[CANCER RESEARCH 64, 6482–6488, September 15, 2004]

Estrogen Enhances Angiogenesis through a Pathway Involving Platelet-Activating Factor-Mediated Nuclear Factor-κB Activation

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ABSTRACT

In this study, we investigated the molecular events involved in estrogen-induced angiogenesis. Treatment of the human endometrial adenocarcinoma cells, HEC-1A, with estrogen up-regulated mRNA expression and protein synthesis of various angiogenic factors such as tumor necrosis factor-α, interleukin-1, basic fibroblast growth factor, and vascular endothelial growth factor. The estrogen-dependent induction of the expression was blocked by the platelet-activating factor (PAF) antagonists, WEB 2170. Estrogen treatment caused the activation of nuclear factor (NF)-κB in HEC-1A cells and was also blocked by PAF antagonist. Inhibitors of NF-κB activation inhibited estrogen-induced mRNA expression and protein synthesis of the angiogenic factors. Estrogen led to a pronounced angiogenesis as assessed by a mouse Matrigel model *in vivo* and endothelial cell sprouting *in vitro*. PAF antagonists or NF-κB inhibitors significantly inhibited this estrogen-dependent angiogenesis. Estrogen caused phospholipase A_2 (PLA_2) gene and protein expression. Estrogen-induced vascular endothelial growth factor mRNA expression and sprouting were significantly inhibited by PLA_2 inhibitors, suggesting PLA_2 expression is the upstream pathway in the estrogen-induced angiogenesis. Taken together, these results suggest that estrogen induces the production of angiogenic factors via a mechanism involving PAF-mediated NF-κB activation.

INTRODUCTION

Angiogenic activity of the female sex hormone, estrogen is associated with physiologic cyclic endometrial angiogenesis (1–4) and other pathological conditions such as endometriosis (5, 6), endometrial cancer (7), and breast cancer (2).

Estrogen induces endothelial cell proliferation and migration (8). Furthermore, multiple lines of evidence suggest that estrogen-induced angiogenesis appears to occur via its ability to induce the expression of various angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (2, 3). However, the molecular mechanisms by which estrogen induces the expression of these angiogenic factors are poorly understood.

Platelet-activating factor (PAF), which is produced by a variety of inflammatory cells, is a potent lipid first messenger involved in cellular activation, fertilization, intracellular signaling, apoptosis, and diverse inflammatory reactions (9–13). We have shown that PAF is a proximal inducer of the transcription factor, nuclear factor (NF)-κB, a key component necessary for the expression of proinflammatory cytokines and many immunoregulatory molecules (14, 15) in response to inflammatory stimuli (16) and microbial infection (17). Recently, we have shown that PAF promotes angiogenesis through the activation of NF-κB, which in turn, promotes various angiogenic factors such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, bFGF, and VEGF (18).

Estrogen increases PAF concentrations, as well as PAF receptors in the uterus (19–21), perhaps through an estrogen-dependent decrease in the expression of the PAF-metabolizing enzyme PAF-acetylhydrolase in the uterus and plasma (19, 22).

Furthermore, estrogen potentates PAF-mediated phospholipase activities in a human endometrial cell line (23). These studies led to hypothesize that PAF plays a role in estrogen-induced angiogenesis.

The results from experiments designed to test this hypothesis demonstrate that, in fact, induction of angiogenesis occurs by increasing the expression of several key angiogenic factors through PAF-dependent NF-κB activation.

MATERIALS AND METHODS

Animals. Specific pathogen-free female BALB/c mice were obtained from the Korean Institute of Chemistry Technology (Daejeon, Korea) and were kept in our animal facility for at least 2 weeks before use. All mice were used at 8 to 10 weeks of age.

Reagents. 17-estradiol 1.3.5(10)-estratriene 3,17-diol, *N*-acetyl-L-cysteine (NAC), (+)-α-tocopherol acid succinate (vitamin E), and 4-hydroxytamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO). ICI 182,780 (ICI) was purchased from Tocris Cookson (Kyoto, Japan). PAF receptor antagonist, WEB 2170, was a gift from Dr. C.K. Rhee (College of Medicine, Dankook University, Cheoan, Korea). NF-κB inhibitor, parthenolide, was purchased from BIOMOL (Plymouth Meeting, PA). Phospholipase A_2 (PLA_2)-nonspecific inhibitor, quinacrine dihydrochloride (quinacrine), secretory (s) PLA_2 inhibitor, 4-bromophenacyl bromide, cytoplasmic (c) PLA_2 inhibitor, AACOCF3, and mitogen-activated protein kinase (MAPK) inhibitors PD 98059 and U 0126 were purchased from Calbiochem Co. (La Jolla, CA). Matrigel, an extract of murine basement membrane proteins, consisting predominantly of laminin, collagen IV, heparin sulfate proteoglycans, and nidogen/entactin, was purchased from Collaborative Research, Inc. (Bedford, MA). Neutralizing antibodies against VEGF and bFGF, as well as a goat IgG, were purchased from R&D Systems (Minneapolis, MN). Neutralizing antibodies against tumor necrosis factor (TNF) and interleukin (IL)-1α were from Endogen (Woburn, MA). Rabbit IgG was purchased from Sigma Chemical Co. Recombinant human flt-1/Fc chimera and KDR/Fc chimera were purchased from R&D Systems. ELISA kits for assaying VEGF and IL-8 were purchased from R&D Systems.

Antisense Oligonucleotides. The following phophorothioate oligonucleotides were synthesized for use in antisense inhibition of gene expression (Peptron, Korea): p65 antisense (p65 AS) of the 5′-end of the NF-κB gene (5′-GAAACAGATCGTCCATGGT-3′) and p65 nonsense (scrambled control, p65 NS) oligonucleotide (5′-GTACTACTCTGAGCAAGGA-3′). The NF-κB antisense oligonucleotide includes the ATG initiation codon.

Cell Culture. Human endometrial adenocarcinoma cell line, HEC-1A, was purchased from American Type Culture Collection (Manassas, VA) and was maintained in McCoy's 5A medium with 10% fetal bovine serum (CAMBREX Co., Walkersville, MD).

Primary Cell Culture. Porcine pulmonary arterial endothelial cells (PPAECs) were prepared by collagenase digestion as described previously (24). PPAECs were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum at 37°C in a 5% CO_2 atmosphere. PPAECs used in this study were between passages 2 and 4.

Received 9/3/03; revised 6/14/04; accepted 7/16/04.

Grant support: Korea Research Foundation, Republic of Korea grant DP0555.

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Note: Drs. K. H. Seo and H-S. Lee contributed equally to this work.

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Endothelial Sprouting Assay. Endothelial sprouting was assessed by a modification of the method used by Kim *et al.* (24). Briefly, microcarrier beads coated with denatured collagen (Cytodex 3; Sigma Chemical Co,) were seeded with PPAECs and embedded in fibrin gels in 24-well plates. For preparation of fibrin gels, bovine fibrinogen was dissolved in PBS at a concentration of 6 mg/mL. Aprotinin was added at a concentration of 32 units/mL. The fibrinogen solution was supplemented with estrogen and inhibitors or neutralizing antibodies. As a control, the fibrinogen solution was supplemented with PBS. The fibrinogen solutions were transferred to 24-well plates together with PPAEC-coated beads at a density of 150 beads/well. Clotting was induced by the addition of thrombin (25 units/mL). After clotting was complete, gels were equilibrated with DMEM containing 2% FBS with or without various treatments at 37°C. After 3 days of incubation with daily changes of the medium, the number of capillary-like tubes formed/microcarrier bead (sprouts/bead) was counted by microscopy, monitoring at least 450 beads for each treatment. Only sprouts greater than a diameter of bead in length and composed of at least three endothelial cells were counted.

Reverse Transcriptase-PCR. RNA was prepared as described previously (17, 18). Reverse transcription was performed using 500 ng of total RNA in a 10 μL reaction mixture (Promega, Madison, WI) containing oligo(dT) and avian myeloblastosis virus reverse transcriptase. cDNA (1 μL) was amplified by PCR in a thermal cycler Perkin-Elmer System 2400 (Norwalk, CT). Reverse transcriptase-PCR products were quantified by densitometric analysis of the ethidium bromide stained gel (Fluor-STM Imager; Bio-Rad, Muncher Germany). The level of expression was quantified by calculating the ratio of densitometric reading of the bands for cytokines and β-actin from the same cDNA. To measure the relative amount of PLA_2, VEGF gene transcripts, amplification of sample cDNA (1 μL), were monitored with the fluorescent DNA-binding dye SYBR Green in combination with the Rotor-Gene 3000 System (Corbett Research, Mortlake, Australia). The primers used are as follows: VEGF, 5′-GCAGAATCATCACGAAGTGG-3′ and 5′-GCAACGCG-AGTCTGTGTTTTTG-3′; TNF-α, 5′-CAGAGGGAAGAGTTCCCCAG-3′ and 5′-CCTTGGTCTGGTAGGAGACG-3′; IL-1α, 5′-GTCTCTGAATCAGAAAT-CCTTCTATC-3′ and 5′-CATGTCAAATTTCACTGCTTCATCC-3′; bFGF, 5′-CAAGCGGCTGTACTGCAAAAAC-3′ and 5′-CAGCTCTTAGCAGAC-ATTGG-3′; IL-8, 5′-ACATACTCCAAACCTTTCCACCC-3′ and 5′-CAAC-CCTCTGCACCCAGTTTTC-3′; sPLA2, 5′-ATGAAGACCCTCCTACTG-3′ and 5′-TCAGCAACGAGGGGTGCT-3′; $cPLA_2$, 5′-ATGCCCAGACCTAC-GATTTA-3′ and 5′-AGGGGTTTTCTTCATACTTC-3′; and β-actin, 5′-CT-GAAGTCACCCATTGAACATGGC-3′ and 5′-CAGAGCAGTAATCTCCT-TCTGCAT-3′.

Electrophoretic Mobility Shift Assay. The nuclear extracts were prepared from the cells as described previously (17, 18). To inhibit endogenous protease activity, 1 mmol/L phenylmethylsulfonyl fluoride was added. As a probe for the gel retardation assay, an oligonucleotide containing the immunoglobulin κ-chain binding site (κB, 5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) was synthesized. The two complementary strands were annealed and labeled with (α-^{32}P) dCTP. Labeled oligonucleotides (10,000 cpm), 12 μg of nuclear extracts, and binding buffer [10 mmol/L Tris-HCl (pH 7.6), 500 mmol/L KCl, 10 mmol/L EDTA, 50% glycerol, 100 ng of poly(deoxyinosinic-deoxycytidylic acid), and 1 mmol/L DTT] were incubated for 30 minutes at room temperature in a final volume of 20 μL. The reaction mixture was analyzed by electrophoresis on a 5% polyacrylamide gel in 0.5 × Tris-borate buffer. Specific binding was controlled by competition with a 50-fold excess of cold κB or cAMP response element oligonucleotide.

Quantitation of Cytokines by ELISA. The quantitative determination of cytokines in culture supernatants was performed by ELISA according to manufacturer's instructions. Briefly, after pretreatment of antisense oligonucleotides or inhibitors, medium containing 0.1 μmol/L estrogen was added, and culture supernatants was prepared after 4 hours.

Western Blot Analysis. Cell lysates were prepared in radioimmunoprecipitation assay buffer (0.1% SDS, 1% IGEPAL, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulphonyl fluoride) from HEC-1A cells. Aliquots of each cytosolic extract containing 50 μg of protein were separated by SDS-PAGE (10% acrylamide), transferred to a nitrocellulose membrane by electroblotting (*trans*-Blot; Bio-Rad, Muncher, Germany) and probed with a rabbit polyclonal antibody recognizing the p65 (sc 372) or the $cPLA_2$ (sc 438; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bands were visualized using alkaline phosphatase-conjugated antirabbit IgG (Santa Cruz Biotechnology).

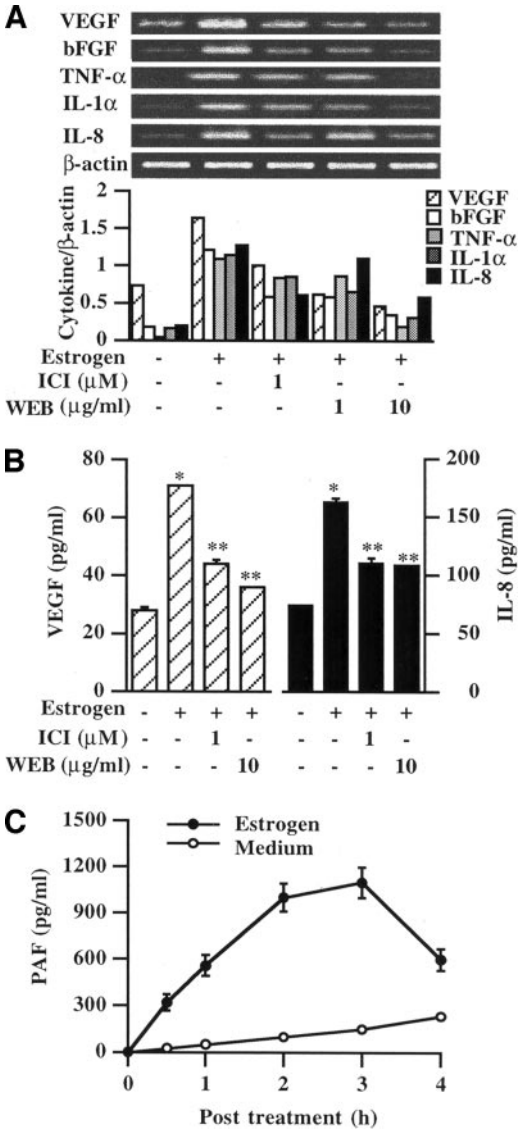

Fig. 1. Involvement of PAF in the induction of angiogenic factor production by estrogen. *A*, HEC-1A were plated at 1×10^6 cells/60-mm dish and were pretreated with the estrogen inhibitor, ICI, or PAF antagonist, WEB 2170 (WEB), at indicated concentrations 30 minutes before estrogen (0.1 μmol/L) treatment. RNA was prepared 4 hours after estrogen treatment. cDNA was reverse transcribed from total RNA of the cell and amplified as described in Materials and Methods. The reverse transcriptase-PCR results are shown in the *top panels* as are the combined densitometric analysis (*bottom panels*). *B*, VEGF and IL-8 proteins were detected by ELISA in the culture supernatants prepared 4 hours after estrogen treatment. *C*, HEC-1A (5×10^6 cells/60-mm dish) were stimulated with estrogen (0.1 μmol/L) for the indicated time periods. PAF contents in the culture supernatants were measured as described in Materials and Methods. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

PAF Assay. HEC-1A cells cultured for 48 hours were recultured for 4 hours with fresh medium. Cells were washed twice and cultured with serum-free medium in the presence of estrogen. PAF contents in the culture supernatants were measured using thin-layer chromatography and SPRIA kit (Amersham) as described previously (25).

Angiogenesis Assay. Angiogenic potential was estimated by measuring hemoglobin content in Matrigel as described previously (18). Matrigel (10 mg/mL), in liquid form at 4°C, was mixed with 64 units/mL heparin plus the experimental substances or vehicle alone and injected (0.5 mL) into the dorsal s.c. tissue of mice. After 6 days, mice were sacrificed, the gels were recovered, and processed for the measurement of hemoglobin content using the Drabkin reagent kit 525 (Sigma Chemical Co.). The results were expressed as mg/dL.

Immunohistochemistry. The Matrigels were removed and immersed in 4% paraformaldehyde and processed and embedded in paraffin by the routine

procedure. Immunoperoxidase staining was performed using Histostain-*plus* kit (ZYMED Laboratory, Inc., San Francisco, CA) as described by the manufacturer's instructions. Briefly, deparaffinized sections were treated with 3% H_2O_2 in methanol for 3 minutes to reduce endogenous peroxidase activity and were incubated with serum block solution for 30 minutes. The sections were incubated with the diluted rabbit anti-von Willebrand Factor (vWF) antibody (Sigma, St. Louis, MO) for 1 hour followed by incubation with biotinylated secondary antibodies for 10 minutes and then horseradish peroxidase-streptavidin complex for 10 minutes. Color was developed with horseradish peroxidase substrate for 3 minutes. After immunostaining, sections were stained with hematoxylin. Photos were taken under microscope at 400-fold magnification.

Statistical Analysis. The data are represented as the mean ± SE. Statistical significance was determined by the Student's *t* test with the statistical software StatView (version 4.5). All experiments were conducted two or more times. Reproducible results were obtained and representative data are therefore shown in the figures.

RESULTS

Estrogen Produces Angiogenic Factors from HEC-1A in a PAF-Induced NF-κB-Dependent Manner. We first determined the ability of estrogen to induce mRNA expression and protein synthesis of the angiogenic factors VEGF, bFGF, TNF-α, IL-1, and IL-8 in the endometrial cell line HEC-1A. Treatment of HEC-1A with estrogen resulted in increased mRNA levels for all of the cytokines examined, and this estrogenic effect was blocked by the estrogen antagonist ICI or the PAF antagonist WEB 2170, suggesting a role for PAF in estrogen-induced angiogenic factor synthesis (Fig. 1*A*). The protein amounts of IL-8 and VEGF but not bFGF, TNF-α, and IL-1 increased in the culture supernatants of HEC-1A cells incubated *in vitro* with estrogen (Fig. 1*B*). We verified the involvement of PAF in the estrogen-induced angiogenesis by measuring the molecule. Stimulation of HEC-1A cells with estrogen resulted in an increase in the PAF contents in the culture supernatants; PAF gradually increased and peaked at 2 to 3 hours and slowly declined thereafter (Fig. 1*C*). Given that PAF has an ability to produce several critical angiogenic factors

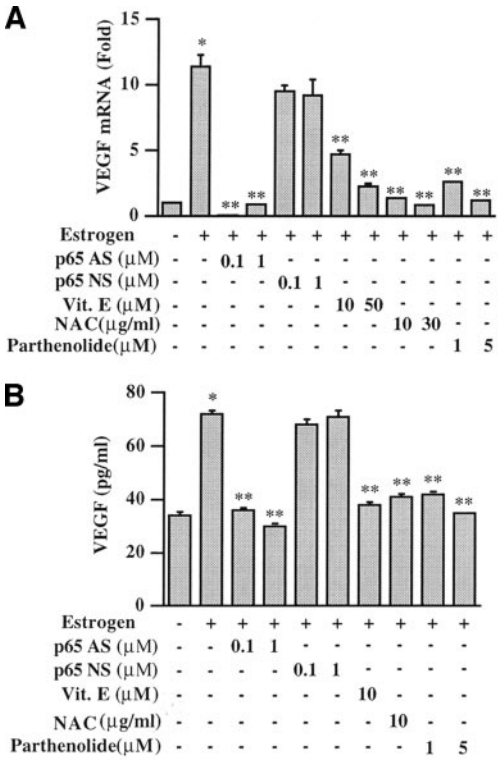

Fig. 3. NF-κB inhibitors block estrogen-induced increases in mRNA levels and protein synthesis of VEGF. HEC-1A were plated at 1×10^5 cells/60-mm dish and were pretreated with p65 AS or p65 NS for 5 days before estrogen (0.1 μmol/L) treatment. HEC-1A were plated at 1×10^6 cells/60-mm dish and were pretreated with the NF-κB inhibitors [vitamin E (Vit. E), NAC, and parthenolide] 30 minutes before estrogen treatment. *A*, RNA was prepared 4 hours after estrogen treatment, and cDNA was reverse transcribed from total RNA. Expression of VEGF level was analyzed by real-time PCR. β-actin was used for endogenous control. *B*, VEGF protein was measured by ELISA in the culture supernatants prepared 4 hours after estrogen treatment. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

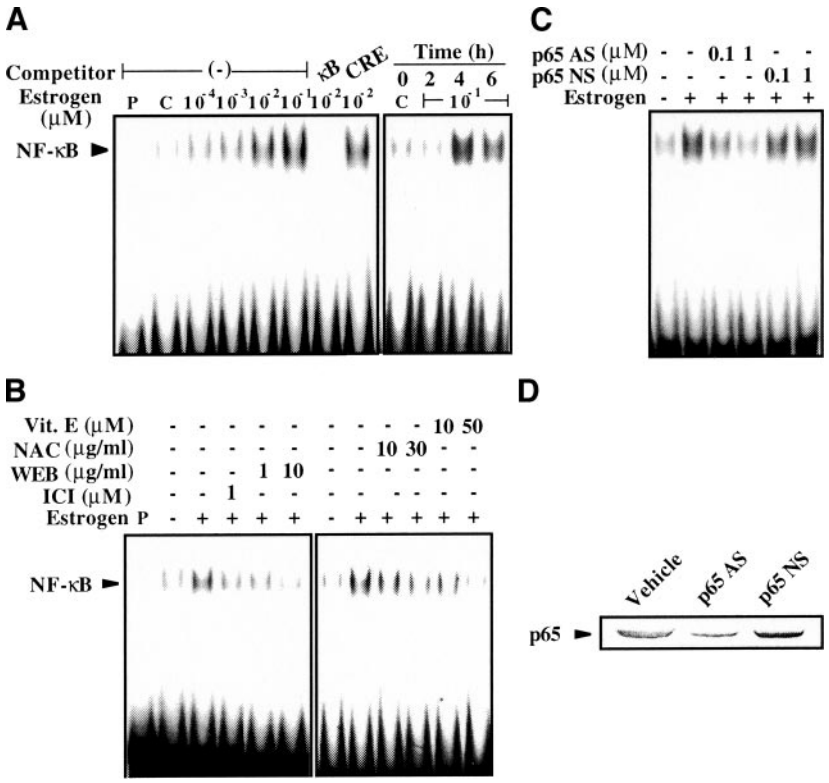

Fig. 2. Estrogen activates NF-κB through PAF. *A* and *B*, HEC-1A were plated at 1×10^6 cells/60-mm dish and were treated with the estrogen (0.1 μmol/L). The cells were pretreated with ICI, WEB, vitamin E (Vit. E), or NAC at indicated concentrations 30 minutes before estrogen treatment. *C*, HEC-1A were plated at 1×10^5 cells/60-mm dish and were pretreated with p65 AS or p65 NS for 5 days before estrogen treatment. Nuclear extracts were prepared 4 hours after estrogen treatment and were incubated with α-^{32}P-labeled κB or cAMP response element (CRE) oligonucleotide and electrophoresed on a 5% polyacrylamide gel. Lane P contained probe incubated without extract. A 50-fold excess of cold κB or CRE oligonucleotide was added as competitor. *D*, HEC-1A were plated at 1×10^5 cells/60-mm dish and were treated with p65 AS or p65 NS for 5 days. Whole cell lysates were prepared, and Western blot was performed as described in Materials and Methods.

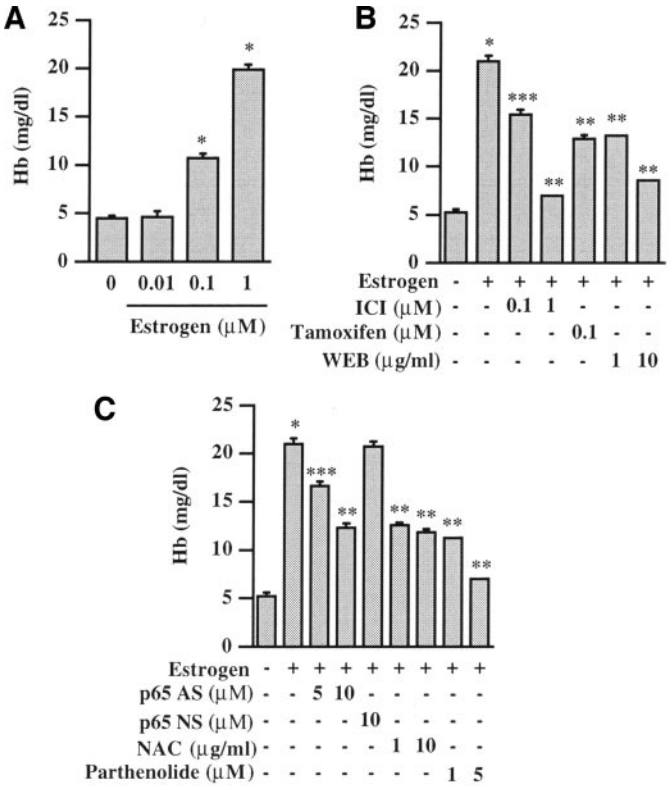

Fig. 4. PAF antagonist or NF-κB inhibitors block estrogen-induced angiogenesis *in vivo*. *A*, Matrigel plugs mixed with indicated concentrations of estrogen were injected s.c. *B*, Matrigel containing estrogen (1 μmol/L) was mixed with indicated concentrations of estrogen inhibitors or PAF antagonist. *C*, Matrigel containing estrogen was mixed with indicated concentrations of NF-κB inhibitors. Matrigel containing 64 units/mL heparin or vehicle alone was used as controls. Matrigel plugs were excised and processed for quantification of angiogenesis by measuring the hemoglobin (Hb) content on day 6. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$; and ***, $P < 0.005$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

through NF-κB activation (18), we next determined whether estrogen could also regulate NF-κB activation. Treatment of HEC-1A with estrogen resulted in the activation of NF-κB in a dose-dependent manner, reaching a maximum at 4 hours (Fig. 2*A*). The estrogen-induced NF-κB activity was blocked by pretreatment with the estrogen inhibitor ICI, PAF antagonist WEB 2170, or the NF-κB inhibitors such as NAC, vitamin E (Fig. 2*B*), or p65 AS (Fig. 2*C*). We confirmed the knockdown of NF-κB p65 protein by Western blotting using the antibody against p65 subunit of NF-κB. An intense band was observed in the whole cell extract prepared from the control or p65 NS-treated HEC-1A cells. However, the expression of p65 was markedly reduced in the p65 AS-treated HEC-1A cells (Fig. 2*D*). The NF-κB inhibitors prevented estrogen-induced mRNA expression (Fig. 3*A*) and protein synthesis of VEGF (Fig. 3*B*). Taken together, these data suggest that estrogen induces the expression of angiogenic factors through a pathway involving PAF-mediated NF-κB activation.

Estrogen Induces Angiogenesis in a PAF-Mediated NF-κB-Dependent Manner. We next determined the role of PAF-mediated NF-κB activation in estrogen-induced angiogenesis. Angiogenic activity was determined using a mouse Matrigel implantation model *in vivo* and the sprouting of PPAECs *in vitro*. Estrogen caused a pronounced angiogenic activity in a dose dependent manner in *in vivo* (Fig. 4*A*). Estrogen inhibitor, PAF antagonist, or NF-κB inhibitors significantly inhibited the estrogen-induced angiogenesis (Fig. 4, *B* and *C*). Immunohistochemistry was also used to assess the angiogenic activity of estrogen. Sections of Matrigel were immunostained with the rat anti-vWF antibody. Erythrocyte containing canalized microvessels and staining of vWF were observed within the Matrigel in estrogen-treated mice (Fig. 5, *B* and *F*) but not in Matrigel of control group (Fig. 5*A*). Estrogen-induced increase in microvessels and vWF staining were not observed in Matrigel of ICI- (Fig. 5*C*), WEB 2170- (Fig. 5*D*) or parthenolide-treated animals (Fig. 5*E*). Estrogen also markedly induced sprouting of PPAECs (Fig. 6*A*), which was inhibited by estrogen inhibitor, PAF antagonist, or NF-κB inhibitors (Fig. 6, *B* and *C*). The results indicate that PAF-mediated NF-κB activation also plays an important role in estrogen-induced angiogenesis.

The extent of the involvement of these cytokines in estrogen-induced angiogenesis was subsequently analyzed through the use of neutralizing antibodies. We performed preliminary experiments and used a concentration of antibody that resulted in the neutralization of 50% maximal angiogenic effect of each cytokine (18). Fig. 7 showed that antibodies against bFGF, TNF-α, and IL-1 showed little inhibitory effect on estrogen-induced angiogenesis. In contrast, anti-VEGF

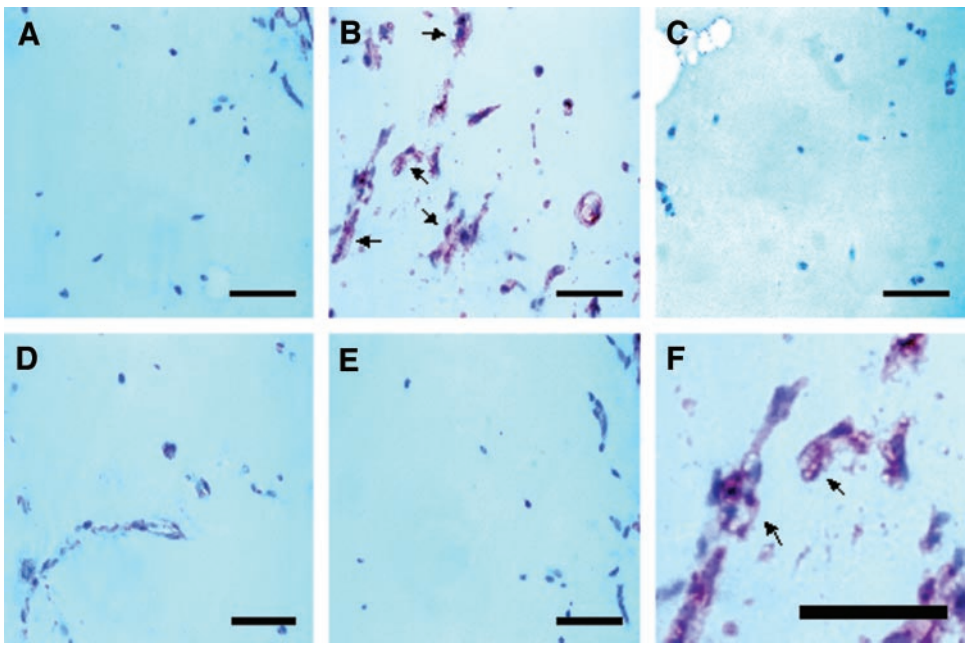

Fig. 5. Immunohistochemistry of Matrigel plugs in estrogen-treated mice. Matrigel plugs were mixed with estrogen (1 μmol/L), with or without ICI (1 μmol/L), WEB 2170 (1 μg/0.5 mL; Matrigel), or parthenolide (1 μmol/L) and injected s.c. On day 6, the Matrigel plugs were excised, and immunoperoxidase staining was performed using anti-vWF antibody. After immunostaining, sections were stained with hematoxylin. Matrigel mixed with vehicle (*A*), estrogen (*B* and *F*), estrogen + ICI (*C*), estrogen + WEB 2170 (*D*), and estrogen + parthenolide (*E*). *F*, magnification of section *B*. *Arrows*, examples of the erythrocyte-containing microvessels and staining of vWF. *Scale bar:* 100 μm.

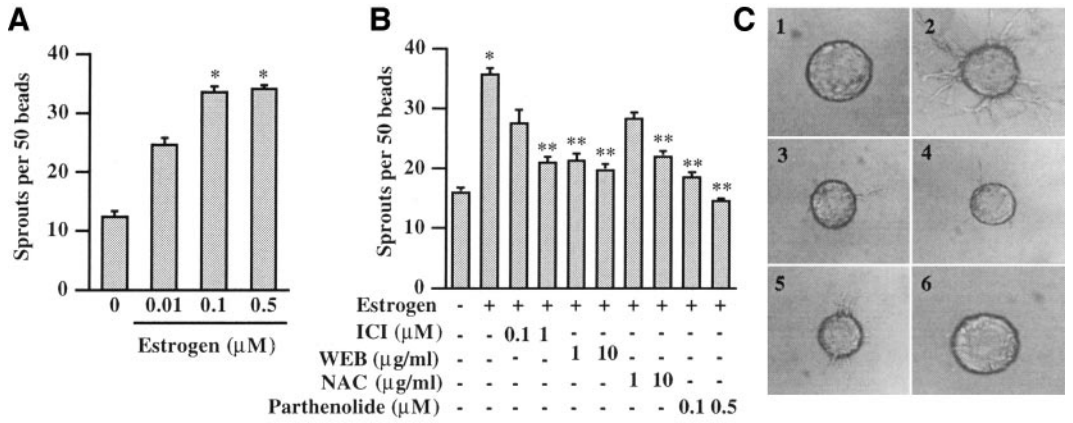

Fig. 6. PAF antagonist or NF-κB inhibitors block estrogen-induced sprout formation. *A*, Fibrin gels containing PPAEC-coated beads were treated with estrogen at indicated concentrations. *B*, The fibrin gels were treated with estrogen (0.1 μmol/L) with or without ICI, WEB 2170 (WEB), NAC, and parthenolide. *C*, macroscopic comparison of sprouting activities. PPAEC-coated control bead (*1*). Beads treated with estrogen (*2*). Beads treated with estrogen plus 1 μmol/L ICI (*3*) or 1 μg/mL WEB (*4*) or 10 μg/mL NAC (*5*) or 0.1 μmol/L parthenolide (*6*). Quantification of sprouting activity was performed on day 3 as described in Materials and Methods. The results were expressed as the number of capillary-like tubes formed per 50 beads. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

antibody exhibited the greatest inhibitory effect. To further clarify the effect of VEGF, the effect of soluble VEGF receptors, sKDR and sflt-1 chimera, was examined. sKDR or sflt-1 almost completely inhibited the angiogenic activity of estrogen at a concentration as low as 1 ng/0.5 mL Matrigel, indicating that VEGF is the most potent effector molecule in estrogen-induced angiogenesis.

PLA_2 Expression Is Involved in Estrogen-Induced Angiogenesis. PLA_2 is an enzyme that generates the precursors of eicosanoids (prostaglandins and leukotrienes), when the cleaved fatty acid is arachidonic acid. PAF, however, is produced by PLA_2 when the *sn*-1 position of the phosphatidylcholine contains an alkyl ether linkage and a bioactive lysophospholipids such as lysophosphatidic acid (lyso-PAF; refs. 26, 27). PLA_2 is activated by submicromolar concentrations of Ca^{2+} ions and by phosphorylation by MAPKs (28–30). Estradiol, by binding to the estrogen receptor, initiates a cascade of protein-protein interactions involving Shc, GRB-2, and SOS to activate MAPK (ref. 31 and references therein). Furthermore, estrogen has been shown to stimulate PLA_2 activity in reproductive organs (32–34). Collectively, these studies suggest that estrogen might be able to enhance the release and/or synthesis of PAF through pathways involving MAPK-induced phosphorylation and activation of PLA_2. We, therefore, examined the possibility. Treatment of HEC-1A cells with estrogen resulted in protein synthesis of $cPLA_2$ and the mRNA expression of $cPLA_2$, as well as $sPLA_2$, which were blocked by ICI (Fig. 8*A*). Estrogen-induced VEGF mRNA expression and sprouting were significantly inhibited by nonspecific PLA_2 inhibitor quinacrine, $cPLA_2$ inhibitor AACOCF3 or $sPLA_2$ inhibitor 4-bromophenacyl bromide (Fig. 8, *B* and *C*). In addition, those estrogen activities were also blocked by MAPK inhibitors (Fig. 8*D–F*), suggesting that PLA_2 expression through MAPK is the upstream pathway of PAF release in the estrogen-induced angiogenesis.

DISCUSSION

Although estrogen-induced angiogenesis, in both physiologic and pathological conditions, appears to be dependent on angiogenic factors such as VEGF and bFGF, the molecular mechanisms underlying the phenomenon are unknown. In this study, we demonstrated that estrogen induces the release of PAF, which subsequently leads to production of several angiogenic factors such as IL-8, bFGF, and VEGF in endometrial cells via the activation of NF-κB. To our knowledge, this is the first report to demonstrate the critical role for PAF-induced NF-κB activation in estrogen-mediated angiogenesis.

In this study, we demonstrate that PAF is involved in estrogen-induced angiogenesis because it was observed that the inhibition of PAF action inhibited the effects of estrogen. We have shown that PAF exerts its biological activity through NF-κB activation (16–18). The transcription factor NF-κB is known to be an important regulator of proinflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules, all of which play a key role in inflammatory process (14, 15). Several investigators have reported a role for NF-κB in angiogenesis (35–37). In fact, regulation of synthesis of the various key angiogenic factors such as IL-1, TNF-α, bFGF, IL-8, and VEGF is controlled via NF-κB activity (38–42). Because PAF is a proximal inducer of NF-κB *in vivo* as well as *in vitro* (16–18), any circumstance in which the release and/or synthesis of PAF is induced abnormally may lead to pathological angiogenesis via a NF-κB-dependent mechanism. Thus, the observations that estrogen increases not only PAF concentrations in the

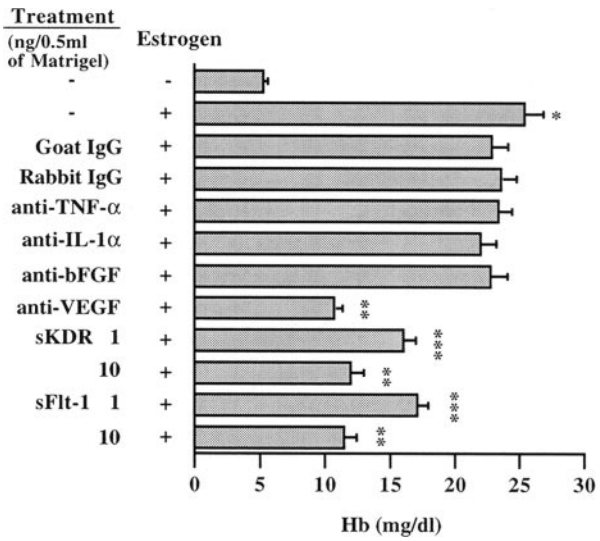

Fig. 7. Effect of angiogenic factor neutralizing antibodies on estrogen-induced angiogenesis. Matrigel were mixed with estrogen (0.1 μmol/L) in the presence or absence of sKDR and sflt-1 and neutralizing antibodies for TNF-α (90 ng/0.5 mL Matrigel), IL-1α (100 ng/0.5 mL Matrigel), bFGF (80 ng/0.5 mL Matrigel), or VEGF (10 ng/0.5 mL Matrigel). Isotype-matched rabbit IgG or goat IgG (100 ng/0.5 mL Matrigel) were used as negative controls. The results were expressed as mg of hemoglobin (Hb)/dL of Matrigel. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$; and ***, $P < 0.005$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

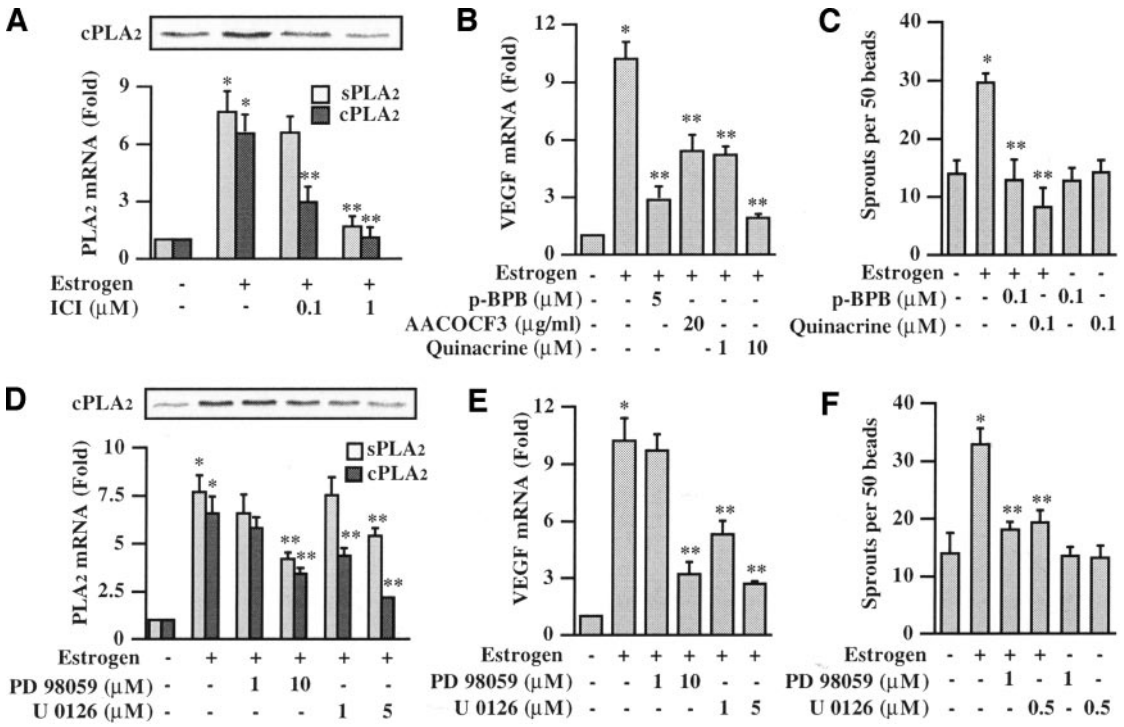

Fig. 8. Involvement of PLA_2 activity in the estrogen-induced VEGF expression and sprouting. *A*, *B*, *D*, and *E*, HEC-1A were plated at 1×10^6 cells/60-mm dish and cultured for an additional 4 hours after estrogen treatment. Levels of protein of $cPLA_2$ (*top panel* of *A* and *D*) and mRNA expression of $cPLA_2$ and VEGF were analyzed by Western blotting and real-time PCR, respectively. *C* and *F*, Sprouting activity was performed as described in Fig. 6. All of the reagents at indicated concentrations were added to the cultures 30 minutes before estrogen (0.1 μmol/L) treatment. *, $P < 0.0001$ compared with control group; **, $P < 0.0001$ compared with estrogen-treated group. Values are expressed as means; *bars*, ± SE.

uterus (19, 20) but also the expression of the PAF receptors in human endometrial glandular cells (21) provide additional support for the concept that PAF plays a role in estrogen-induced angiogenesis. In this study, we have demonstrated that estrogen induces NF-κB activation through PAF synthesis in endometrial cells. Experiments concerning the influence of estrogen on NF-κB activation had rather controversial results. Estrogen has been reported to inhibit NF-κB activation in many cells or tissues such as osteoblast (43), muscle cells (44), liver (45), and keratinocytes (46). Although information about the effects of estrogen on NF-κB activation in endometrial cells is quite limited, Shyamala and Guiot (47) reported that estrogen activates κB-specific protein. This information suggests that estrogen differently regulates NF-κB activation depending on cell types. This is additionally supported by the fact that estrogen receptor regulates NF-κB activity in a cell-specific manner (48).

How can estrogen function to increase PAF levels *in vitro* (Fig. 1*C*) as well as *in vivo* (the uterus; refs. 19–21)? PAF is not stored in the cells but is present in the form of an inactive precursor (alkylacylglycero-3-phosphorylcholine) in the membrane (49). Upon an inflammatory stimulus, PLA_2 becomes activated and cleaves the phospholipid at the 2(R) position, leading to the formation of the lysophospholipid derivative, lyso-PAF (27). Lyso-PAF is, in turn, acetylated by acetyl-transferase into PAF (50). PLA_2 is activated by MAPKs (28–30). We here demonstrated that PLA_2 expression via MAPK is a possible upstream pathway in the estrogen-induced angiogenesis. This conclusion came from the observations that estrogen caused an up-regulation of PLA_2 expression in the HEC-1A cells, and estrogen-induced VEGF mRNA expression and sprout formation were both inhibited by the PLA_2 inhibitors or MAPK inhibitors. Our data provide evidence to support the findings that estrogen has been shown to stimulate PLA_2 expression in reproductive organs (32–34) and explains how PLA_2 functions in the estrogen-induced angiogenesis. The upstream pathway of MAPK activation by estrogen requires additional studies.

In summary, our results indicate that estrogen induces the synthesis of several key angiogenic factors in endometrial cells through PAF-mediated activation of NF-κB. This information provides new insight not only into the understanding of estrogen-induced angiogenesis but also establishes a potential therapeutic target for estrogen-dependent pathological conditions such as breast cancer.

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Cancer Res 2004;64:6482-6488.