases eNOS activity and NO production in HUVECs. HUVECs were treated with PGE_2 or DB-cAMP in presence or absence of 100 nM wortmannin, 3 M PKI, and 2 mM NMA. (A) Cells were harvested, washed twice with ice-cold PBS, and lysed by three cycles of freeze and thaw. The lysates were used to assay for eNOS activity as measured by the production of L-[U-^{14}C]citrulline from L-[U-^{14}C]arginine. (B) After 36 h-incubation, the levels of NOx were determined in the culture medium using a chemiluminescent NO analyzer. Cell migration (C) and tube formation (D) were determined by the same method as described in the legend of Figure 1. Data showed are the mean ± SD of three independent experiments. $^{**}P < 0.01$ versus control.

PGE_2 could regulate eNOS activity and NO production. Cell lysates from HUVECs stimulated with PGE_2 or DB-cAMP showed an increase in eNOS activity compared with the control cell lysate (Figure 4A). The increased enzyme activity was reduced in the lysates from HUVECs co-treated with PKI or wortmannin. To measure NO production by HUVECs, cells were treated with PGE_2 or DB-cAMP and NO production was determined in the cultured media. Treatment with PGE_2 or DB-cAMP significantly increased NO production, and this increase was inhibited by co-treatment with PKI, wortmannin or the NOS inhibitor NMA (Figure 4B). These results indicate that PGE_2 and DB-cAMP elevated NO production in HUVECs by increasing phosphorylation-dependent eNOS activity. To further examine a functional role of increased NO production in PGE_2- and DB-cAMP-mediated angiogenesis, HUVECs were treated with PGE_2 or DB-cAMP in the presence or absence of NMA, and cell migration and tube formation were measured. PGE_2- and DB-cAMP-dependent increases in cell migration and tube formation were significantly inhibited by NMA (Figure 4C, D), indicating that NO is a critical mediator for PGE_2-induced angiogenesis of HUVECs.

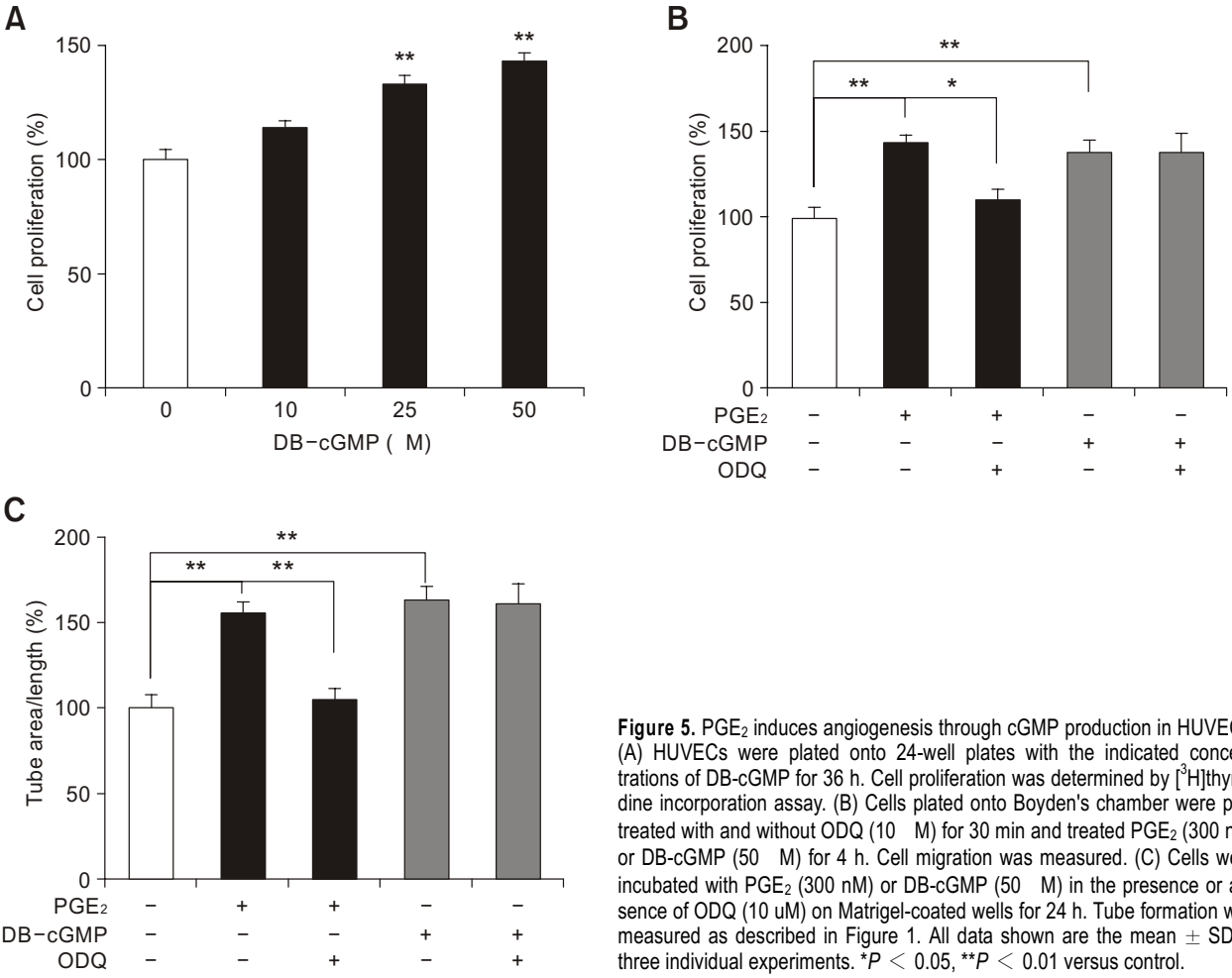

Figure 5. PGE_2 induces angiogenesis through cGMP production in HUVECs. (A) HUVECs were plated onto 24-well plates with the indicated concentrations of DB-cGMP for 36 h. Cell proliferation was determined by [^{3}H]thymidine incorporation assay. (B) Cells plated onto Boyden's chamber were pretreated with and without ODQ (10 M) for 30 min and treated PGE_2 (300 nM) or DB-cGMP (50 M) for 4 h. Cell migration was measured. (C) Cells were incubated with PGE_2 (300 nM) or DB-cGMP (50 M) in the presence or absence of ODQ (10 uM) on Matrigel-coated wells for 24 h. Tube formation was measured as described in Figure 1. All data shown are the mean ± SD of three individual experiments. *$P < 0.05$, **$P < 0.01$ versus control.

PGE_2 requires NO-dependent cGMP production for its angiogenic activity

Earlier studies have shown an important role of NO in the activation of soluble guanylate cyclase, resulting in the increased intracellular cGMP level (Katsuki *et al.,* 1977; Chung *et al.,* 2001). We examined a role of cGMP in PGE_2- and cAMP-mediated endothelial cell proliferation. As shown in a previous study (Yamahara *et al.,* 2003), treatment of HUVECs with the membrane permeable cGMP analogue DB-cGMP increased cell proliferation in a dose-dependent manner (Figure 5A). To examine the possible involvement of cGMP in PGE_2-mediated angiogenesis, cells were treated with PGE_2 or DB-cGMP in the presence or absence of the soluble guanylate cyclase specific inhibitor ODQ and then angiogenic activity was measured. Treatment with PGE_2 or DB-cGMP increased migration and tube formation of HUVECs, and the addition of ODQ inhibited only the angiogenic activities of PGE_2, but not of DB-cGMP (Figure 5B, C). These findings suggest that cGMP is involved in angiogenesis of HUVECs stimulated with PGE_2.

PGE_2 increases vessel sprouting *ex vivo* by NO/cGMP pathway

The aorta model of angiogenesis has gained wide acceptance as a tool to study angiogenesis and its mechanism (Nicosia *et al.,* 1990). To determine whether PGE_2 could enhance vessel sprouting *ex vivo*, rat aortic rings were placed in Matrigel and incubated with PGE_2 in the presence or absence of PKI, wortmannin, NMA, and ODQ. The sprouting of vessels was increased in the cut edge of rat aortic rings incubated with PGE_2 compared with the untreated controls. This angiogenic response was inhibited by co-treatment with PKI, wortmannin, NMA, and ODQ (Figure 6A). It indicates that PGE_2 increases vessel sprouting *ex vivo* via the NO/cGMP pathway. We next used aortic rings from eNOS-deficient mice to investigate a role of NO/cGMP

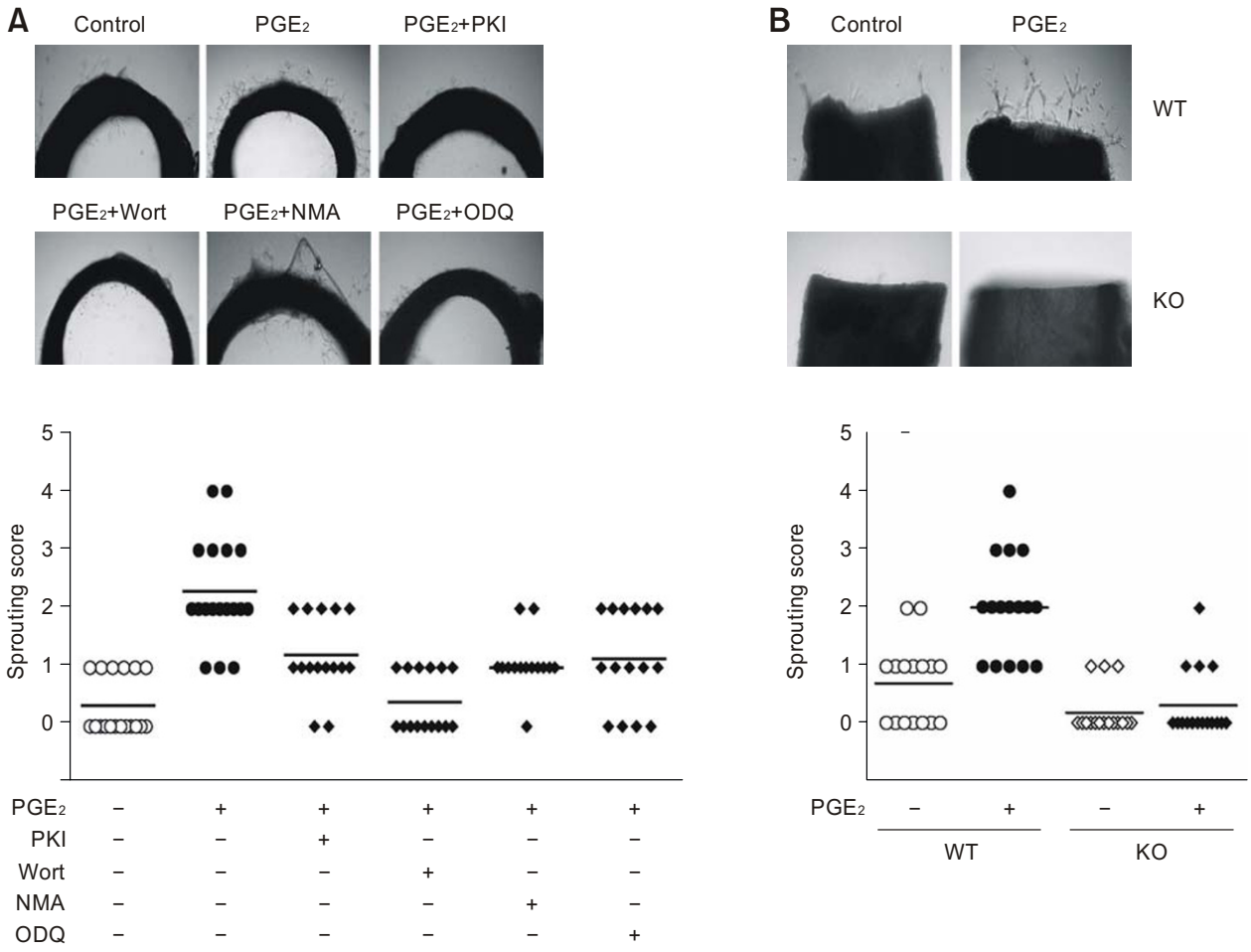

Figure 6. PGE_2 induces vessel sprouting from aortic ring segments of wild type rats and mice, but not of eNOS-deficient mice. Aortic segments were harvested from rats or mice as well as wild type (WT) and $eNOS^{-/-}$ (KO) mice. Vessels were embedded in Matrigel and cultured for 5 days with PGE_2 (300 nM) in the presence or absence of 100 nM wortmannin, 3 M PKI, 2 mM NMA, or 10 M ODQ. Upper panels of (A) and (B) are the representative photographs for endothelial cell sprouts formed from the margin of vessel segments. Low panels of (A) and (B) are the sprouting scores quantitated from 0 (least positive) to 5 (most positive). Results are expressed as the mean ± SD three individual experiments in sextuplets.

pathway in PGE_2-mediated vessel sprouting. As shown in Figure 6B, treatment with PGE_2 significantly increased vessel sprouting in aortic ring segments from wild type mice, but not from eNOS-deficient mice. These results indicate that PGE_2 increased angiogenesis by increasing endothelial NO/cGMP production via PKA- and PI3K/ Akt-dependent eNOS activation.

Discussion

The present study was undertaken to elucidate the potential effect and molecular mechanism of PGE_2 on angiogenesis of HUVECs. We found that PGE_2 significantly increased angiogenesis of endothelial cells *in vitro* as well as vessel sprouting in aortic rings from wild type rats and mice *ex vivo*, but not from eNOS-deficient mice. The membrane permeable cAMP analogue DB-cAMP mimicked the angiogenic effect of PGE_2. Both PGE_2 and DB-cAMP increased phosphorylation of Akt and eNOS, resulting in increases in eNOS activity and endothelial NO production. Furthermore, the angiogenic activity of PGE_2 was suppressed by the PKA inhibitor PKI, the PI3K inhibitor wortmannin, the NOS inhibitor NMA, and the soluble guanylate cyclase inhibitor ODQ. These data indicate that PGE_2 induces angiogenesis through PKA/PI3K/Akt-dependent activation of the NO/cGMP signaling pathway.

PGE_2, which is a major product of COX, exerts its biological action by binding to specific receptors known as four subtypes of EP receptors, such as EP_1, EP_2, EP_3, and EP_4. These receptor subtypes have been classified based on their distinct genes and signal transduction pathways with cell type-

specificity. EP_1 receptor activates phospholipase C and mobilization of the inositol trisphosphate pathway and Ca^{2+}, EP_2 and EP_4 receptors activate adenylate cyclase and the cAMP/PKA pathway, whereas EP_3 receptor activation can both inhibit adenylate cyclase and activate phospholipase C (Coleman *et al.,* 1994). Although not shown, EP_2 and EP_4 were specifically expressed in HUVECs as determined by reverse transcriptase-polymerase chain reaction analysis. This data suggests that EP_2 and EP_4 increase the intracellular cAMP level in HUVECs and that cAMP may play a role as a key mediator in the angiogenic process of HUVECs treated with PGE_2. Our data also showed that the cell-permeable cAMP analogue DB-cAMP significantly induced proliferation, migration, and tube-like structure in HUVECs, which represent *in vitro* phenotype of angiogenesis for endothelial cells. Therefore, PGE_2-dependent increase in the intracellular cAMP level causes PKA activation, which is involved in PGE_2-mediated angiogenesis. Furthermore, we showed that the angiogenic activity of PGE_2 was blocked by PKA inhibitor, but not by phospholipase C inhibitor. These results indicate that PGE_2 induced angiogenesis of HUVECs by PKA-dependent signaling through an increase in EP_2 and EP_4-mediated cAMP production.

It has been demonstrated that cAMP causes PI3K-dependent Akt activation in hepatocytes and hepatic WIF-B9 cells, probably in a PKA-dependent manner (Li *et al.,* 2000; Kagawa *et al.,* 2002). The PI3K/Akt signaling pathway can be induced by VEGF (Kosmidou *et al.,* 2001; Laughner *et al.,* 2001) and sphingosine 1-phosphate (Morales-Ruiz *et al.,* 2001) and plays a central role in angiogenesis by promoting endothelial sprouting, proliferation, migration, and vessel-like structure formation (Deregibus *et al.,* 2003; Licht *et al.,* 2003). These evidences suggest that many angiogenic factors activate the PI3K/Akt signaling pathway, which is a central mediator of angiogenesis (Zachary, 2001). The present data showed that the PGE_2 and DB-cAMP induced Akt phosphorylation and angiogenesis, which were inhibited by PKI and wortmannin. This data indicates that PGE_2 induces angiogenesis by PKA-dependent activation of the PI3K/Akt signaling pathway. It has been previously demonstrated that PKA increased Akt activation and then promoted neovascularization *in vivo* in Matrigel plugs (Filippa *et al.,* 1999; Miyashita *et al.,* 2003). We here found that PGE_2- and cAMP-induced Akt activation in HUVECs was significantly blocked by the inhibition of PI3K and PKA, indicating that PKA is an important mediator upstream of PI3K/Akt. This result suggests that PI3K/Akt pathway is critically involved in PGE_2- and cAMP-mediated angiogenesis of HUVECs.

There are increasing evidences indicating that activation of PKA and Akt stimulates phosphorylation of eNOS at Ser^{1177} and then increases endothelial NO production (Michell *et al.,* 2001; Boo *et al.,* 2002). NO produced from the endothelium seems to play essential roles in vessel relaxation, angiogenesis, and inhibition of endothelial cell apoptosis and vascular inflammation. Although PGE_2 has been shown to stimulate angiogenesis and vasodilation (Form and Auerbach, 1983; Kennedy *et al.,* 1999), the molecular mechanism by which PGE_2 regulates vascular function is unknown. Our results clearly showed that PGE_2 increased eNOS activity by phosphorylating its Ser^{1177} residue, leading to the enhancement of endothelial NO production, which is critically involved in promoting endothelial cell proliferation, migration, and tube formation (Ziche *et al.,* 1994; Lee *et al.,* 1999) as well as vasodilation (Ignarro, 1989). The PI3K inhibitor wortmannin completely suppressed Akt phosphorylation, but revealed partial inhibition of eNOS phosphorylation; the PKA inhibitor PKI, however, inhibited the phosphorylation of both Akt and eNOS, indicating that PKA may be upstream of the PI3K/Akt pathway in PGE_2-dependent NO production and angiogenesis and that eNOS can be directly activated by both PKA and Akt (Michell *et al.,* 2001).

The angiogenic activity of PGE_2 was strongly suppressed by the inhibitors of PKA and PI3K as well as the NOS inhibitor NMA. This result indicates that NO produced by eNOS phosphorylation via the PKA- and PI3K/Akt-dependent signaling pathways is an important mediator in PGE_2-induced angiogenesis. NO can interact with a variety of biomolecules. Of them, soluble guanylate cyclase is activated by interaction of its cofactor heme with NO and increases the intracellular level of cGMP, acting as intracellular signal mediator for vasodilation, anti-apoptosis, and angiogenesis (Forstermann *et al.,* 1986; Pipili-Synetos *et al.,* 1994; Kim YM *et al.,* 1997). In the present study, we showed that the soluble guanylate cyclase inhibitor ODQ inhibited the angiogenic activity of PGE_2 in cultured HUVECs and murine aortic ring segments, whereas PGE_2 could not stimulate endothelial sprouts in aortic ring segments from eNOS-deficient mice. We also showed that the membrane-permeable cGMP analogue DB-cGMP caused an increase in HUVEC proliferation. These results indicate that PGE_2 stimulated angiogenic process by increasing endothelial NO production through the PKA-dependent PI3K/Akt signaling pathway. Our data also suggest that the vasodilatory activity of PGE_2 may be linked to the activation of the NO/cGMP signaling pathway.

We here showed that PGE_2 stimulated an increase

in endothelial sprouting from aortic rings of wild type mice, which was suppressed by NMA, but not from those of eNOS-deficient mouse. Similarly, previous study has demonstrated that eNOS plays a significant role in endothelial proliferation, migration, and differentiation in response to growth factors and eNOS KO mice significantly delayed *in vitro* angiogenesis compared with wild-type controls (Lee *et al.,* 1999). These findings indicate that eNOS-dependent NO production is involved in PGE_2-dependent angiogenesis.

In conclusion, we have demonstrated that PGE_2-mediated elevation of intracellular cAMP level leads to an increase in eNOS activation and endothelial NO and cGMP production by PKA-dependent PI3K/Akt activation and then promotes angiogenesis *in vitro* and *ex vivo*. These results indicate that the NO/cGMP pathway is a critically important factor for PGE_2-dependent angiogenesis during inflammatory wound healing.

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